Topics in Medicinal Chemistry 25

Jed F. Fisher
Shahriar Mobashery
Marvin J. Miller *Editors*

Antibacterials

Volume I

Springer

25
Topics in Medicinal Chemistry

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Jed F. Fisher • Shahriar Mobashery •
Marvin J. Miller
Editors

Antibacterials

Volume I

With contributions by

C.J. Balibar · G.S. Basarab · K. Bush · M. Chang ·
J.F. Fisher · S.H. Lee · J.C. Malinverni · J.E. Meisel ·
C. Melander · R.J. Melander · S. Mobashery · T. Roemer ·
D.M. Shlaes · L.L. Silver · H.A. Sutterlin

Springer

Editors
Jed F. Fisher
Department of Chemistry & Biochemistry
University of Notre Dame
Notre Dame, Indiana
USA

Shahriar Mobashery
Department of Chemistry & Biochemistry
University of Notre Dame
Notre Dame, Indiana
USA

Marvin J. Miller
Department of Chemistry & Biochemistry
University of Notre Dame
Notre Dame, Indiana
USA

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Preface

An essential – that is to say, life-saving – component of modern medicine is the reliable ability to suppress bacterial infection. The chemical entities entrusted with this responsibility correspond to exceeding structural diversity, mostly of natural product origin but increasingly as well of synthetic origin. They occupy a chemical space that is distinct in key respects as compared to the entities used in other therapeutic areas of medicinal chemistry [1–6]. This distinction when combined with the perpetual increase in bacterial resistance mechanisms [7–16], the seeming sparseness of valid antibacterial targets [17–23], and the belief that antibacterial discovery offers a poor return-on-investment [10, 24–36] support a widespread concern as to the future reliability of antibacterial chemotherapy [37–46]. While the assertion that the antibiotic apocalypse has yet to arrive is certainly correct [47] and while considerable reasons for optimism exist [47–49], we must be mindful both that the harbingers of possible apocalypse will arrive first elsewhere (in the third world) [50] and that successful drug discovery and development is emphatically noninstantaneous [51]. The fourteen chapters of these two volumes on antibacterial drug discovery capture this urgency, and add to its dimension the challenge, perspicacity, and ingenuity of contemporary antibacterial discovery. The compounds represented within these chapters include the antibacterials of Nature (the antibiotics) – both as starting material and as inspiration – and de novo structures. The chapters emphasize antibacterial target selection, emerging concepts for antibacterial discovery and structure-activity refinement, and antibacterial clinical development and utility.

All medicinal chemistry efforts begin with a hypothesis as to an intimate interconnection among a structure, a target, and a disease. While one does not need to have at the outset both the structure and target, a recurring discussion point in antibacterial discovery is whether the universe of antibacterial targets extends beyond those targets already known. In the opening chapter of the first volume, Sutterlin et al. [52] address antibacterial target selection from the vantage of screening methodology and the relationship between conditional essentiality and synthetic lethality among intersecting bacterial pathways. A complementary

perspective on targets – especially with respect to the different resistance mechanisms used by the Gram-positive and Gram-negative bacteria, and including both multitargeting and antibacterial combinations – is provided by Silver [53]. Bush critically assesses the possibility of synergistic antibiotic combinations to address the clinical emergence of multidrug-resistant bacteria [54]. Melander and Melander [55] extend the concept of mechanistic synergy by judicious selection of structural pairs as antibacterial adjuvants. Given the proven value of allosteric modulation in other therapeutic areas of medicinal chemistry, Meisel et al. [56] address allosteric modulation of bacterial targets as a new antibacterial strategy. In addition to standards for efficacy, all drugs must meet rigorous standards of safety. The unique challenges presented by the antibacterials with respect to clinical evaluation for safety and efficacy are discussed by Shlaes [57]. In the last chapter of the first volume, Basarab [58] summarizes the diversity of the exploratory structural classes that act against a classic antibacterial target, the topoisomerases.

The second of these two volumes on antibacterial drug discovery gives further exemplification of the astonishing diversity of antibacterial structure. Bugg provides a perspective on the structure-activity relationships of the nucleoside antibiotics that target the MraY translocase catalyst of cell wall biosynthesis, a class that represents a possible solution to the pressing need for efficacious Gram-negative antibacterials [59]. Kleijn and Martin review our current understanding of the structurally complex, and mechanistically enigmatic, cyclic lipopeptide antibiotics [60]. The bacterial ribosome is the target of numerous antibacterial structural classes. Sun and Ciao [61] demonstrate the power of synthetic chemistry, as inspired by the tetracycline structures of Nature, to secure even more powerful and selective antibacterial structures. The oxazolidinone class of synthetic structures (also targeting the ribosome) have transformed the treatment of recalcitrant Gram-positive-caused infection in the twenty-first century and, as described by Barbachyn [62], are poised to continue in this capacity with new structures having improved safety and efficacy. The opportunities for both empirical and rational drug design, at the interface between natural and synthetic structures, are explored for the antifolates by Scocchera and Wright [63]. They remind us of the important historical role of the antifolates in antibacterial chemotherapy and the value of contemporary structure-based design to the preservation of this importance.

The two final chapters of the second volume address emerging strategies in antibacterial drug discovery. Bacteria have a rapacious need for iron and have devised extraordinary pathways for its sequestration and importation. Wencewicz and Miller [64] explore the exciting potential of incorporating siderophore (iron-chelating) structures into antibacterial design, as an enabling strategy for antibacterial delivery. The virtue of attenuating bacterial virulence as a means of control of bacterial infection is discussed by Kamal et al. [65], using the example of "pathoblocker" interference with the quorum sensing mechanisms of the notorious Gram-negative pathogen, *Pseudomonas aeruginosa*.

The collective value of these perspectives, as inspirational studies in antibacterial discovery, is the accomplishment of the authors of these chapters.

We thank them for their willingness to share not just with us, but with you as the readers of this volume, their reflections and guidance for advancing this most demanding, and most critical, realm of medicinal chemistry.

Notre Dame, IN, USA — Jed F. Fisher
Notre Dame, IN, USA — Marvin J. Miller
Notre Dame, IN, USA — Shahriar Mobashery
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Antibacterial New Target Discovery: Sentinel Examples, Strategies, and Surveying Success

Holly A. Sutterlin, Juliana C. Malinverni, Sang Ho Lee, Carl J. Balibar, and Terry Roemer

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

Contents

H.A. Sutterlin, J.C. Malinverni, S.H. Lee, C.J. Balibar, and T. Roemer (✉)
Merck Research Laboratories, Kenilworth, NJ 07033, USA
e-mail: terry_roemer@merck.com; troemer@prokaryotics.com

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

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

NA not applicable
ND not determined
[a]Target pathogen: *P. aeruginosa*
[b]Demonstrated in vivo efficacy
[c]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)
[d]Co-crystal structure available
[e]Target pathogen: *E. coli*
[f]Target pathogen: *S. aureus*
[g]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

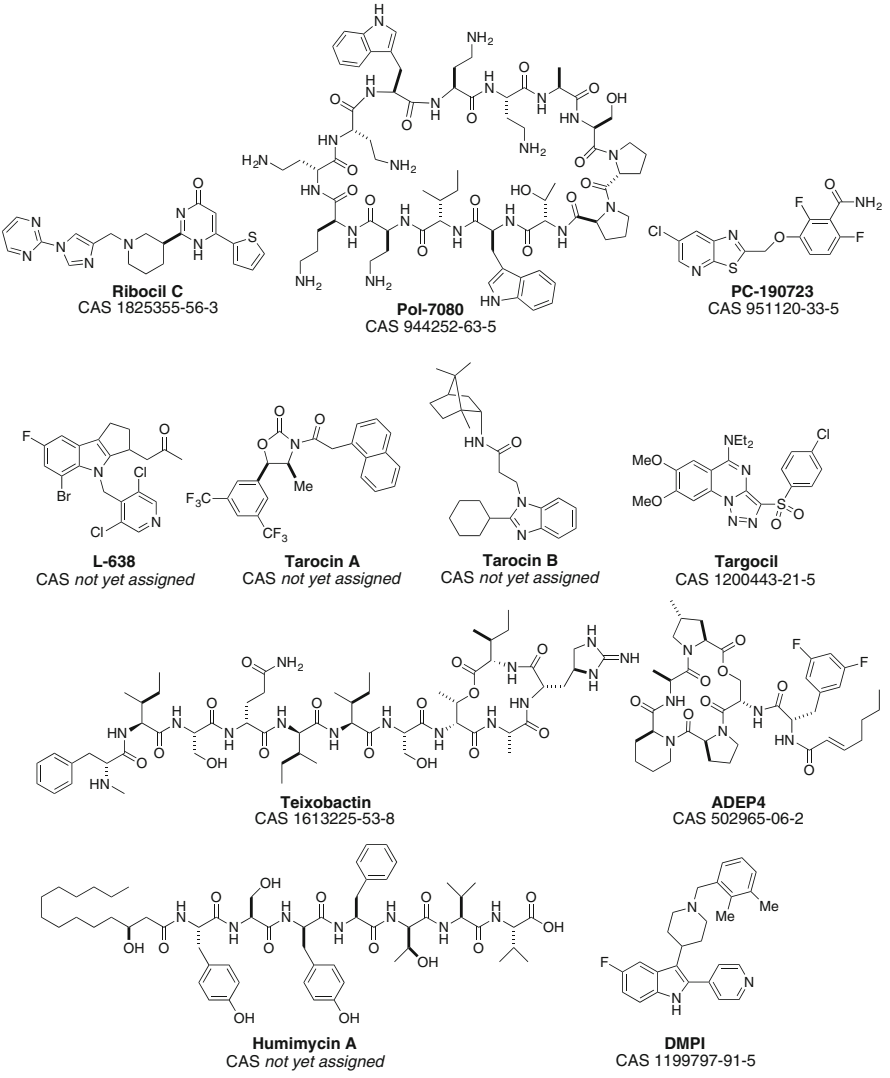

Fig. 1 Structures of representative compounds cited in this review

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 β-lactam efficacy against otherwise β-lactam resistant *Staphylococci* [47, 75, 76]. Here, a β-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 β-lactam antibiotics [76]. Interestingly, many of these β-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 β-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 β-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 β-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 β-lactamase inhibitors [80], as a result of its ability to restore the efficacy of β-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 identity 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 μg/mL). Moreover, tarocins resensitize MRSA and MRSE to a broad diversity of β-lactams in vitro below the clinical breakpoint drug concentration defining β-lactam resistance and provide synergistic efficacy when paired with β-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 β-lactamase inhibitors used to restore β-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 β-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 β-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 β-lactam potentiation adjuvant that restores β-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 μg/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, β-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 β-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 β-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 β-barrel assembly. Of note, Bakelar et al. found that when the lipoprotein subcomplex BamCDE binds, the essential β-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 β-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 β-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 β-jellyroll N-terminal domain of LptD and an enormous 26-β-stranded C-terminal barrel domain, the largest β-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 β-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-β-lactam inhibitor which not only inhibits the classic targets of penicillin, PBP1, but also allosterically inhibits the target responsible for β-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 β-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 β-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 β-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 β-lactam potentiator.

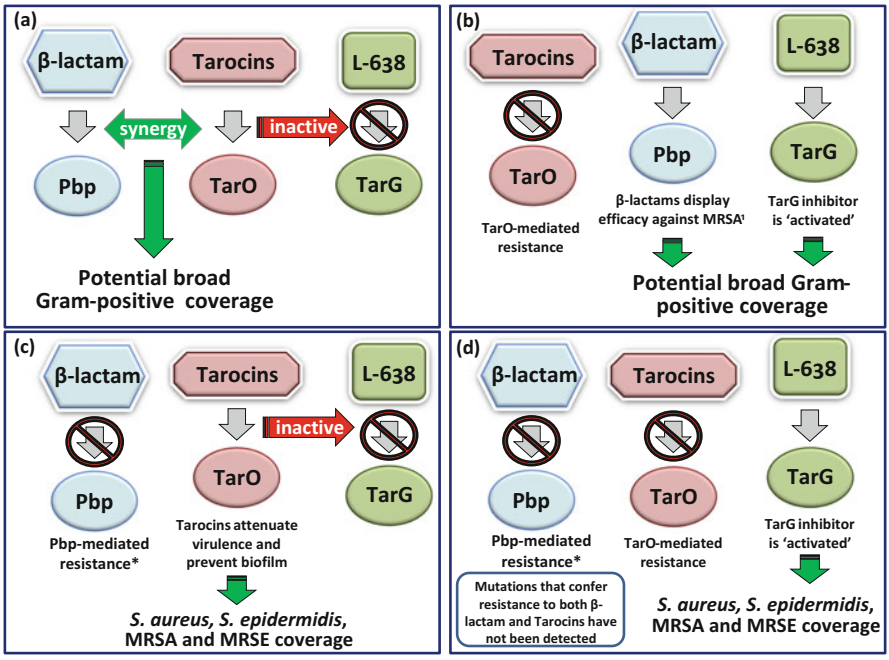

Fig. 2 Triple combination strategy provides an interdependent mechanism of mitigating target-based resistance. The illustration depicts multiple scenarios in which of a triple combination of TarO and TarG inhibitors paired with a β-lactam antibiotic overcomes various potential mechanisms of resistance. (**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 antibiotic 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

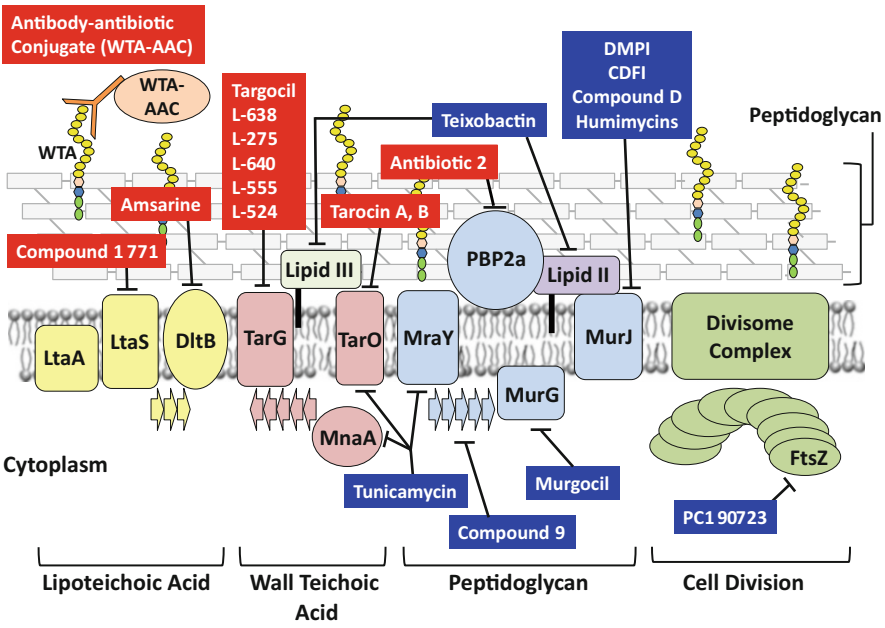

Fig. 3 (**a**) Select Gram-positive cell-wall associated targets and cognate inhibitors. Representative diagram of a prototypical *S. aureus* bacterial cell surface displaying color-coded biologically relevant biosynthetic pathways: lipoteichoic acid (*yellow*); wall teichoic acid (*salmon*); peptidoglycan (*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 β-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 β-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

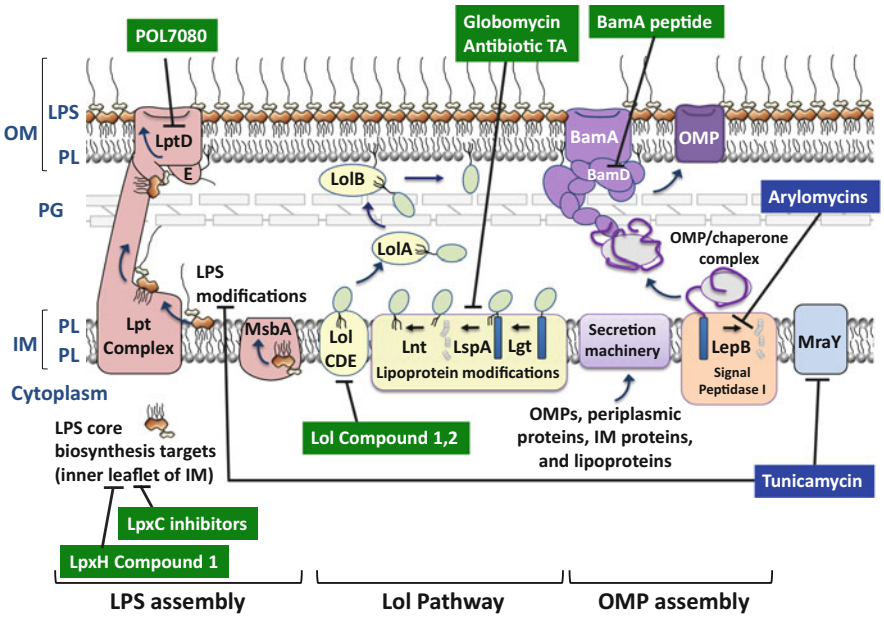

Fig. 3 (continued)

(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, Welcome 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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The Antibiotic Future

Lynn L. Silver

Abstract Will the future of antibacterial therapy rely on an ongoing pipeline of new small molecule, direct-acting antibacterial agents that inhibit or kill bacterial pathogens, referred to here as antibiotics? What role will these small-molecule antibiotics have in the control of the bacterial infections of the future? Although there is today increased activity in the field of new antibiotic discovery, the history of this field over the past 30 years is a history of low output. This low output of new antibiotics does not encourage confidence that they can be central to the future control of bacterial infection. This low productivity is often blamed upon financial disincentives in the pharmaceutical industry, and on regulatory difficulties. But I believe that a critical underlying reason for the dearth of novel products is the fundamental difficulty of the science, coupled with a failure to directly grapple with the key scientific challenges that prevent forward motion. The future fate of antibiotic discovery will depend upon the degree to which the rate limiting steps of discovery are fully recognized, and the discovery technology turns to overcoming these blockades.

Keywords Antibiotic chemical space, Antivirulence, Combination therapy, Druggability, Entry barriers, Frequency-of-resistance, Hollow-fiber infection model, Hypersensitive screening, Monotherapy, Multi-targeting, Natural products, Synergy

Contents

L.L. Silver (✉)
LL Silver Consulting, Springfield, NJ 07081, USA
e-mail: silverly@comcast.net

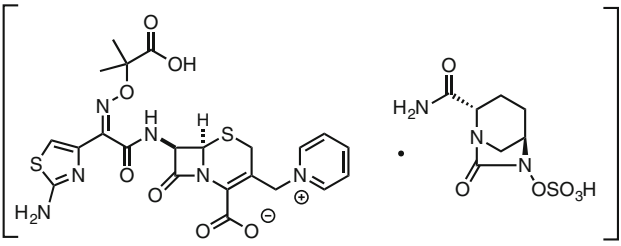
1 Introduction

The rising tide of antibiotic resistance has compromised the usefulness of existing antibiotics against many human pathogens. While there usually are appropriate drugs still available for most infections, there are a growing number of problematic pathogens that are resistant to most antibiotics. In response to the need for new drugs and new paradigms to address the problem of antibiotic resistance, policy makers and funders have proposed that directing monetary incentives, rewards and support to small companies in the area will increase "innovation" [1–3]. The realization of innovation in this case is the introduction of new antibiotics (or other therapies) to combat rising resistance. That is, innovation is directly connected to the end product. But innovation is also a process involving the application of new technology and creative ideas at the level of individual innovative scientists. Can there be innovation without ultimate success? Will money drive innovation of either sort? Clearly financial incentives can bring more players to the table, but ultimate success will require efforts to forcefully confront the reasons for failure.

Much of antibiotic discovery over the past 20 years has focused on the discovery and exploitation of novel targets for new drugs, with little success. The challenges of this approach have been much discussed [4–10] and, in my opinion, are mainly due to the focus on inhibiting targets that are subject to rapid resistance selection and to the poverty of chemical libraries, which lack compounds with properties correlated with antibacterial activity and entry. Without an effort to seriously address these limitations, it is hard to see a rosy future for direct-acting small-molecule antibiotics.

Even though many large pharmaceutical companies have cut back their antibiotic discovery programs over the past 10 years or so, there has been a great deal of ongoing industrial and academic work in the field of antibiotic discovery. These efforts indeed have involved a great deal of innovation – creative individuals using new technologies to solve problems – of the sort mentioned above. Often, this work has been directed toward improving old classes of antibiotics in order to render them less susceptible to resistance mechanisms. A major success of current antibacterial discovery is the ongoing discovery of new combinations of often novel β-lactams and β-lactamase inhibitors. This effort has only recently produced

registered compounds which will allow the treatment of some very hard-to-treat pathogens including MDR *P. aeruginosa* (ZERBAXA®) and many carbapenem-resistant Enterobacteriaceae (CRE) (AVYCAZ®). A new aminoglycoside, plazomicin, in late clinical development is also targeted against CRE. But sometimes the efforts toward finding novel agents have been misguided: jumping head first into "genomics as panacea" without first considering the rate-limiting steps in discovery was a mistake. Can new technologies open up the field, or will they lead us astray? We must take hype with a grain of salt and continue to probe the assumptions underlying the application of new technology for its own sake. That said, it is important to bring in new viewpoints and different scientific backgrounds to attack these problems. But those new players must familiarize themselves with the field, its aims, its history, what has been tried and, importantly, what the roadblocks are.



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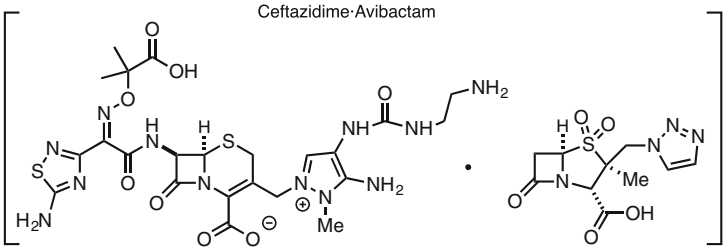

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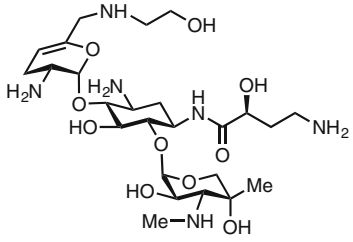

Plazomicin
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ACHN 490

Will antibiotics, small molecules that directly inhibit the growth of or kill bacterial pathogens, be the mainstay of antibacterial therapy in the future? Is there a path to replenishing the failing pipeline? Will other modalities take the place of direct acting antibiotics? If there are to be innovative breakthroughs, then they will be, perforce, difficult to predict. Thus, it seems more productive to focus on the problems whose innovative solutions will lead to those breaks in the logjam. This focus necessitates a more historical discussion than perhaps envisioned by the editors of this book. Nonetheless, I maintain only by understanding the history of this field will we will make progress. After a small excursion this chapter will focus on the obstacles to antibacterial discovery that must be overcome in order to discover and develop small-molecule, direct-acting antibacterial agents: the antibiotics of our future.

2 Excursion: Alternatives to Antibiotics

It may be that novel, direct-acting antibiotics will continue to elude us and that the way forward for the treatment or prevention of bacterial infection lies in alternatives to antibiotics [11–16]. Indeed, interest in this field has been growing apace with strong emphasis on antivirulence approaches focused on antibodies [14] as well as small-molecule inhibitors of virulence mechanisms [15]. In any case, alternatives must be pursued.

A review [16] commissioned by the Wellcome Trust evaluated the pipeline of alternative antibacterial therapies. The authors limited their study of alternatives to "non-compound approaches that target bacteria or approaches that target the host". For example, they included antibodies but excluded small-molecules targeting virulence factors. There is clearly a great deal of effort in these "alternates-to-antibiotics" areas: antibodies and vaccines, both prophylactic and therapeutic; drugs inhibiting or modifying the activity of host factors involved in responses to bacterial infection; adjuvants that enhance the activity of antibiotics by enhancing permeability or inhibiting efflux pumps; inhibitors or blockers of antibiotic resistance functions. It is likely that some of these will take their place in the armamentarium, but most likely as adjuncts to existing antibiotics and to the small-molecule antibiotics of the future. It must be noted that many of these areas have been under study for many years with little progress. As the Wellcome review concluded, the need in these alternative approaches is not so much for discovery efforts but for "enhanced translational expertise" [16], including guidance in clinical development and regulatory paths for such alternate therapies. A recent review of antivirulence efforts, specifically including small molecules, seems quite upbeat [14]. The latter review discusses several antibodies that have been approved for prophylaxis or therapy against bacterial toxins, including those for *C. botulinum* BoNT toxins, *B. anthracis* PA toxin, and *C. difficile* toxin B (TcdB). Five antibodies (four monoclonals against toxins, one engineered bispecific antibody against two *P. aeruginosa* surface proteins) are listed as being in clinical trials and a large

variety of small molecules targeting various virulence factors and functions are under preclinical study. While antitoxin therapy has a long history [17], the development of non-antibacterial small molecules, those acting indirectly to affect infectious processes in the host, is a path less traveled.

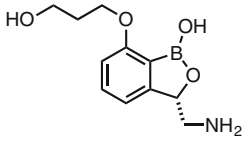
There are a number of such indirectly-acting small molecules under study for antivirulence, as described in a recent review [15]. Many of these have been uncovered in innovative screens or by in silico selection through genetic and structural study of the desired targets. In most cases, antivirulence activity is measured via surrogate in vitro assays that involve specific engineering of reporter bacterial strains. In order to develop such therapies for clinical use would require simple diagnostic tools to estimate the presence of the virulence mechanism in populations of clinical isolates (a surrogate for an MIC_{90}) as well as testing susceptibility to the compound of specific pathogens in the laboratory. Implementation of these tools may not be straightforward. It is often claimed that antivirulence approaches would discourage resistance development due to lack of selective pressure. That may be true in some cases, but resistance mutations would undoubtedly arise. Their amplification in the population would then be dependent upon the degree of selection. But resistance mutations can be useful in defining the actual molecular targets of a compound, in order to support the identification of the proposed antivirulence function as the critical target of inhibitor action. Since desirable antivirulence compounds lack antibacterial activity, it is generally difficult to select in vitro for resistance. Hence there is little evidence (for most small-molecule antivirulence compounds) that they are acting solely through the supposed target.

Other possibilities for therapeutic intervention may involve visionary synthetic biology and genome engineering approaches, including the very interesting possibility of using methods based on RNA-guided nucleases of the CAS/CRISPR (clustered, regularly-interspaced, short palindromic repeats) type [18–22]. Such proposed systems that recognize unique DNA sequences could narrowly target and kill specific pathogens or inactivate resistance mechanisms. The limitation for the CRISPR-based approach resides in design of suitable delivery methods which generally involve bacteriophage vectors. Such vectors would have to infect virtually every infecting pathogen.

3 The Resistance Problem with Single Targets

I subscribe to the view that inhibitors of single enzymes have a high likelihood of selecting for single-step resistance due to changes in the target molecule that result in large increases in MICs [9, 23–25]. Although there are exceptions (see below) I predict that most such inhibitors will fail in monotherapy, unless the resistance mutations lead to much lowered fitness. Conversely, successful monotherapeutic systemic antibiotics are those which have multiple targets, or targets that are the products of multiple genes or a pathway [23, 25]. While the potential peril of single-

targeted antibiotics was first hypothesized ~25 years ago [24], the first clear example of this was the failure due to rapid resistance development in a Phase II clinical trial of Epetraborole (GSK2251052) an inhibitor of leucyl-tRNA synthetase with excellent activity against Gram-negative pathogens [26–28]. This failure is discussed more fully below.



Epetraborole
CAS 1093643-37-8
GSK 2251052

Sutterlin et al. discuss [29] "Antibacterial New Target Discovery" and note the potential for single-targeted compounds to yield single-step resistance in vitro. In the footnote to Table 2 of that chapter, an acceptable starting point for frequency of resistance (FoR) is stated as 10^{-8}. But what is an acceptable final FoR in vitro (and in vivo)? They note that the initial FoRs observed for a lead structure may be modulated favorably by chemical optimization, often by increasing target affinity via additional compound-target interactions. While the optimism voiced by Sutterlin et al. that in vitro results showing potentially high rates of resistance may not translate in vivo is sometimes true, there are definitely exceptions, as discussed below. However, each compound must be evaluated separately, and care taken that compounds are not mistakenly maintained in a development pipeline as a result of neglecting to test rigorously for resistance potential.

3.1 Measurement of Resistance

With advances in technology, one might think that methodologies for predicting the likelihood of clinically-important rapid resistance were well in hand. In vitro measures to ascertain the frequency of single-step resistance (FoR) generally involve plating a large inoculum of a bacterial strain on agar plates containing increasing amounts of test compound [30]. The rate of resistance (number of resistance mutations per bacterium per generation) can be measured by a Luria-Delbruck-type fluctuation test (or variations thereof) [31–35]. It is also useful to measure FoR in hypermutable (mutator) strains, where such frequencies may be increased 1,000-fold or more [36]. Such mutators occur at significant rates among clinical isolates of many pathogens and so are likely to play a role in resistance development [37–40]. There are also a variety of methods for selecting mutations by serial passage at sub-inhibitory levels of compound, which might be thought to mimic some clinical condition. In addition to revealing stable mutations that can give rise to incremental steps in resistance, serial passage can uncover unstable

adaptive changes leading to transient phenotypic resistance that requires maintenance of selective pressure. These adaptive changes may involve induction of resistance functions or target gene amplification [41]. Andersson et al. discuss the place of serial passage and resistance selection at sub-MIC levels in laying a predicate for clinical behavior [42–44].

Of course, none of these methods takes into account the horizontal genetic transfer which accounts for much clinical resistance [44]. Furthermore, in vivo growth rates, nutrient limitation, varying drug levels and the immune status of the host certainly contribute to the survival and propagation of resistant mutants. Indeed, the value of in vitro methods to predict resistance occurrence in the clinic in treatment of human infections is largely untested. While there are animal models for antibacterial efficacy that are highly predictive of clinical results, animal models for development of resistance are few and not standardized. One reason for this difference is that efficacy models are normally run with relatively low bacterial inocula, generally 10^6 or fewer infecting pathogens, and even fewer when virulent pathogens are used. This inoculum yields populations that are too small to contain pre-existing resistant mutants. The level of pre-existing mutants is likely critical to the rapid increase in resistance during therapy. Nevertheless, efforts to tie in vitro results to predictions of clinical performance should continue. An important in vitro approach, the hollow-fiber infection model (HFIM), can demonstrate the efficacy of antibiotics and the appearance of resistance under conditions of dynamic variation of drug concentration, mimicking human discontinuous dosing pharmacokinetics [45–48]. HFIM can play a useful role in relating in vitro findings to clinical outcomes, as discussed below.

What FoR determined in vitro is bound to yield rapid resistance in vivo? As noted above, while in vitro methods of FoR determination are feasible and should be routine tools in antibacterial discovery programs, there is a disconnect between in vitro results and clinical reality. This disconnect is due to the fact that there has been little clinical experience with single-targeted agents in the treatment of standard bacterial pathogens. Put another way, there is little experience with antibacterials that select rapidly for high level resistance. For the mainstays of systemic monotherapy that are multi-targeted, there may be single-step FoRs in vitro that, in the main, lead to modest increases in MICs. These single-step FoRs are often due to changes in permeability or efflux, and not to mutational changes in targets. Often there are claims in the literature that a new inhibitor has a low FoR – say a value of 10^{-8} – which is said to be in the range of that seen with standard drugs. But the amplitude of change in MIC for those standard drugs is usually not very large, while the amplitude of MIC increase for single-step resistance for a single-targeted agent can be >100-fold. In a fulminant infection, the bacterial burden is likely to be high enough to contain spontaneously-resistant mutants. Thus, while it is likely that an FoR of 10^{-6} (which might be seen if a genetic deletion can give rise to a resistant phenotype) would show overnight resistance in a clinical situation, it is not clear how low a frequency would have to be to insure that rapid resistance would not arise.

In the clinical trial, mentioned above, for complicated UTI infection of the leucyl tRNA inhibitor, GSK2251052, an in vitro resistance frequency of $\geq 10^{-8}$ yielding mutants with good fitness led to a significant rate of high-level rapid resistance in 3 of 14 patients, after 1 day of treatment [26, 27]. The mutants had MICs as high as 2,000-fold the initial MIC. This result was modeled retrospectively in an HFIM experiment [28] which showed that with a starting inoculum of 10^8 *E. coli*, resistant mutants took over the population within 24 h. This result had not been seen in preclinical animal-efficacy models, again most likely because the infecting inoculum was low.

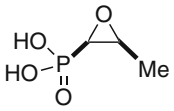
3.2 Overcoming Single-Target Resistance

There is a strong likelihood that single-step mutations leading to resistance against single targeted agents can and will arise before challenge with the agent, given that the inverse of the FoR is less than the population size. Does this result obviate the exploitation of any single target? The future of antibiotics research and development may well involve creative solutions for the exploitation of single-target inhibitors in ways that reduce resistance potential. These solutions may lie in target-inhibitor exceptions to the rapid resistance problem, including low virulence of resistant mutants; low toxicity allowing high dosing to prevent survival of resistant mutants; focus upon narrow spectrum agents which could allow design of very high affinity, high-potency inhibitors that are insusceptible to target-based resistance; and the use of combinations of single-target inhibitors to lower the probability of resistance selection. There are clearly exceptions to the "no single-targets" dictum that might be explained by special circumstances. Can we learn anything from those exceptions?

3.2.1 Low Fitness

Single-step mutations to resistance to certain antibiotics can reduce growth rates, decrease virulence, and otherwise lower the competitive fitness of the pathogen in the host milieu. These changes can counteract the effect of pre-existing resistant mutants and reduce their contribution to the infecting population. Thus, fosfomycin, an inhibitor of the MurA (UDP-*N*-acetylglucosamine-enolpyruvyl-transferase) enzyme, the first committed step of peptidoglycan synthesis, has been used traditionally as a single dose oral treatment for uncomplicated urinary tract infections (uUTI). As fosfomycin has a broad antibacterial spectrum including many MDR pathogens, is active both orally and parenterally, and has an excellent safety profile, there has been recent interest in expanding its indication. Rapid resistance through transport loss, albeit occurring at high frequency in vitro, yields lowered fitness in vivo, in the urinary tract setting. Thus, fosfomycin has been quite successful in treatment of uUTI. Very few target-based resistance mutations, which

likely arise at low frequency, have been detected in the clinic, possibly due to this lowered fitness. It is not clear whether mutational resistance, which has not been problematic for uUTI, will translate to low fosfomycin resistance in other tissue sites [49]. Furthering this caveat is a study of fosfomycin resistance development in a mouse lung model of *P. aeruginosa* infection showing that mutations did arise during treatment and that the mutants (in the *glpT* transporter) were not compromised in fitness [50]. Importantly, recent reports point to the rise of transmissible resistance due to modification of fosfomycin, including (in China) plasmids encoding both resistance to fosfomycin and the presence of the β-lactamase KPC-2 [49, 51–55].



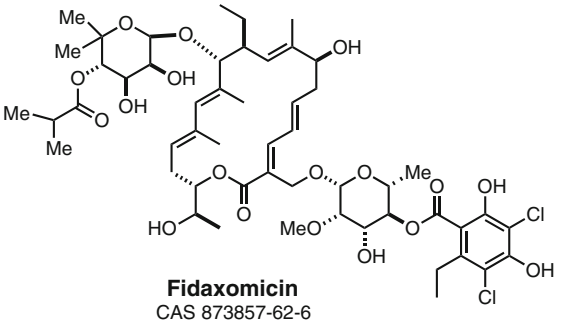
Fosfomycin
CAS 23155-02-4

3.2.2 Dosing above the Mutant Prevention Concentration (MPC)

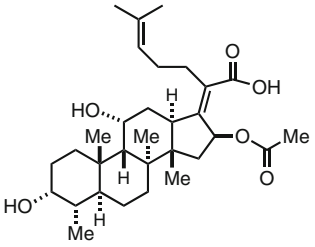
The idea of the mutant-prevention concentration, MPC, was developed in studying resistance to the fluoroquinolones [56]. Single-step mutations with increased MICs could be selected in vitro up to a threshold concentration, above which the frequency fell dramatically (to less than 10^{-11}). This pattern is due, in the case of the fluoroquinolones, to the occurrence of mutations in the most sensitive target (DNA gyrase, GyrA, in *E. coli*) that increased the MIC to that threshold concentration, above which concentration a second mutation, in a second target (topoisomerase IV, ParC, in *E. coli*), would be required to raise the MIC further. Thus, the MPC is the concentration of drug above which a single mutation cannot afford resistance. In some cases the increase in MIC due to a single target mutation can be >100-fold, as with rifampicin (targeting RNA polymerase) in *S. aureus*.

Fidaxomicin is an inhibitor of RNA polymerase with a significant FoR in vitro [57, 58], nonetheless successfully treats *C. difficile*-associated disease since very high concentrations can be maintained in the gut [59]. Essentially, this is an example of dosing above the MPC. Such dosing can be done when the drug is sufficiently safe so as to achieve very high concentrations at the infection site. Fusidic acid is an inhibitor of protein synthesis elongation factor G (Ef-G) that has been in use in Europe for many years in the oral treatment of *S. aureus* infections. Mutations in the target gene *fusA* can arise and yield resistance. Cempra Inc., in a trial for fusidic acid treatment of ABSSSi, is investigating the use of a loading dose of fusidic to kill off the pre-existing resistant mutants. Dosing above the MPC has been endorsed by many experts [60–63]. This dosing requires careful attention to the pharmacokinetic/pharmacodynamic (PK/PD) parameters for each drug, as well

as the reimagination of the concept of MIC breakpoints, perhaps evolving to an MPC breakpoint.



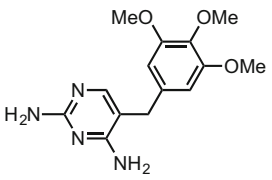
Fidaxomicin
CAS 873857-62-6
Dificid, Dificir



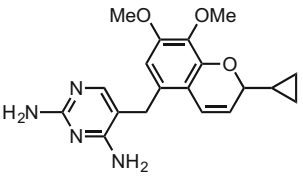
Fusidic Acid
CAS 6990-06-3
Flucidin

3.2.3 Avoiding Resistance by Multiple Target-Ligand Interactions

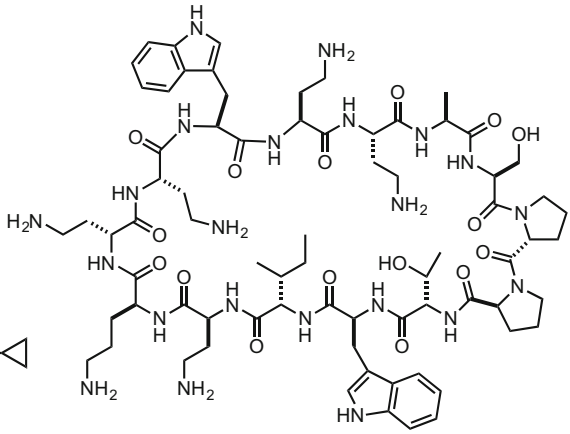
If resistance due to changes in the target-binding site is significant, it may be possible to reduce the FoR by increasing binding affinity for example by adding ligand-target interactions. This outcome was the aim in the development of iclaprim, a derivative of trimethoprim, an inhibitor of dihydrofolate reductase (DHFR), which is not yet registered. POL7080 (murepavidin) is a peptide mimetic targeting LptD of *P. aeruginosa*, a protein involved in translocation of lipopolysaccharide to the outer membrane, and is also in development. Murepavidin has an FoR of 10^{-10} [64]. Mutants contain an 18-bp duplication that apparently prevents compound binding. Presumably the lack of single-base-change resistance mutations is due to the multiple target-ligand interactions of this peptide mimetic.



Trimethoprim
CAS 738-70-5



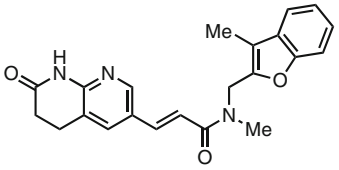
Iclaprim
CAS 192314-93-5



Murepavadin
CAS 944252-63-5
POL 7080

3.2.4 Narrow Spectrum Agents

Other single-target agents are under development including those inhibiting FabI and LpxC. Inhibitors of LpxC (UDP-3-*O*-[acyl]-*N*-acetyl glucosamine deacetylase, the first committed step in Lipid A synthesis) are subject to various resistance mechanisms depending upon the species being inhibited [65, 66]. However, it appears that resistance in *Pseudomonas aeruginosa* is infrequent and due either to increased efflux or increased LpxC expression, generally leading to MIC increases of two- to fourfold (with exceptions) [67]. It may be possible to engineer compounds less susceptible to efflux and/or increase potency such that the compound can, if sufficiently safe, be dosed above the MPC. Thus, *P. aeruginosa*-directed LpxC inhibitors are a stated goal of Achaogen [68]. FabI, enoyl-ACP reductase, an essential enzyme in fatty acid synthesis in some bacterial species, is the target of the anti-*S. aureus*-compound, Debio1452 (AFN-1252) [69]. As this compound is being developed for a single species, the potency attained can be very high, due to the possibility of developing very tight binding inhibitors to the single isoform of the enzyme rather than to the many homologues requiring coverage for broad spectrum targets. Indeed, the MIC of this compound against *S. aureus* is ~3.9 ng/mL. Resistant mutants map solely to two sites in *fabI* and can raise MICs significantly, from 3.9 ng/mL to 250–500 ng/mL [70, 71]. These mutants appear fit. However, these MICs are easily covered by standard dosing regimens of the compound and so should not give rise to rapid resistance in the clinic.



Debio 1452
CAS 620175-39-5
API 1252

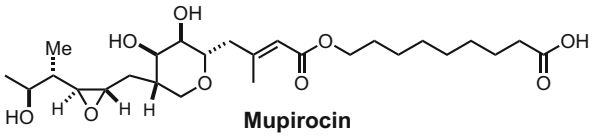
3.3 Leveraging Single Targets by Truly Assessing Combinations

In the areas of *M. tuberculosis*, HIV, HCV, and cancer, treatment with combinations of multiple single agents has become the norm. In the case of the infectious agents, while such combinations may be directed toward covering important subpopulations (such as persisters or non-growing MTB cells), these therapeutic regimens have been optimized over time, by addition of new classes of drugs, to reduce rapid selection of resistant mutants to which each of the individual drugs is subject. This process proceeded with the serial introduction of the first and following drugs for each of these pathogens, followed by appearance of resistant

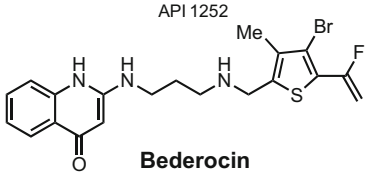
organisms. The histories of these drug regimens against HIV [72–76], HCV [77–80] and TB [81] show a progression to the use of three or four moieties as standard therapy. This progression is in contrast to the history of therapy of more "standard" (non-mycobacterial) bacterial pathogens, where the systemic agents available were not subject to rapid high-level target-based mutational resistance in the pathogen. Those standard antibiotics (including β-lactams, vancomycin, the ribosomally-targeted natural product antibiotics such as tetracycline, chloramphenicol, erythromycin, as well as the synthetic fluoroquinolones) have multiple targets or inhibit the products of multiple genes or a pathway. I believe this multitargeting is responsible for the low frequency of high-level target-based resistance [9, 23, 25]. As the discovery paradigm for standard pathogens changed in the late 1990s to wholesale pursuit of novel essential enzyme targets (as theoretically discovered through genomics), the benefits of multi-targeting were underemphasized. While not proven for every such inhibitor of single enzymes, single-targeted inhibitors (when tested) do select resistance in a single-step. Thus, in light of successful combinations of single-targeted agents for therapy of TB, HIV and HCV, it would seem reasonable to test this approach for standard pathogens, treating with combinations of single-targeted agents. If it is possible to evolve successful combinations of two or three single-targeted drugs to treat the ESKAPE pathogens, for example, then the whole of antibacterial discovery could return to the pursuit of single-targeted agents with a rational path to development and deployment – albeit one which would require regulatory buy-in and most probably, cooperation of inventors to pool the agents.

There is a great deal of literature on the clinical use of combinations of the classical (mostly multi-targeted) antibiotics to treat problematic, even MDR, infections, with varying results. Success may be based on synergistic activity, sensitization by one sub-inhibitory drug of another, or other, not well understood, mechanisms. But the idea of developing and using combinations of single-target agents against important standard bacterial pathogens in order to prevent resistance development has not been tested much. Exceptions include the use in animals and humans of combinations of various single targeted-agents, like rifampicin, fusidic acid, trimethoprim or novobiocin to treat MRSA infections [82–85], and the long standing use of combinations of trimethoprim and sulfamethoxazole, the combination of two inhibitors of the bacterial folate pathway [86].

While the hypothesis that combinations of single-targeted antibiotics should reduce resistance development in standard pathogens has been much discussed, there has been little direct testing of this in vitro with suitable controls. Recently, however, it was shown that targeting two different tRNA synthetases with a combination of inhibitors does lead to a great reduction in resistance frequency in vitro, as expected [87]. The authors showed that inhibitors of isoleucine, leucine and methionine tRNA synthetases (mupirocin [88], epetraborol (AN3365/GSK2251052) [26], and bederocin (REP8839/CRS3123) [89], respectively) which have high (10^{-7} to 10^{-8}) resistance frequencies, show $<10^{-12}$ frequencies when combined pairwise.



Mupirocin
CAS 12650-69-0
API 1252



Bederocin
CAS 757942-43-1
REP 8839

There have been a large number of HFIM studies of PK/PD and resistance development of combinations of single targeted agents in TB, for example [90–92]. Drusano et al. [63, 93] argue cogently for the use of PK/PD methods to approach ways of suppressing resistance development during monotherapy and in combination therapy. They consider both mouse models and hollow-fiber infection models (HFIM). It is explicitly proposed [93] to study combinations of antibiotics in order to suppress resistance emergence, using both standard classes of antibiotics but also preemptively studying combinations which might be used with new agents under development. I would expand this to include testing by HFIM (and, if possible, animal models) of as many of the published, validated single-target inhibitors as are available, in combinations both pairwise and in higher multiples. Table 1 lists a number of validated inhibitors subject to single mutations that significantly raise MICs. They could be used for testing in combinations against, for example, *S. aureus*, *E. coli*, *H. influenzae*, *P. aeruginosa* and permeable strains of *E. coli* and *P. aeruginosa*.

3.3.1 Problems that could Compromise Combinations

It should be noted that the feasibility of combination therapy in standard pathogens is not a given. The concept behind the use of combinations to avoid rapid resistance selection is based on initial killing (or growth inhibition) of pre-existing resistant mutants in the starting population, such that one drug will kill off the mutants resistant to the other. Theoretically, if the pre-existing rate is 10^{-8} per cell per generation, then for a combination of two drugs, a pre-existing double mutant should arise at the rate of 10^{-16}. As noted above, mutators are enriched among clinical isolates and can lead to increased resistance [37, 39, 40, 124]. This increase could require that more than two components be used in the combination. Two drugs might suffice to prevent rapid resistance during therapy, but over time if the level of either drug becomes insufficient, single mutations could arise in the surviving population (perhaps among persisters) that would then provide a

Table 1 Compounds subject to single step mutations to resistance for testing in combinations

Target	Inhibitor	Mini spectrum[a]	Resistance mechanism	Resistance frequency	In vivo?[b]	Reference for resistance
Cell wall						
MurC	Pyrazolopyrimidine	Pa effΔ, Ec tol	Target	10^{-9}	Unk[c]	[94]
MurA	Fosfomycin	Ec, Sa, Pa	Uptake	10^{-7} to 10^{-8} (Ec)	Yes	[55]
MraY	Pacidamycin	Pa	Uptake	2×10^{-6} to 10^{-8}	Yes	[95]
MurG	Murgocil	Sa	Target	10^{-7}	Unk	[96]
PBP2	Mecillinam	Ec	Target	NA[d]	Yes	[97]
Dxr	Fosmidomycin	Ec	Uptake	NA[e]	Yes	[98]
TarG	Targocil	Sa	Bypass	10^{-6}	Yes	[99]
Cell division						
FtsZ	PC190723; TXA-709	Sa	Target	10^{-8} to 3×10^{-8}	Yes	[100, 101]
MreB	CBR-4830	Pa effΔ	Target	5×10^{-9}	Unk	[102]
Lipoprotein/lipid						
LolC E	Compound 2	Ec, Ec tol, Hflu	Target	10^{-6} to 2×10^{-7} (Ec tol)	Unk	[103]
LspA	Globomycin	Ec	Bypass	10^{-7}	Yes	[104]
Fab I	Debio-1450	Sa	Target	10^{-10} to 2×10^{-9}	Yes	[105]
CoaD (PPAT)	Cycloalkyl pyrimidines	Sa	Target	10^{-7} to 10^{-9}	Yes	[106]
AccA AccD	CPD2	Sa, Ec	Target	$<10^{-9}$	Yes	[107, 108]
AccC	Pyridopyrimidines	Ec tol, Hflu	Target	10^{-8} to 10^{-9}	Yes	[109]
LpxC	Pfizer LPXC-4	Ec, Pa	Bypass, efflux, upcopy	1.5×10^{-8} to 3.4×10^{-8} (Pa)	Yes	[67]
DNA/RNA						
DHFR	Trimethoprim	Ec Sa	Target/ overexpression	10^{-9} to 2.3×10^{-10} in Sa	Yes	[110]
Tmk	Compound 39	Sa	Target	6×10^{-9}	Yes	[111]

DnaE	Nargenicin	Sa, Ec tol	Target	10^{-9}	Yes	[112]
PolC	EMAIPU	Sa	Target	NA[d]	Yes	[113]
Lig	Adenosine analogs	Sa, Hflu	Target	NA[d]	Yes	[114]
RpoB	Rifampicin	Sa, Ec lpx	Target	10^{-7} to 10^{-8}	Yes	[115]
Protein synthesis						
Tuf (Ef-Tu)	LFF571	Sa	Target	1.2×10^{-9} to $<3 \times 10^{-11}$[f]	Yes	[116]
FusA (Ef-G)	Fusidic acid	Sa, Ec tol	Target	10^{-7} to 10^{-8}	Yes	[115, 117]
RplK (L11)	Nocathiacin	Sa	Target	10^{-7} to 10^{-9}	Unk	[118]
Leu-RS	GSK2251052	Ec, Pa	Target	1×10^{-7} to 4×10^{-7}	Yes	[26]
IleRS	Mupirocin	Sa, Ec tol	Target	8×10^{-8}	Topical	[88]
ProRS	PPD-2	Ec	Target	3.3×10^{-9}	Unk	[119]
metRS	REP3123	Sa	Target	10^{-7} to 10^{-8}	Unk	[120]
PDF	GSK1322322	Sa	Bypass	5×10^{-7} to 6×10^{-8}	Yes	[121]
Other						
ClpP	ADEP4	Sa	Target	1×10^{-6}	Yes	[122]
FMN riboswitch	Ribocil	Ec lpx tol	Target	1×10^{-6}	Yes	[123]

[a]Mini spectrum: Active on *E. coli* (Ec), *P. aeruginosa* (Pa), *H. influenza* (Hflu) and permeable strains: tolC *E. coli* (Ec tol), *P. aeruginosa* efflux deletion (Pa effΔ), *E. coli* lpxC (Ec lpx), *E. coli* tolC lpx C (Ec lpx tol)
[b]Reported in vivo activity
[c]Unknown whether compound has in vivo activity
[d]Not available but target mutations obtained
[e]Not available but should be same as fosfomycin
[f]In *C. difficile*

background in which resistance to the second drug was possible. It seems important, but remains to be tested and proven, that combinations to retard resistance development would require matched pharmacokinetics of all drugs in the combination such that there is no time in which coverage is by a single drug only. A fixed dose of all components would be desired for both physician ease-of-use and patient compliance.

Treatment with multiple compounds could have further liabilities: possible increased toxicity, deleterious drug-drug interaction, and direct antagonism due to incompatible mechanisms of action. Antagonism between inhibitors may not be evident at the MIC level but could be seen in reduction in killing. For example, the killing effect of β-lactams is counteracted by drugs that inhibit protein synthesis. Projan has noted [125] that successful drug targets are not necessarily enzymes (which require very strong inhibition) but may be those functions that, if inhibited, interfere in a dominant way with cell viability – that is, they act as poison complexes. He notes that these targets include those whose inhibition leads to "stimulating autolysis, causing protein misfolding, stalling ribosomes on mRNA". I would add to this list DNA breakage, as exemplified by the fluoroquinolones acting on topoisomerases. These activities would still be subject to resistance, but their action might be subject to antagonism by inhibitors of other pathways that prevent those killing activities. Thus the effects of combinations on MBC and on killing must also be taken into account.

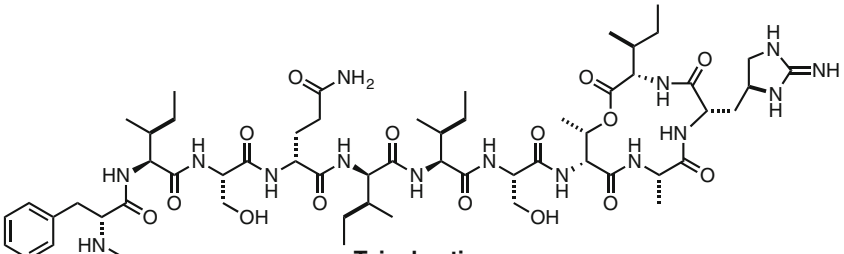
A tendency in considering the use of combinations of antibiotics is to favor combinations synergistic in terms of potency. But for this resistance-prevention use, synergy might add to selective pressure for resistance mutations, as the synergistic increase in activity would be reduced by mutations yielding resistance to one member of the combination. The idea that synergy can select for resistance mutations has been explored by Kishony's group [126]. In fact, that group demonstrated that antagonistic (as opposed to synergistic) interactions can even suppress resistance selection [127, 128]. Thus, choice of drugs in a combination must take into account intracellular drug interactions. Perhaps systems biology can uncover the right combinations of targets to be hit in order to effect sufficient antagonism to retard resistance. On the other hand, "right combinations" could be identified by empirical means.

4 Old Targets new Chemical Matter

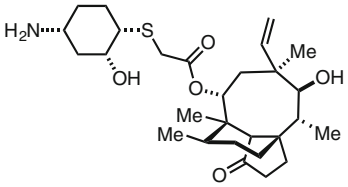
The emphasis in antibacterial discovery since 1995 has been the pursuit of inhibitors of "new targets", previously "unexploited" by marketed drugs. The motivation behind this emphasis is that inhibitors of such targets should not be subject to cross-resistance with drugs in the clinic. While resistance to the drugs in current use is in some cases target-based, it is more often based on the chemical nature of the compound. Further, since much of the history of antibacterial discovery involved empirical (kill-the-bug) screening, all possible killing targets were ostensibly

screened. What resulted was not obviously biased against or for specific targets but, in the case of natural products screening, was biased towards those targets (or receptors) selected by nature. Nevertheless, the advent of whole genome sequencing led to the search for new targets. Criteria for target suitability have traditionally included (1) essentiality to the pathogen, (2) non-homology with mammalian enzymes/targets, (3) "druggability", and (4) low likelihood of cross-resistance with existing classes. This list should be expanded to include (5) preference for an accessible location (see below, text on the entry problem) and (6) low probability of rapid resistance selection. Such rapid resistance can include resistance mutations in the target itself, or the appearance of bypasses and redundancies that might be accessed by regulatory changes or single mutations. As noted above, good antibacterial targets (as identified by successful monotherapeutic systemic drugs) are generally not the product of a single gene. It has been proposed that the already-exploited targets for antibacterials are likely to be privileged and thus are already so-called "multitargets". These targets are different from the 160 or so broad-spectrum essential gene products that have been noted as targets of interest [7]. If we accept that the 30–40 established targets [5] are privileged, it seems reasonable to continue to search for inhibitors of these targets among novel chemical matter with the purpose of finding new inhibitors that are not cross-resistant with old, and that do not themselves select for single-step resistance.

The classical targets worth attacking with new chemical matter include the cell wall pathway, rRNA of ribosomes, Lipid II, and Gyrase/Topoisomerase IV. Targets in the cell wall pathway have been reviewed [129–132]. The penicillin-binding proteins (PBPs) are demonstrated and the Mur-ligases are potential multitargets. Recent work from the Mobashery-Chang group has focused on novel oxadiazole compounds, unrelated to β-lactams, that inhibit PBPs and are active in vitro and in vivo against *S. aureus* including MRSA [133, 134]. Work toward finding developable inhibitors of multiple Mur-ligases has been in progress for many years, notably in the Tomasic group. Reported multi-Mur-ligase inhibitors – for an example, see [135] – have been discovered, but show only very limited whole-cell activity. Lipid II is an intermediate in the synthesis of peptidoglycan and is the target of the glycopeptides. It is the product of a single pathway. No single mutations in pathway members are likely to yield high-level resistance. A new Gram-positive inhibitor, teixobactin, targets both Lipid II and Lipid III, the lipid involved in teichoic acid synthesis. As expected, resistance to teixobactin has not been observed in vitro [136, 137]. Ribosomal targets have been reviewed recently [138] and chapters in this book cover the oxazolidinones and tetracyclines. Novel ribosomal inhibitors in the clinic include the systemic compound lefamulin being developed by Nabriva [139, 140]. Under preclinical study are the 50S subunit targeted ESKAPE pathogen compounds of Melinta therapeutics [141] and the Gram-negative 30S ATI-1503 (a negamycin derivative) of Appili Therapeutics [142].

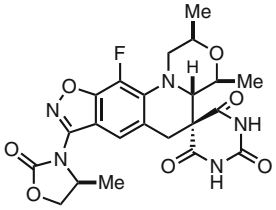


Teixobactin
CAS 1613225-53-8

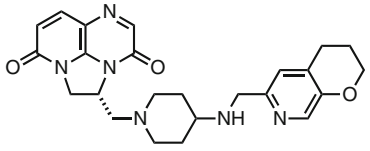


Lefamulin
CAS 1061337-51-6
BC-3781

Probably the most popular set of multitargets are the bacterial type II Topoisomerases, DNA Gyrase and Topoisomerase IV. The fluoroquinolones target the A subunits of both enzymes (GyrA and ParC). Efforts toward finding new inhibitors of these enzymes that target sites separate from those targeted by the fluoroquinolones have been widespread and are addressed [143]. Non-fluoroquinolone Inhibitors of the topoisomerases that are in the clinic include zoliflodacin (ETX0914/AZD0914), a spiropyrimidinetrione being developed by Entasis for treatment of uncomplicated gonorrhea [140, 144]; and gepotadacin (GSK2140944), a triaza-acenaphthylene being developed by GSK for cUTI, uncomplicated gonorrhea and community acquired bacterial pneumonia [140, 145].



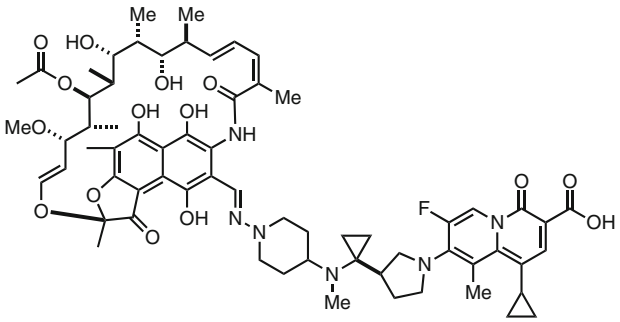
Zoliflodacin
CAS 1620458-09-4
ETX 0914



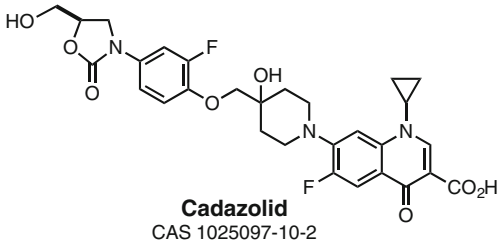
Gepotidacin
CAS 1075236-89-3
GSK 2140944

The concept of conjugating two antibiotics to make a hybrid molecule, active against the targets of each of the components and potentially less subject to resistance than either component, has been explored for many years [146]. One compound, originally discovered by Cumbre Pharmaceuticals, is a hybrid of

rifampicin (an RNA Polymerase inhibitor) and a quinolizinone (a topoisomerase inhibitor, similar to a fluoroquinolone). The compound (TNP-2092) is now under preclinical development by TenNor Pharmaceuticals for the treatment of prosthetic-joint infections [147]. A hybrid of a quinolone and an oxazolidinone, Cadazolid, is being developed by Actelion for treatment of *C. difficil*e-associated disease [58, 140].



TNP-2092
CAS 922717-97-3



Cadazolid
CAS 1025097-10-2
ACT 179811

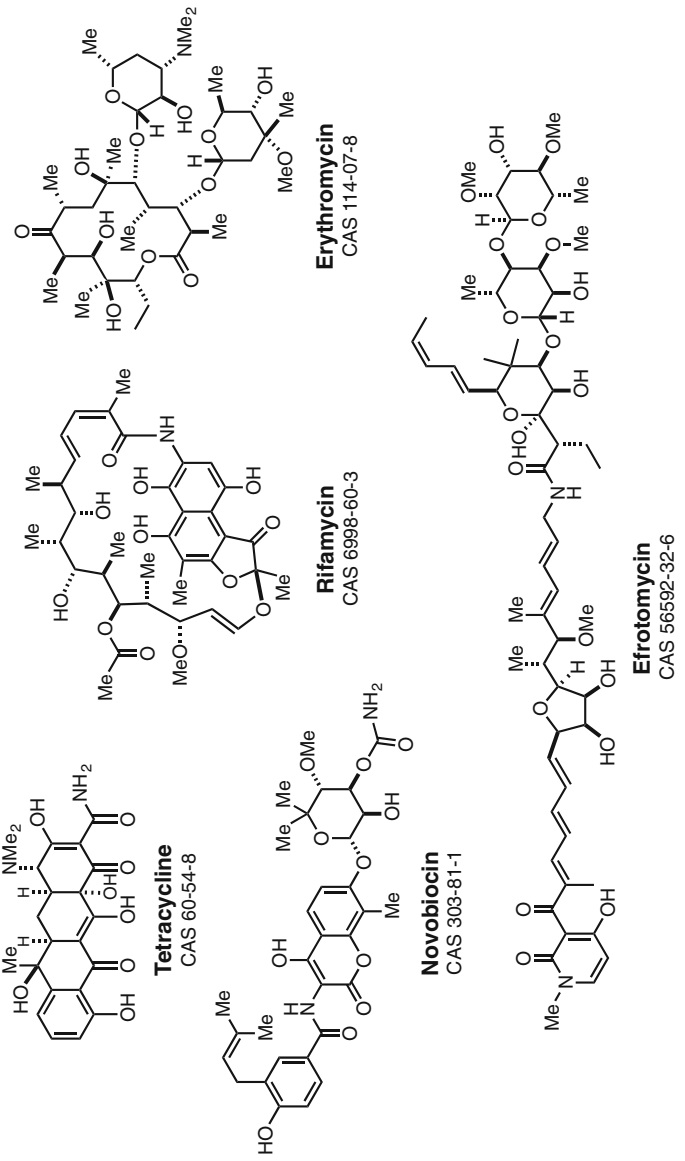
5 The Entry Problem

5.1 Gram Positives: Cytoplasmic Membrane Transit

Some classes of Gram-positive antibiotics, such as the β-lactams and glycopeptides, have extracellular targets and hence do not require membrane passage for activity. However, most of the catalogued potential genomic targets are located in the cytoplasm, as are the targets of many antibiotics in clinical or veterinary use. Obviously, to reach the cytoplasm requires properties that allow permeation of the cytoplasmic membrane. Chemical properties associated with diffusion through membranes have been uncovered, mostly based on studies with mammalian cells, synthetic membranes and liposomes. Diffusion through lipid bilayer membranes is correlated with optima of size and lipophilicity, and it is the neutral species of ionizable compounds that are preferred for entry [148].

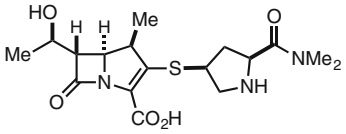
Lipinski and co-authors formulated a set of guides (the Rule of 5 or Ro5) [149] for the properties of drugs that are orally bioavailable due to permeability through intestinal membranes via diffusion. Oral absorption is more likely with compounds that have fewer than 5 H-bond donors, 10 H-bond acceptors, a molecular weight (MW) less than 500 Da, and calculated Log P (CLogP) that is less than 5 [149]. Variations of this rule also specify fewer than 10 rotatable bonds and polar surface area equal to or less than 140 $Å^2$ [150]. These "rules" have heavily influenced the composition and physicochemical characteristics of many/most industrial chemical libraries. Lipinski noted that the outliers (drugs that do not follow the Ro5) among the 2,245 orally active compounds used to generate the rule were antibiotics, antifungals, vitamins, and the cardiac glycosides. He postulated that this result was due to their ability to act as substrates for (intestinal) transporters. Later publications note that "substrates for transporters and natural products are exceptions" to the Ro5 [151, 152]. It has been argued that focus on oral bioavailability in drug discovery and expansion of chemical screening libraries to follow the Ro5 has perhaps retarded the process of drug discovery in general, as has avoidance of natural products and their semisynthetic derivatives, as they form a large percentage of successful drug classes (34% as of 2007) [152]. But the Ro5 may well be useful in identifying compounds capable of diffusion through lipid bilayer membranes in general, even though the lipid composition of membranes can vary greatly from species to species.

Clearly, there are orally-active, cytoplasmically-targeted, often large, natural product antibiotics that are among Lipinski's outliers and do not obey the Ro5, but appear to enter cells by diffusion, without the intercession of active transporters. These include erythromycin, fusidic acid, tetracycline, rifamycin, novobiocin, efrotomycin, and others. Although there may be mammalian transporters for these compounds (as proposed by Lipinski), it appears that these non-Ro5 compounds can diffuse through Gram-positive cytoplasmic membranes. Thus, some natural products have evolved to solve the membrane diffusion problem in ways outside the Ro5. For example, erythromycin and tetracycline [153, 154] although possessing many H-bond donors and acceptors (5 and 7 H-bond donors and 14 and 10 H-bond acceptors for erythromycin and tetracycline, respectively) may undergo intramolecular H-bonding [155]. Furthermore, the pK_a values of these protonatable groups are such that the molecules are ionizable to the extent that neutral species can exist at pH 7.4 in aqueous solution [153], and it is these species that can diffuse through the bilayer. Perhaps further rules could be derived for these larger natural products from QSAR studies of their permeability characteristics.



On the other hand, if molecules do adhere to Ro5, do they automatically enter the cytoplasm? Not necessarily. As an example, meropenem has no Lipinski exceptions but is unlikely to enter the cytoplasm due to its hydrophilicity [156]. It would be useful to formulate rules, or to compile exceptions, that could guide optimization of compounds for Gram-positive entry. Historically, if activity against the isolated cytoplasmic antibacterial target is measurable, then a rough estimate of relative permeation of the cytoplasmic membrane may be obtained for a series of inhibitors

by inspecting the ratio of MIC to IC_{50} or K_i of the inhibitor. However, in the absence of measurable activity against the target (or complete impermeability), obviously no ratio is obtained. Thus, there has not been much work measuring the rate or extent of uptake of a compound in the absence of activity. Recently, a number of methods have been described for this evaluation using liquid chromatography-mass spectrometry (LC-MS) [157, 158] and Raman spectroscopy [158, 159]. As noted below, the measurement of entry into Gram-negatives is complicated by the necessity of determining the compartment of accumulation.



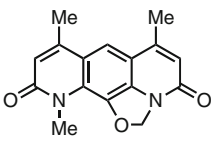
Meropenem
CAS 96036-03-2

5.2 *Gram Negative Entry Barriers*

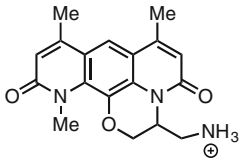
Many reviews have discussed the barrier functions of Gram-negative bacteria [160–164]. In simplest terms, Gram negatives (like Gram-positives) are bounded by a cytoplasmic membrane (CM) but in addition a second "outer membrane" (OM), which consists of an asymmetric bilayer comprising an inner phospholipid leaflet and an outer leaflet of lipopolysaccharide. The sieving properties of these two membranes are largely orthogonal. The cytoplasmic membrane favors passage of neutral lipophilic compounds, while the outer membrane allows passage of small hydrophilic, charged compounds through water-filled protein channels called "porins". This orthogonality makes it problematic to create compounds with physicochemical properties suitable for crossing both barriers. Additionally, powerful efflux pumps can remove compounds from the cytoplasm or periplasm. Their action is synergized by the low permeability of the outer membrane [165]. The variety of pumps and the relative promiscuity of some of them make it difficult to rationally avoid efflux, while allowing accumulation in the cytoplasm and/or periplasm. Clearly, there are compounds that have the right combination of properties to allow cytoplasmic entry by passive diffusion, but that collection of antibiotics arriving in the Gram-negative cytoplasm is quite small (in the low hundreds, representing few chemical classes). Do these compounds share chemical characteristics that enable their entry? The search for rules or guidelines for endowing small molecules with entry ability has been frustrating [164, 166].

Recently, the Innovative Medicines Initiative (IMI) TRANSLOCATION consortium and a number of academic groups have been working on the problem of Gram-negative entry, attempting to dissect the various contribution to barrier function of efflux, porins, and membrane bilayers [162, 167–169]. It may be that there is a set of physicochemical parameters that favor the diffusion of compounds

into the cytoplasm, past the various barriers [163, 170–172]. In a recent article I suggested that the set of compounds which appeared to enter the cytoplasm by diffusion were characterized as having relatively high polarity, with a cLogD between −4 and +2, and MW less than ~500 Da. They are either neutral, or have a net charge of −1 [163]. This analysis must be extended to include more physicochemical descriptors. However, the paucity of compounds known to arrive in the Gram-negative cytoplasm limits meaningful analysis. To approach the problem productively requires methodology to measure the accumulation of a large number of compounds in various compartments by activity-independent means. Published methods for analysis of compound localization include an LC/MS method for measuring accumulation of ciprofloxacin [157] and two single-cell methods, one employing tunable UV excitation combined with light microscopy [173] and another using C_{60}-secondary ion MS [174]. The exquisite sensitivity of LC-MS analysis was applied recently to an ensemble of antibacterial structures to identify the structural similarities among the compounds that accumulate within the *E. coli* bacterium [175]. Accumulation coincided generally with compounds that were rigid, had low globularity, and that paired hydrophobic structure with a sterically unencumbered amine (thus, having positive charge at physiological pH). Application of these principles to the selective Gram-positive gyrase inhibitor, deoxynybomycin (left structure), gave an analog (right structure) now having Gram-negative activity (MIC values for the racemate of 0.5–16 μg/mL, depending on strain). Moreover, the analog retained the Gram-positive activity [175]. The basis for attacking the problem of Gram-negative entry, and proposals for solutions, is reviewed in the Pew Scientific Roadmap for Antibiotic Discovery [176, 177].



Deoxynybomycin
CAS 27259-98-9



5.2.1 Other Routes of Gram-Negative Entry

It seems that some antibiotics do arrive in the cytoplasm by passive diffusion. But other antibiotics use other various routes that could be emulated. Figure 1 summarizes routes of cytoplasmic entry, including the route of transit of the outer membrane by porins and the cytoplasmic membrane by diffusion (Route A in Fig. 1). Many natural product antibiotics can enter the Gram-negative cytoplasm via the active transporters that the cell deploys for the uptake of small, generally hydrophilic, molecules and to which the cytoplasmic membrane is minimally permeable. These natural products may cross the outer membrane through porins, or by use of facilitated diffusion pores (Route B). As described in an earlier section, fosfomycin

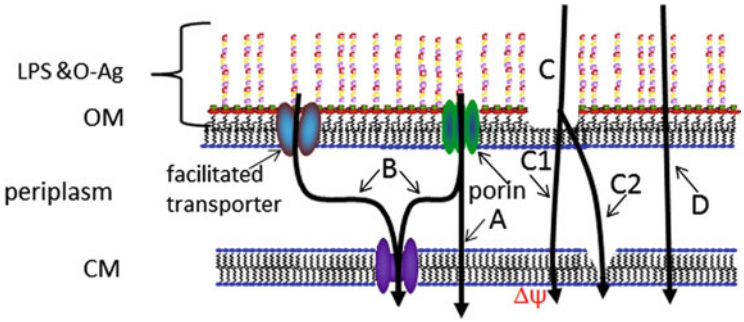
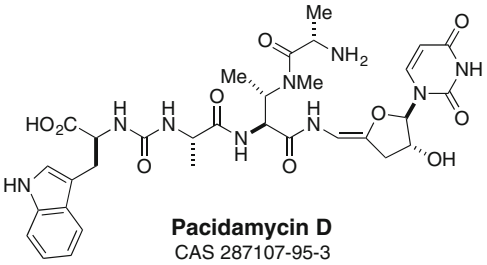


Fig. 1 Routes to the cytoplasm. *A* Entry through OM via porins and diffusion through CM. *B* Diffusion through porins, active transport through CM. *C* Self-promoted uptake of cations through OM; *C1* CM passage via Δψ or *C2* clustering of anionic lipids with passage at cluster-edges. *D* Diffusion of hydrophobic molecules through both OM and CM. *LPS* lipopolysaccharide, *O-Ag O* antigen, *OM* outer membrane, *CM* cytoplasmic membrane

is transported to the cytoplasm by either of two permeases, GlpT or Uhp. Loss of permease function leads to rapid resistance: but, in this case, the resistant mutants are not fit in UTIs. The oligopeptide permease, Opp, can transport pacidamycin, an inhibitor of the MraY enzyme, but high-level resistance to pacidamycin arises at very high frequency via mutations in *opp* [95].



Pacidamycin D
CAS 287107-95-3

Facilitated transport across the outer membrane, notably via siderophore re-uptake mechanisms has been exploited to import into the periplasm antibiotics to which iron-binding moieties such as catechol have been attached. Thus far a number of such efforts have demonstrated rapid resistance development, adaptive or mutational, due to its use of the siderophore uptake mechanism [178, 179]. However, at least two such agents using this method for periplasmic entry are still under active study [180, 181]. It is certainly worth pursuing the avenue of endowing antibiotics with the ability to use natural permeases and uptake pathways, (so-called Trojan horse approaches). Again, care should be taken to critically test for resistance emergence, not only in vitro but also in vivo. Permease loss could affect fitness of resistant mutants in the host and high FoR in vitro might discourage development of a useful compound.

A different route of cytoplasmic entry is by certain cationic compounds which can mediate their own passage through the outer membrane by means of "self-promoted uptake" [182] (Route C) and then cross the cytoplasmic membrane by less well understood mechanisms, such as that promoted by the $\Delta\psi$ component of the proton motive force (Route C1) or via clustering of anionic lipids in the cytoplasmic membrane leading to disruptions at the edge of such domains [183] (Route C2). Aminoglycosides are among the compounds using this route. The precise structural and physicochemical characteristics of such compounds are ripe for study. An understanding of the requirements for their entry could add another set of rules for entry.

Finally, it has been noted that early work showing very poor penetration of the outer membrane by hydrophobic molecules was likely compromised by the existence of efflux pumps, which were unknown at the time. Thus Plesiat and Nikaido have presented data showing that highly hydrophobic molecules can transit the outer membrane bilayer via the "hydrophobic route", not via porins, albeit slowly [184]. Such compounds, if they could avoid efflux, should easily enter the cytoplasm by diffusion.

5.2.2 Make new Gram-Negative Libraries

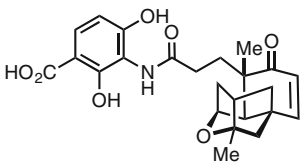
If chemical rules can be derived for any routes of entry, it should, from this microbiologist's point of view, be possible to create chemical libraries with the desired characteristics within which to screen for antibacterial activity, whether phenotypically or against in vitro targets. However, compounds in the cLogD range of -4 to $+2$ are poorly represented in corporate compound libraries, as this cLogD range does not match that for eukaryotic drug targeting. Initially such compounds might be gleaned from existing libraries industrial and commercial sources.

6 Natural Products

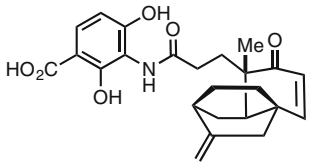
In the foregoing sections I've highlighted the target and entry problems which I feel are pre-eminent and which, if surmounted, will lead us to future antibiotics. Now let us address briefly the old source, natural products (NPs). But first a caveat: it is easy to kill bacteria with agents both synthetic and natural, but it is rare to find compounds whose toxicity is sufficiently selective to produce an acceptable therapeutic index. Microbial NPs have indeed been the source of the majority of our antibiotics, and were discovered for the most part by empirical means. While penicillins are indeed fungal products, the β-lactamase synthetic genes were apparently imported from Actinomycetes [185], which are the major source of antibacterials and in my opinion should remain the preferred producers to be exploited. Antibacterial agents are produced by many Kingdoms, but I would propose that the bulk of non-bacterial NPs are generally toxic to species unrelated to the producer,

while bacteria produce at least some antibiotics that are more selectively toxic, perhaps so the producers can co-evolve resistance mechanisms.

The value of NPs lies in the possibility of novel chemistry that has evolved through selection by forces not well understood by us to create optimized ligands for receptors we are left to define. One may regard NPs as just another chemical library, but a library incorporating chirality, fused rings, 3-dimensionality, and polar nitrogen and oxygen-containing functional groups. In short, a library that can be used to identify inhibitors of one's favorite antibacterial target. On the other hand, commercial development requires that structural novelty is found amidst the commonly found antibiotics. The process of narrowing the hits to those hits having novelty is called dereplication. It is the rate-limiting step of the process of NP antibacterial discovery. Dereplication has been addressed traditionally by biological and chemical methodologies [186–189] and also by the use of hypersensitive-phenotypic screens [190–193]. Hypersensitive screens are whole-cell screens designed to detect active compounds at concentrations below the MIC. Numerous recent reviews have addressed new methodologies for detection and discovery of novel antibiotics, especially by genome mining and by accessing previously unculturable producing organisms [194–199]. The subject is too vast for further discussion here, except to note the discovery of teixobactin, a novel inhibitor of Lipid II and III, through screening of unculturable organisms [136, 137] and of platensimycin and platencin [200, 201], by hypersensitive screening for inhibitors of FabF. They have not been developed as yet, but they represent the possibility of finding novelty among NPs.



Platensimycin
CAS 835876-32-9



Platencin
CAS 869898-86-2

7 Conclusion

Recently, the recognition of increasing antibiotic resistance has led to strong interest in the area of antibacterial discovery, yielding many policy statements from governments and NGOs promoting the need for discovery of new antibacterials to replenish the pipeline. This recognition has spurred increases in funding and a variety of efforts, mostly in academe and small companies, toward discovery of new ways to attack resistant pathogens. While many such efforts have been directed toward improvements to old antibiotic classes, there are inroads into novel areas, some new targets and new chemistry. This progress is illustrated by the recently revealed first round of funding by the CARB-X initiative supporting preclinical

development of a variety of molecules which include "3 potential new classes of antibiotics, 4 innovative non-traditional products and 7 new molecular targets" [202]. In this chapter, rather than reviewing in detail the current discovery programs (as appear in the literature, at meetings, and on websites) I have instead approached the subject of future antibiotics by noting the scientific problems that must be overcome in order to produce new successful drugs. While I have trodden this ground repeatedly in previous reviews (copiously referenced herein), it has been my experience that defining the problem is the important first step to solving the problem. I have emphasized the need to address the probability of rapid resistance development early on in the discovery process, preferably at the stage of target choice. I view the optimization of combinations of single-target inhibitors as a path to readdressing target-based discovery in the antibiotic field. Additionally, the problem of entry of compounds into the bacterial cytoplasm, especially of Gram-negatives, must be solved in order to fully address the need for new Gram-negative agents. Finally, I believe antibacterial natural products still offer a wealth of novel possibilities, which should be addressable by new methods along with hypersensitive screening. The future of antibiotics lies in addressing these obstacles.

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Synergistic Antibiotic Combinations

Karen Bush

Abstract Synergy between antibiotics is a strictly defined microbiological phenomenon, requiring two bioactive agents to exhibit enhanced bacterial killing when the two are combined. Because of increasing antibiotic resistance, and few new drugs to treat multidrug-resistant bacteria, combination therapy is often used in the clinical setting. Frequently, these combinations have demonstrated synergistic activity both in vitro and in animal models before being used therapeutically. Antibiotic combinations are more likely to be used in patients with drug-resistant staphylococcal or enterococcal infections, as well as in patients whose diseases are caused by carbapenem-resistant *Enterobacteriaceae*, *Pseudomonas aeruginosa*, or *Acinetobacter* spp. Although well-defined combinations have been approved by regulatory authorities as single agents, such as trimethoprim–sulfamethoxazole or β-lactamase inhibitor combinations, many combinations are used empirically with no clinical data to support their use. Because combination therapy will continue to be used in the absence of supportive clinical data, it will be important in the future to investigate mechanistic principles that may lead to predictive models for successful patient outcomes.

Keywords Antibiotic, Combination, Multidrug-resistant, Resistance

Contents

K. Bush (✉)
Indiana University Bloomington, Bloomington, IN 47405, USA
e-mail: karbush@indiana.edu

1 Introduction

Bacterial infections occur every day, in every country, in every age group, in every ethnic population. For thousands of years the human race has struggled to combat these infections, with limited success. The introduction of the sulfonamides in the 1930s [1], followed shortly thereafter by penicillin [2] and aminoglycosides [3], began to make the world complacent about the ability to overcome bacterial disease. However, following the use of these antibiotics, resistance arose more rapidly than expected [4], beginning with yearly increases in penicillin resistance in staphylococci in the 1940s [5, 6]. As novel resistance mechanisms to all antibiotics continue to emerge, resistant bacteria are becoming one of the most critical threats to human health worldwide. According to the Centers for Disease Control and Prevention (CDC) "Antibiotic resistance has been called one of the world's most pressing public health problems" [7]. In 2016 the United Nations issued a declaration addressing antibiotic resistance, with the UN Secretary General stating that "Antimicrobial resistance (AMR) poses a fundamental, long-term threat to human health, sustainable food production and development" [8].

In response to these concerns, the CDC provided a listing of those antibiotic-resistant pathogens deemed to be serious or urgent threats to human health in 2013 [9]. Among the most prominent are the urgent threat of carbapenem-resistant *Enterobacteriaceae* (CRE) and the serious threats of multidrug-resistant (MDR) *Pseudomonas aeruginosa*, *Acinetobacter* spp., and methicillin-resistant *Staphylococcus aureus* (MRSA) [9]. This document was followed in 2017 by the World Health Organization (WHO) report identifying the three most critical priorities as carbapenem-resistant *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacteriaceae*, including extended-spectrum β-lactamase (ESBL)-producing as well as carbapenemase-producing isolates [10]. The WHO noted specifically that *Mycobacterium tuberculosis* was not included because it had already been identified as a global priority for which new treatments are urgently needed.

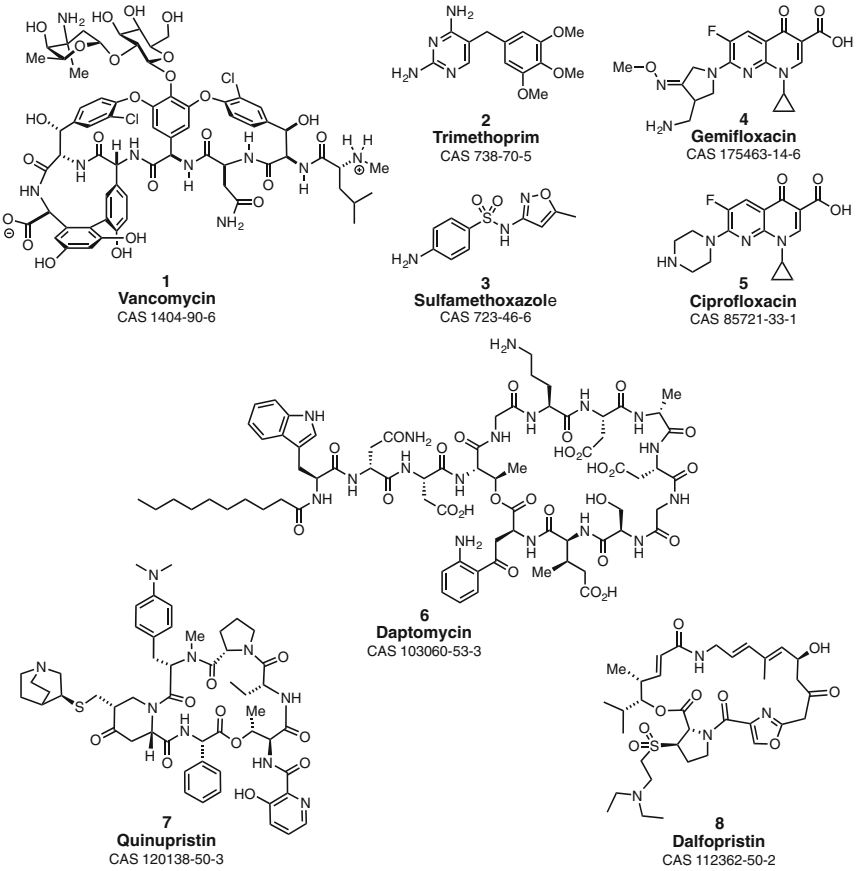
As the race between man and bug continues, fewer therapeutic options remain for the treatment of antibiotic-resistant infections. Although novel antibacterial drugs are both in development and have been introduced recently to the market, many of these agents encounter resistance within a short time following their introduction. Even with the new agents, monotherapy may not be sufficiently effective to treat serious infections caused by pathogens that are multidrug- or pan-resistant [11]. As a result, physicians have been relying on combination therapies to address these issues. Curiously, in a large meta-analysis of 12 recent

clinical studies that enrolled 3,571 patients treated empirically for ventilator-associated pneumonia (VAP), no statistical difference was observed in outcomes in patients treated with monotherapy compared to those who received combination therapy when following the American Thoracic Society guidelines [12]. However, these studies did not look at a subpopulation of patients with infections caused by MDR pathogens. Many experts believe that combination therapy should always be used as empiric therapy against MDR infections, especially when caused by CRE, *P. aeruginosa,* or *Acinetobacter* spp. [13]. This approach is based on the well-established principles used in the treatment of tuberculosis where monotherapy is never indicated due to the rapid selection of resistance, and new combinations of drugs are continually being tested in the clinic [14]. In addition to the enhanced antibacterial effects that can be gained by using more than one agent, combinations of known antibiotics may have the potential to reduce selection of resistance [15–17]. However, a recent study by Vestergaard et al. showed that the combination of ciprofloxacin and ceftazidime, when tested in vitro against *P. aeruginosa*, tended to select for broad-spectrum resistance due to mutational inactivation of *mex*R, the repressor gene that regulates expression of the multidrug efflux pump MexAB-OprM [18].

In this chapter, combinations of antibacterial agents are discussed using as the primary focus the literature from 2015 to 2017 describing combinations of agents shown to demonstrate microbiological synergy against pathogens of serious medical concern. The emphasis is on combinations of approved antimicrobial drugs, rather than proposed combinations with investigational agents such as the addition of the novel oxadiazoles to potentiate the activity of β-lactams against MRSA [19], or with agents that do not possess antibacterial activity. The use of adjuvants to improve pharmacological properties of an active agent is not covered.

2 Microbiological Synergy

Synergy is a well-defined concept in microbiological terminology. It is defined as the inhibition of microbial growth by two bioactive agents that exhibit a positive interaction [20]. According to a consensus in the clinical microbiology field [20–22], drug combinations may act in "synergy," may show "antagonism," or may have "no interaction" or "indifference." Investigators may test for synergism in vitro using checkerboard assays in which the concentrations of the two drugs are varied in a two-dimensional array and minimum inhibitory concentrations (MICs) are recorded. Although disk-diffusion synergy testing has also been described as a method to test for synergy, Sy et al. showed that broth dilution assays were more predictive of synergy than assays using disk diffusion, based on validation in time-kill studies of the combination of vancomycin (**1**) (Scheme 1) and β-lactam antibiotics against MRSA [23]. Most microbiologists validate checkerboard synergy results by monitoring the microbial growth of the target organism in the presence of each agent alone and in combination over a 24 h period



Scheme 1 Structures of the glycopeptide vancomycin; the folate antagonists trimethoprim and sulfamethoxazole; the fluoroquinolones gemifloxacin and ciprofloxacin; the cyclic lipopeptide daptomycin, and the synergistic inhibitors of the streptogramin-class, quinupristin–dalfopristin

in time-kill studies. Interpretations of the results from checkerboard assays are calculated using the "fractional inhibitory concentration" (FIC) index (FICI), as shown in the following consensus agreed upon in the early 2000s.

FIC = MIC for drug in combination/MIC for drug alone
FICI = FIC for drug A + FIC for drug B

FICI $\leq$ 0.5, Synergy
FICI $>$ 0.5–4.0, No interaction/nonsynergistic/nonantagonistic
FICI $>$ 4.0, Antagonism

These interpretations were accepted in order to avoid terms such as "additivity," "indifferent," or "partial synergy" that were previously used to describe data ranging from 0.5 to 4.0, within experimental error of an FICI value [20]. MIC

values that are determined in assays using drug concentrations in a series of twofold dilutions exhibit an inherent reading error of +/− one doubling dilution. Thus, valid data may fall into a fourfold range of being accurate, e.g., an MIC of 1 μg/mL may actually be 0.5 or 2 μg/mL and experimental variations of one twofold dilution for the MICs for each drug would give FICI values that remained in the "no interaction" range.

Data from killing curves, or time-kill assays, also have strict definitions for the interpretation of synergy, and are often used to validate synergistic combinations identified from checkerboard assays [24]. In these studies bacteria growing in log phase are incubated in media containing each drug alone or the drugs in combination and compared to a growth control that has no drug added to the medium. The concentration of at least one of the drugs should be low enough so as to not affect the growth of the organism when tested alone. At selected time points aliquots are removed and colony forming units (CFUs) are counted. Synergism is observed if these two criteria are met: [1] a decrease of at least 2 $\log_{10}$ CFU/mL is observed compared to the CFU/mL of the more active drug after 24 h; and [2] the final bacterial count at 24 h must be at least 2 $\log_{10}$ CFU/mL lower than the starting inoculum.

3 Approved Antibiotic Combinations

3.1 Folate Pathway Inhibitors

Relatively early in the history of antibiotic development, trimethoprim (**2**), a dihydrofolate reductase (DHFR) inhibitor, was shown to potentiate the activity of sulfonamide drugs that block the conversion of *p*-aminobenzoic acid into dihydrofolic acid [25]. Combination of trimethoprim with sulfamethoxazole (**3**) results in broad-spectrum, synergistic, bactericidal activity against a wide range of pathogens, which include MRSA, streptococci, *E. faecalis*, *Neisseria* spp., and many enteric bacteria. This combination is a well-prescribed and orally active therapy for the treatment of common infections such as urinary tract infections (UTI) and otitis media, particularly in patients with allergies to other antibiotics [26]. Although strong synergy is observed in vitro for the combination, clinical practice suggested that this synergy did not always carry over to the treatment of patients [27], except for the treatment of toxoplasmosis, brucellosis, nocardiosis, chancroid, and pneumonia due to *Pneumocystis carinii* [28]. Resistance to trimethoprim can occur as a result of several different events, including the acquisition of a plasmid encoding a DHFR that confers high-level resistance [29]. Unfortunately, the use of the combination did not tend to reduce the emergence of trimethoprim resistance, but trimethoprim appears to reduce the incidence of sulfonamide resistance [28]. Triple combinations including trimethoprim–sulfamethoxazole have also been considered. Gemifloxacin (**4**) in combination with trimethoprim–

sulfamethoxazole has demonstrated synergistic bactericidal activity against community-acquired-MRSA (CA-MRSA) in both time-kill studies and animal models [55]. Combinations of vancomycin or ciprofloxacin (**5**) tested in vitro in time-kill assays with trimethoprim–sulfamethoxazole were also synergistic against vancomycin-intermediate *S. aureus* (VISA) or heterogeneous vancomycin-intermediate *S. aureus* (hVISA) [56]. In a clinical study (Table 1), the combination of daptomycin (**6**) and trimethoprim–sulfamethoxazole resulted in microbiological cures of 24 of the 28 patients infected with either daptomycin-susceptible or daptomycin-resistant MRSA; 17 of the 17 isolates that could be recovered demonstrated synergistic behavior in time-kill assays [54].

3.2 Streptogramins

Quinupristin–dalfopristin represents the only approved streptogramin combination. Quinupristin (**7**) is a cyclic depsipeptide analog of the naturally occurring pristinamycin IA, and dalfopristin (**8**) is a polyunsaturated cyclic macrolactone derivative of the natural product pristinamycin IIA, all members of the streptogramin family [57]. Combined in a molar ratio of 30:70, the combination has potent synergistic activity against Gram-positive bacteria, including MRSA and MDR-streptococci. In contrast to the behavior of many agents with Gram-positive activity, *E. faecalis* demonstrates intrinsic resistance due to production of an ABC (ATP-binding cassette) homologue, Lsa(A), whereas the generally more resistant *E. faecium* is naturally susceptible. Resistance to the streptogramins in *E. faecium* has been reported both in vitro and in vivo due to production of a variant of Lsa (A) with a point mutation [58]. Over time, the drug combination has not been used extensively in the clinic, due in part to a relatively high incidence of localized phlebitis during infusion, and observed elevations in serum aminotransferase levels in a small percentage of patients [57].

3.3 β-Lactamase Inhibitor Combinations

Probably the most commonly used antibiotic-combination therapy involves the addition of a β-lactamase inhibitor (BLI) to a β-lactam that is labile to hydrolysis by β-lactamases. Prescribing information reported for the years 2004–2014 shows that 65% of all United States hospital prescriptions are for β-lactam antibiotics, and of these over half are for BLI combinations [59]. Because this set of combinations has been reviewed extensively in the past few years [59–61], particularly with respect to newer combinations in clinical development, the following discussion is centered on FDA-approved BLI combinations (Scheme 2).

Beginning in 1986 and proceeding until 2014, three BLIs were approved for therapeutic use, with all the inhibitors matched with a penicillin counterpart:

Table 1 Empiric antibiotic combinations using FDA-approved antibacterial agents

Antibiotic	Antibiotic in combination	Organism affected	Studies to support synergy	Reference
Colistin	Azithromycin	*Acinetobacter baumannii*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*	Time-kill curves	[30]
	Chloramphenicol	*Klebsiella pneumoniae*	Time-kill curves	[31]
	Doripenem	*Pseudomonas aeruginosa*	In vitro, hollow fiber studies	[17]
	Rifampin	*Acinetobacter baumannii*	Checkerboard; time-kill curves	[32]
	Tazobactam	*Acinetobacter baumannii*	Time-kill curves	[33]
	Tigecycline	*Acinetobacter baumannii*, CRE[a], *Klebsiella pneumoniae*	Checkerboard; time-kill curves clinical data	[34–37]
	Vancomycin	*Acinetobacter baumannii*	Checkerboard; time-kill curves	[32]
Daptomycin	Ceftaroline	MRSA	Bacteremic patients	[38]
	β-Lactams	MRSA, enterococci	Checkerboard; time-kill curves	[39–41]
	Dalbavancin	MRSA	Checkerboard	[42]
	Gentamicin	MRSA, enterococci	Checkerboard; time-kill curves (variable results)	[40, 43–45]
	Linezolid	MRSA	Checkerboard	[42]
	Sulbactam, tazobactam	MRSA, hVISA, VISA[b]	Time-kill curves	[33]
	Tigecycline	MRSA	Checkerboard; time-kill curves, surgical site infection model	[46]
Levofloxacin	Linezolid	*Bacillus anthracis*	Synergy in checkerboard against Sterne strain; indifference or antagonism in models	[47]
Vancomycin	β-Lactams	MRSA	16 studies based on in vitro and in vivo animal models	Summarized in [43, 48, 49]
	Ceftaroline	MRSA	In vitro PK/PD model; six clinical case studies	[50, 51]
	Flucloxacillin	MRSA	Bacteremic patients	[48]

(continued)

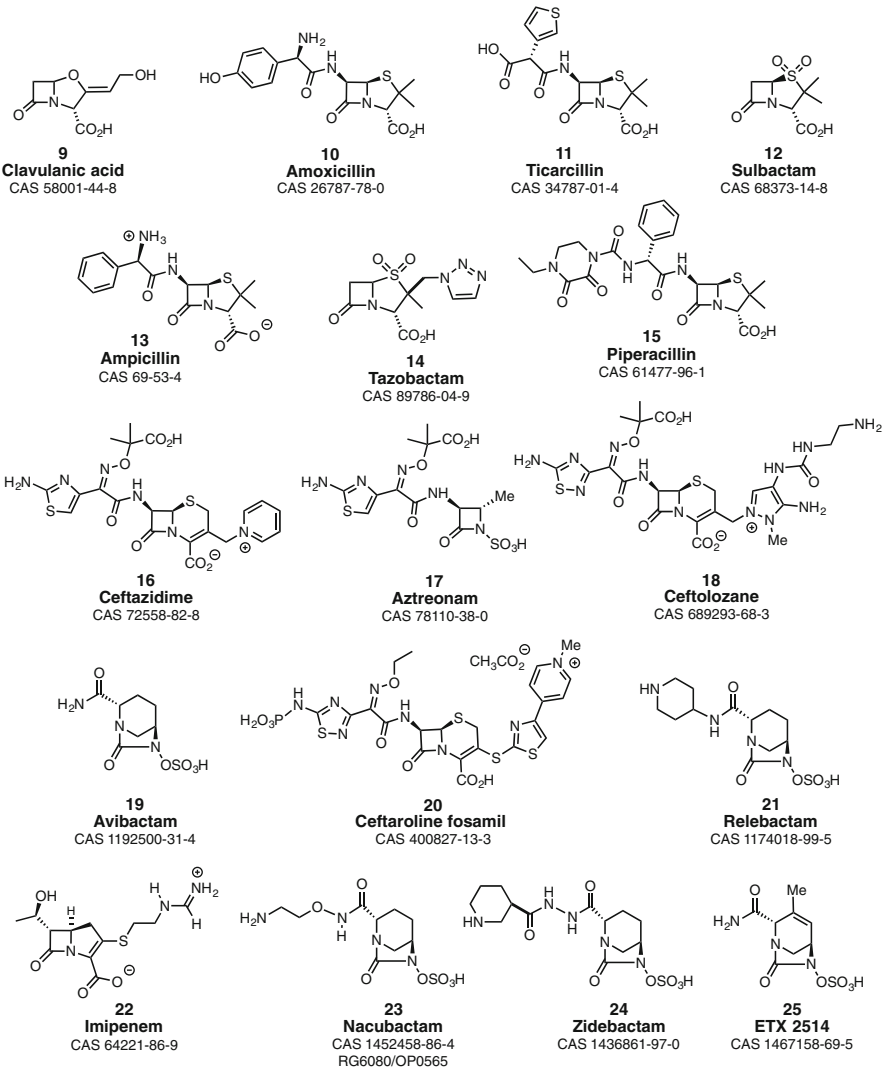
Table 1 (continued)

Antibiotic	Antibiotic in combination	Organism affected	Studies to support synergy	Reference
	Gentamicin	MRSA	Checkerboard; time-kill curves; not supported by clinical studies	[52, 53]
	Trimethoprim–sulfamethoxazole	MRSA	Clinical studies in daptomycin-resistant patients	[54]

[a]*CRE* carbapenem-resistant Enterobacteriaceae
[b]*MRSA* methicillin-resistant *Staphylococcus aureus*, *hVISA* heteroresistant vancomycin-intermediate *S. aureus*, *VISA* vancomycin-intermediate *S. aureus*

clavulanic acid (**9**) with amoxicillin (**10**) or ticarcillin (**11**); sulbactam (**12**) with ampicillin (**13**); and tazobactam (**14**) with piperacillin (**15**). These inhibitors act as suicide inactivators with inhibitory activity against class A penicillinases and broad-spectrum β-lactamases that do not hydrolyze carbapenems or β-lactams with aminothiazole oxime side chains such as ceftazidime (**16**) or aztreonam (**17**) [62]. These BLI-penicillin combinations had broad-spectrum bactericidal activity against MSSA, streptococci, and enteric bacteria. Piperacillin–tazobactam also was efficacious against pseudomonal infections, primarily due to the antipseudomonal activity of piperacillin. The three BLIs were developed during the time that ESBLs were not known (clavulanic acid and sulbactam combinations), or during the time that ESBLs were considered to be rarities in clinical practice (piperacillin–tazobactam). However, that situation changed during the late 1990s when ESBLs became global problems. Although the inhibitors usually demonstrated inhibitory activities against most ESBLs when tested in vitro in isolated enzyme assays, they fell down in efficacy when the combinations were tested against ESBL-producing organisms that harbored additional β-lactamases. As early as 2000 in Canada, 71% of organisms that produced an ESBL were reported to produce at least one other β-lactamase, resulting in <31% susceptibility to either amoxicillin-clavulanic acid or piperacillin–tazobactam [63]. In addition to the ESBLs, the emergence of carbapenemases in the early 2000s posed additional problems for the inhibitors; none of the inhibitors affected the activity of metallo-β-lactamases (MBLs), and had poor activity when tested in penicillin combinations in whole cell assays with organisms that produced serine carbapenemases such as the KPC enzymes [64], in spite of comparable tazobactam concentrations that effectively inhibited either isolated KPC or broad-spectrum TEM enzymes [65, 66]. Notably, almost all carbapenemase-producing organisms also produce additional β-lactamases in a similar manner as seen with the ESBLs [67], thus exacerbating the situation.

In an attempt to address the decreased response to BLI combinations in ESBL-producing organisms, in 2014 the FDA approved the combination of ceftolozane (**18**), a potent antipseudomonal cephalosporin, with tazobactam, using a different tazobactam dosing regimen from that used for the piperacillin–tazobactam



Scheme 2 Structures of the penicillin β-lactams amoxicillin, ticarcillin, ampicillin, and piperacillin; the cephalosporins β ceftazidime and ceftolozane; the anti-MRSA cephalosporin pro-drug ceftaroline fosamil; the monobactam aztreonam; the carbapenem imipenem; the classical β-lactamase inhibitors clavulanic acid, sulbactam, and tazobactam; and the non-classical DBO-class β-lactamase inhibitors avibactam, relebactam, nacubactam, zidebactam, and ETX2514

combination to allow for more favorable pharmacodynamics [68, 69]. Although the combination had high susceptibility rates when tested against *E. coli* producing a single CTX-M-14 or CTX-M-15 ESBL [70], the agent is probably most useful as an antipseudomonal drug, exhibiting >90% susceptibility in contemporary meropenem-resistant *P. aeruginosa* isolates [71].

The argument may be made that these combinations do not fit the classical definition of synergism, in that a β-lactamase inhibitor is not necessarily considered to have antibacterial activity in its own right. However, many BLIs are known to bind to essential PBPs and may exhibit at least some weak growth inhibition. Examples include clavulanic acid with MICs as low as 0.1 μg/mL for *Neisseria gonorrhoeae* and 6.3 μg/mL for *Haemophilus influenzae* [72] and sulbactam that inhibits PBPs 1 and 3 in *A. baumannii*. [73]. Some of the more recent BLIs have even greater antibacterial activity on their own, thereby qualifying as legitimate synergistic agents when combined with a companion β-lactam.

In 2015 the FDA and EMA approved the combination of ceftazidime with avibactam (**19**), a non-β-lactam BLI with weak antibacterial activity due to binding to PBP4 in *S. aureus* and PBP2 in Gram-negative bacteria [74]. Although avibactam MICs as low as 4 μg/mL against *E. coli* have been reported, MICs >64 μg/mL against non-enteric bacteria and *S. aureus* have been also detected [74, 75]. Avibactam is a potent, covalent, reversible inhibitor of most class A, C, and D β-lactamases [76, 77], and an irreversible inhibitor of the KPC-2 carbapenemase [77]. Combinations of avibactam at subinhibitory concentrations were capable of potentiating ceftazidime such that MICs could be reduced as much as 1,000-fold in enteric bacteria producing KPC and/or ESBL enzymes [78, 79]. Avibactam may also be combined with the anti-MRSA cephalosporin ceftaroline (**20**), potentially to provide efficacy against mixed infections that include Gram-positive pathogens as well as ESBL- or KPC-producing Gram-negative bacteria [80]. Avibactam is also being studied in combination with aztreonam (a monobactam with stability against MBL hydrolysis) in Phase 2 clinical studies (https://clinicaltrials.gov/ct2/home), thus potentially providing at least some coverage of MBL-producing organisms [81].

After confirmation of the potent β-lactamase-inhibitory activity of avibactam, its diazabicyclooctane (DBO) structure was modified extensively by medicinal chemists at multiple pharmaceutical companies to provide "second generation" DBO derivatives such as relebactam (**21**), being developed in combination with imipenem (**22**) [82]. Some of these newer DBOs have enhanced antibacterial activity, such as nacubactam (RG6080/OP0595) (**23**) [83], zidebactam (**24**) [84], or ETX2514 (**25**) [85], again due to binding to PBP2 to provide a dual mechanism of action in Gram-negative bacteria. The combination of the PBP2-binding inhibitors with cephalosporins or monobactams has been proposed to offer a selective advantage in terms of the emergence of resistance [83]. The high affinity of these β-lactams for PBP3 drives their antibacterial activity, so it is possible that resistance due to target modifications will require mutations in both PBP2 and PBP3 to achieve high-level resistance. However, in the short time in which the newer combinations have been used clinically, resistance has been reported in patients treated with ceftazidime–avibactam. Mutations have been reported in KPC-3 with multiple point mutations in different patients [86], conferring resistance to avibactam combinations, but restoring susceptibility to meropenem. Mutations in *E. coli* PBP3 have also been reported in historical clinical isolates, appearing as

gene duplications resulting in four amino-acid insertions that result in 4- to 32-fold increases in MICs for ceftazidime, as well as for ceftaroline and avibactam [87, 88].

4 Empirical Antibiotic Combinations

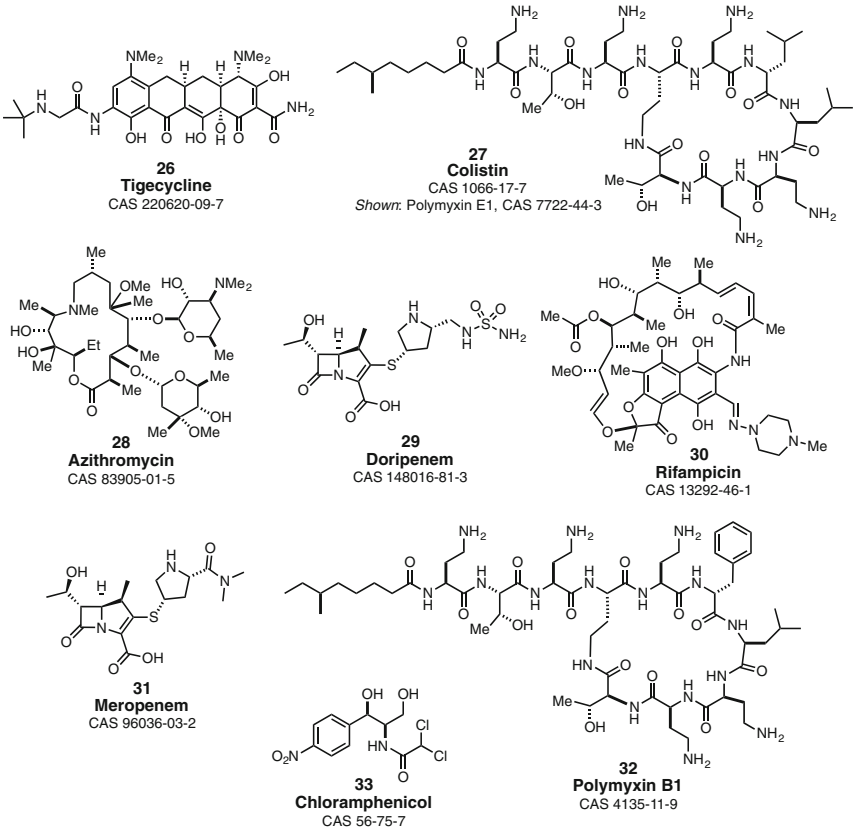
Empirical antibiotic combinations refer to combinations used clinically in the absence of an approved indication by a regulatory agency. In vitro microbiological synergy data may exist to support the use of combination, but there are few, if any, controlled clinical trials that support the use of these agents as effective therapies. These combinations are listed in Table 1.

4.1 Gram-Negative Infections

Gram-negative bacteria are frequently named among the greatest threats to human health [9, 10]. Among the most worrisome are the nonfermentative bacteria *Acinetobacter* spp. and *P. aeruginosa*, as well as the carbapenem-resistant Enterobacteriaceae (CRE). Combination therapy is frequently recommended for initial empiric treatment of patients infected with these organisms [13, 89]. In vitro studies have even suggested that two carbapenems may be synergistic against KPC- or OXA-48-producing Enterobacteriaceae [90]. Limited clinical data based on retrospective data have suggested that a carbapenem (meropenem) in combination with another sensitive drug may be successful in treating CRE infections if the meropenem MIC was <8 μg/mL [91]. In another set of retrospective clinical data from 26 published studies, CRE-infected patients treated with a tigecycline (**26**) combination were statistically more likely to have lower mortality, both in the ICU and at a 30-day follow-up evaluation, compared to patients treated with monotherapy [34] (Scheme 3).

Antimicrobial peptides, especially colistin (**27**), a member of the polymyxin class, have become drugs of last resort for the treatment of infections caused by MDR Gram-negative pathogens. However, liabilities associated with colistin are the perception of increased nephrotoxicity compared to the β-lactams and macrolides, and increasing colistin-resistance [17, 30]. For this reason, combinations with other, safer antibiotics have been examined. One of the more unusual combinations involves a colistin-azithromycin (**28**) duo that demonstrated synergistic activity by time-kill assays against *A. baumannii*, *K. pneumoniae,* and *P. aeruginosa*. The macrolide with poor antibacterial activity against Gram-negative bacteria is presumed to be synergized due to the membrane-permeabilizing properties of colistin [30].

Other colistin combinations that have been studied in vitro include combinations with β-lactams against *A. baumannii* (tazobactam) [33] and *P. aeruginosa* (doripenem) (**29**) [17]. In the latter combination, suppression of resistance was



Scheme 3 Structures of the glycylcycline tigecycline; the polymyxins E1 (primary structural component of colistin) and B1; the macrolide azithromycin; the carbapenems doripenem and meropenem; the rifamycin-class, rifampicin; and chloramphenicol

noted for both colistin and doripenem [17]. Against *A. baumannii*, colistin also synergized the antibacterial activity of rifampicin (**30**) or vancomycin [32] or tigecycline [35, 36]. The latter combination also demonstrated synergy against *K. pneumoniae*, both in vitro and in clinical studies [37]. Triple combinations of colistin with meropenem (**31**) and tigecycline demonstrated synergistic activity against MDR *K. pneumoniae*, but it was no greater than that observed with double colistin combinations with either agent alone [92]. Similar to the observed behavior with colistin, polymyxin B (**32**) was able to synergize the activity of chloramphenicol (**33**) in vitro against MDR *K. pneumoniae* [31]. However, the additive possibilities for toxicity probably do not warrant serious consideration for clinical usage [93]. Clinical data to support combinations therapy to treat infections caused by MDR Gram-negative bacteria are still sparse, especially with regard to controlled trials [34, 89], and further efforts to correlate in vitro synergy with clinical outcomes are needed.

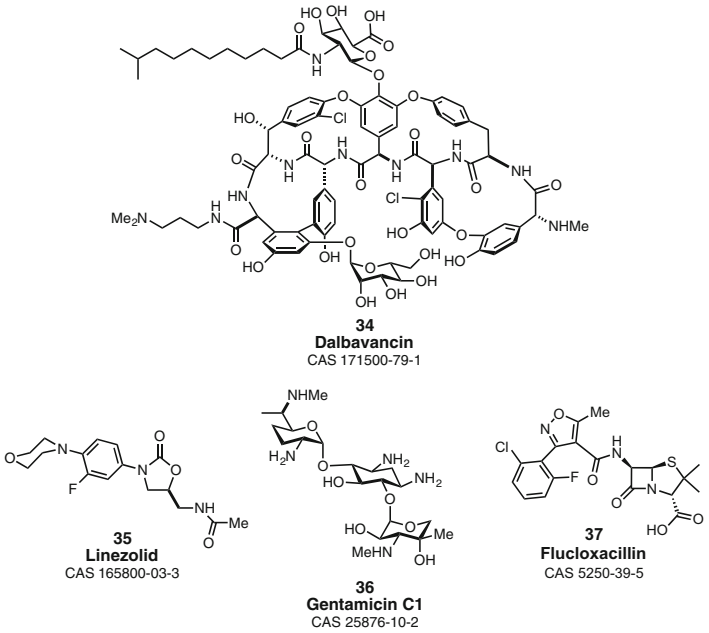
4.2 Gram-Positive Infections

Combination therapy for treatment of Gram-positive infections has been discussed extensively in the literature, with recent reviews tackling the issue with respect to MDR infections caused by MRSA [43] and vancomycin-resistant enterococci (VRE) [94]. Combinations that have been studied either in vitro or in clinical studies are summarized in Table 1. The anti-MRSA cephalosporin ceftaroline has been used successfully as a companion to vancomycin, based on in vitro pharmacodynamic studies and on retrospective case reports [50, 51]. Ceftaroline in combination with daptomycin to treat 20 patients with MRSA bacteremia resulted in a shortened time to eradication compared to standard therapy; in addition, the combination caused a sensitization to bacterial killing by neutrophils [38]. Mechanistically ceftaroline has been shown to bind to a specific allosteric site as well as the active site of PBP2a in MRSA, thus allowing for the possibility of ceftaroline allosteric binding to enhance the binding of other β-lactams to the active site [95].

Daptomycin combinations with drugs in various antibacterial classes have also been studied. Synergy has been observed in time-kill studies with daptomycin combinations containing sulfone-containing β-lactamase inhibitors against MRSA [33, 96] or for combinations with other β-lactams [39–41], especially β-lactams such as meropenem and imipenem that bind preferentially to PBP1 [39]. The combination of daptomycin with dalbavancin (**34**), molecules with similar chemical structures and functions with respect to bacterial killing, was synergistic for MRSA using checkerboard assays; likewise, the protein synthesis inhibitor linezolid (**35**) was also synergistic in the same study [42]. For the synergistic daptomycin-gentamicin (**36**) combination, resistance rates were lower in vitro when tested against MRSA [44]. The daptomycin–tigecycline was synergistic in vitro in checkerboard and time-kill assays, in addition to a surgical site infection model [46] (Scheme 4).

Other antibiotics that kill bacteria by interfering with cell-wall assembly include vancomycin and the β-lactam antibiotics, agents with variable activity against MDR Gram-positive bacteria. Vancomycin, a commonly prescribed agent for treatment of infections caused by MRSA and vancomycin-susceptible enterococci, has demonstrated synergy against these organisms with a number of agents both in vitro and in clinical trials to treat the most serious of these infections. In clinical studies, vancomycin combined with the antistaphylococcal flucloxacillin (**37**) shortened the duration of bacteremia caused by MRSA [48] and was successfully combined with trimethoprim–sulfamethoxazole to treat patients infected with daptomycin-resistant MRSA [54]. Triple β-lactam combinations with in vitro synergistic activity against MRSA include meropenem–piperacillin–tazobactam, a combination shown to suppress the emergence of resistance [49]. This finding is notable in that each of these β-lactams individually has limited anti-MRSA activity.

Linezolid (**35**), a bacteriostatic protein synthesis inhibitor, has been studied in combinations with bactericidal drugs for potential treatment of infections caused by toxin-producing or spore-forming Gram-positive bacteria. These combinations are



Scheme 4 Structures of the lipoglycopeptide dalbavancin; the oxazolidinone linezolid; the aminoglycoside gentamicin C1; and the antistaphylococcal penicillin flucloxacillin

based on the hypothesis that linezolid could inhibit the formation of toxins or spores, at the same time that the organism is being killed by the companion drug. However, the results for these combinations have been mixed. The combination of vancomycin with linezolid has resulted in conflicting reports about synergistic activity against staphylococci [43]. Although in vitro studies demonstrated that linezolid could inhibit toxin production by *S. aureus* [52, 53], possibly serving to decrease virulence, this result has not been validated in animal infection models [43]. Similarly, in studies with *Bacillus anthracis*, linezolid inhibited toxin production when used alone, but the combination with levofloxacin that was synergistic in time-kill studies did not significantly affect spore or toxin formation compared to linezolid alone [47].

Combinations of the aminoglycoside gentamicin with a variety of other agents have also been examined against MRSA. However, clinical data based on patients who received gentamicin together with vancomycin showed no significant improvement in 6-month recurrence rates [45]. When low dose gentamicin was administered with either vancomycin, daptomycin, or an antistaphylococcal penicillin to treat patients suspected to have *S. aureus* native valve endocarditis, a significant decrease in creatinine clearance was reported [97]. Thus, this combination is not clinically advisable.

5 Future Directions

Antibiotic combinations will continue to be used to treat seriously ill patients, because any delay in providing appropriate therapy increases morbidity and mortality [98, 99]. Many of these combinations will be used empirically based on in vitro synergy testing or sporadic case reports, because of the lack of controlled randomized clinical trial data. In vitro synergy testing of newer agents with reduced antimicrobial activity against resistant organisms will undoubtedly lead to the identification of effective combinations with established antibiotics. However, at this time, mechanistic explanations for many of the synergistic combinations are lacking. It is hoped that further studies delineating the biochemical or microbiological explanations for the observed synergies will be undertaken, so as to guide the identification of additional useful combinations of drugs that may be used to treat the most deleterious and life-threatening pathogens. Perhaps, in the process, the selection of resistance to these agents will be diminished as a result of multiple targets that must be mutated in order for resistance to emerge. The study of antibiotic combinations, therefore, will continue to be of high interest in the pursuit of treatment options for MDR and pan-resistant bacteria.

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Antibiotic Adjuvants

Roberta J. Melander and Christian Melander

Abstract Bacteria are becoming increasingly resistant to currently available antibiotics, and the development of new antibiotics is not keeping pace. Alternative approaches to combatting drug-resistant bacteria are sorely needed. One such approach is the development of small-molecule antibiotic adjuvants. Adjuvants that thwart resistance mechanisms and render bacteria susceptible to antibiotics have the potential to prolong the life span and also to extend the spectrum of our current armamentarium of drugs. Several approaches to the development of potential adjuvant therapeutics have been investigated, based upon combatting various resistance mechanisms, and have identified promising adjuvant classes. These classes include adjuvants that inhibit modification or degradation of the antibiotic by enzymes (such as β-lactamases or the aminoglycoside-modifying enzymes), adjuvants that increase the intracellular concentration of the antibiotic by inhibiting efflux or facilitating antibiotic uptake, adjuvants that interfere with bacterial signaling systems that drive or coordinate resistance mechanisms, and finally adjuvants that target nonessential steps in bacterial cell wall synthesis. The antibiotic adjuvant approach is a promising orthogonal strategy for the development of new antibiotics to combat drug-resistant bacteria.

Keywords Adjuvant, Antibiotic-modifying enzymes, Antibiotics, Efflux, Multidrug-resistant bacteria

Contents

R.J. Melander and C. Melander (✉)
Department of Chemistry, North Carolina State University, Raleigh, NC 27695, USA
e-mail: rjworthi@ncsu.edu; ccmeland@ncsu.edu

1 Introduction

The development of new antibiotics that are active against multidrug-resistant (MDR) bacteria is not keeping pace with the ever-increasing emergence of resistance, and there are few truly novel classes of antibiotics in clinical development [1]. While the traditional approach of developing new antibiotics remains a vital tool in the fight against MDR bacteria, one major issue with that approach is that bacteria inevitably evolve resistance to single-entity therapeutics that rely on a bacteriostatic or bactericidal mechanism. For example, resistance to the first-in-class antibiotics daptomycin and linezolid was observed after only 1 year of clinical use [2–4], and this resistance will likely continue to increase as their clinical use is prolonged.

There is, therefore, a pressing need to develop alternative new approaches to combat antibiotic resistance. One approach that is receiving increasing attention is the development of antibiotic adjuvants [5, 6]. This approach involves the combination of an antibiotic with a non-microbicidal compound that increases the activity of the antibiotic, for example by blocking the mechanism of resistance. Compounds that are not of themselves microbicidal are termed antibiotic adjuvants. This approach differs from the identification of synergistic antibiotic combinations involving two or more microbicidal agents that target essential gene products [7]. The development of antibiotic adjuvants has one potential advantage. Because they do not typically inhibit bacterial growth when administered alone, they may exert a lower evolutionary pressure on bacteria to evolve resistance. This outcome is in contrast to the evolution of resistance to synergistic antibiotic combinations, which has been demonstrated to be dependent on the evolutionary response to the constituent drugs. For example, if the mutational response to one drug results in increased resistance to the second drug, enhanced resistance evolution is likely. Conversely, a response to one drug that confers increased susceptibility to the other drug (known as collateral sensitivity) will likely result in reduced or slower evolution of resistance to the drug combination [8]. Though there are challenges associated with the use of combination therapies, such as optimizing dosing regimens, these drugs have the potential to allow the continued use of clinically approved antibiotics that may otherwise be rendered obsolete by increasing bacterial resistance.

This chapter describes the identification of compounds that inhibit genotypic bacterial resistance mechanisms (as opposed to phenotypic drug tolerance such as that conferred by the formation of biofilms or persister cells). Genotypic antibiotic

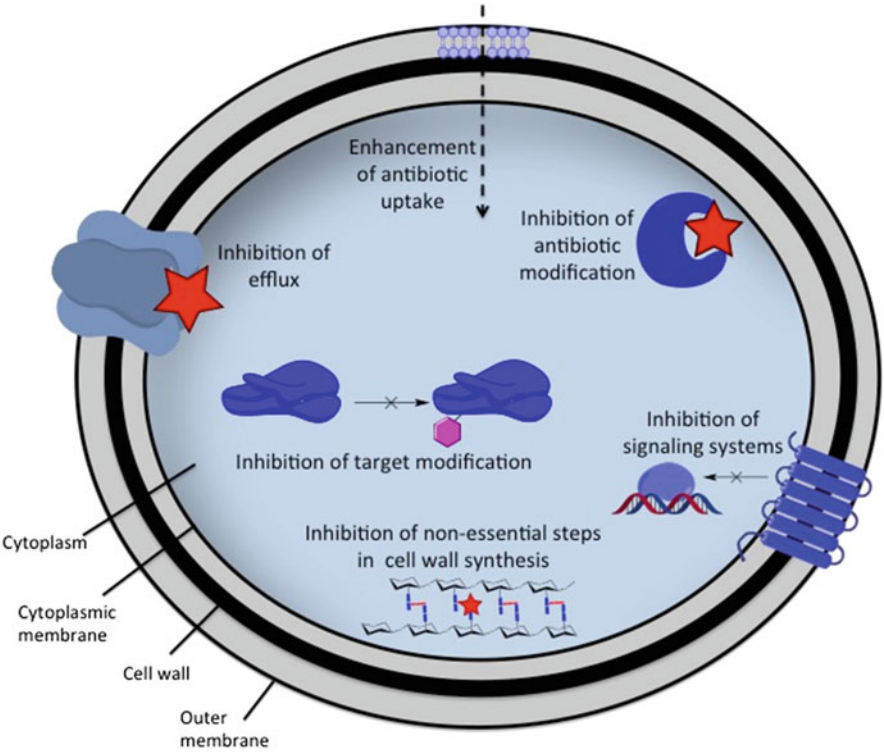

Fig. 1 Targets of small-molecule adjuvants that suppress antibiotic resistance

resistance occurs predominantly through one of three mechanisms [9]: (1) inactivation of the antibiotic by degradation or modification, (2) decreased accumulation of the antibiotic within the bacterial cell as a result of increased efflux or decreased uptake of the antibiotic, or (3) modification of the antibiotic target leading to reduced affinity for the antibiotic. Proteins involved in these resistance mechanisms are therefore attractive potential targets for the development of adjuvant drugs. Additionally, the signaling and regulatory pathways that control the activation of these resistance mechanisms are also potential adjuvant targets. A summary of potential adjuvant targets is shown in Fig. 1.

2 Inhibition of Antibiotic-Modifying Enzymes

The production of enzymes that modify antibiotics such that they no longer have the required affinity for their target and thus render the antibiotic inactive is a common mechanism by which bacteria evade the action of these drugs. In chemical terms, one modification frequently used by bacteria is hydrolysis, for example,

hydrolysis of the lactam bond of β-lactam antibiotics by β-lactamase enzymes, the hydrolysis of the lactone bond of macrolides by macrolide esterases, and the ring opening of the epoxide moiety of fosfomycin [10]. Another way in which bacteria modify antibiotics is to add a group to a key site of the molecule, the most well-known examples of which are the addition of an acetyl, adenyl, or phosphoryl group to aminoglycosides by the aminoglycoside-modifying enzymes (AMEs) [11]. Other examples of antibiotic-modifying enzymes include chloramphenicol acetyl-transferases, macrolide kinases and macrolide glycosyltransferases [10]. Antibiotics can also be inactivated through redox reactions, as in the case of the oxidation of tigecycline by the monooxygenase TetX [12].

The classical examples of adjuvants that work by inhibiting modification of the antibiotic are β-lactamase inhibitors [13]. This class of adjuvants has been reviewed extensively [14–17], and as such only a brief overview is given in this chapter. Augmentin is a clinically approved combination therapy that consists of a β-lactam antibiotic (amoxicillin) and a β-lactamase inhibitor (clavulanic acid **1**) (Fig. 2). The co-dosing of clavulanic acid with amoxicillin inhibits β-lactamase activity in vivo and allows amoxicillin to inhibit cell wall biosynthesis in strains that would be otherwise resistant. This combination ultimately has allowed the continued use of what may otherwise have become an obsolete antibiotic [18]. In 2001 Augmentin was the best-selling antibiotic, demonstrating the effectiveness of combining an antibiotic and an adjuvant in clinical settings [19]. Clavulanic acid, along with the other early β-lactam-containing β-lactamase inhibitors sulbactam **2** and tazobactam **3**, is largely specific for class A β-lactamases and is not active against the class C

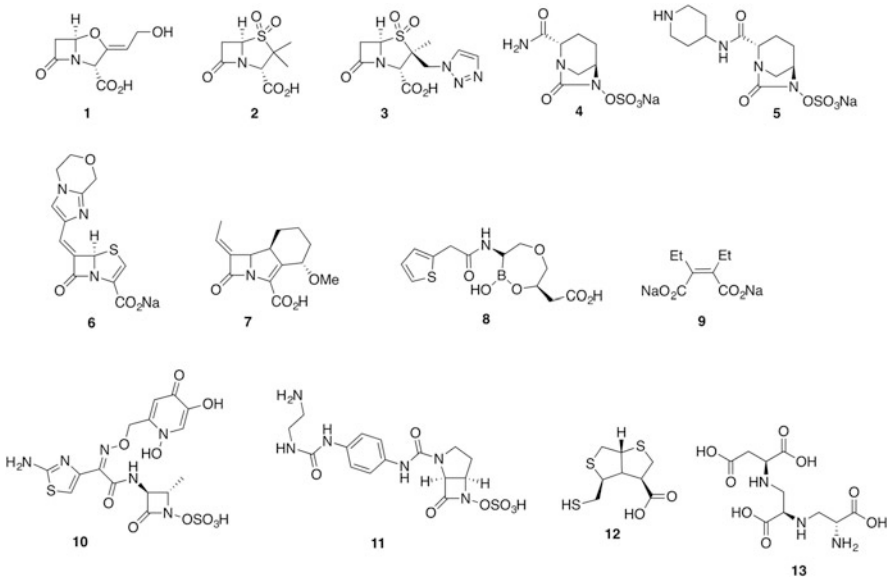

Fig. 2 Adjuvants that inhibit β-lactamases

β-lactamases or the *Klebsiella pneumoniae* carbapenemases (KPCs) or against the metallo-β-lactamases (MBLs) [20].

Recent focus has shifted to the development of non-β-lactam-derived β-lactam inhibitors. One such class is the diazabicyclooctanes (DBOs). This class exhibits a more potent and broader spectrum of activity than earlier inhibitors as it is active against the KPCs and the class C β-lactamases [20]. One member of this class is avibactam **4**, which unlike β-lactam-derived inhibitors is not susceptible to hydrolysis upon binding to the β-lactamase, as the deacylation mechanism releases the intact inhibitor [21]. When examined against a collection of *Enterobacteriaceae* clinical isolates enriched for resistant strains possessing serine β-lactamases, minimum inhibitory concentrations (MICs) of the combination of ceftazidime-avibactam were significantly lower than those of piperacillin-tazobactam, cefotaxime, ceftriaxone, or cefepime and similar or superior to those of imipenem [22]. Avibactam was approved in 2015 in combination with ceftazidime as AVYCAZ® for the treatment of complicated intra-abdominal infections and complicated urinary tract infections [23]. Avibactam has also completed Phase I trials in combination with aztreonam. Another member of the DBO class of β-lactamase inhibitors is relebactam (MK-7665) **5**, which is in Phase III clinical trials in combination with imipenem/cilastatin. Other β-lactamase inhibitors that have demonstrated promising activity against serine β-lactamases, including against several extended-spectrum β-lactamases (ESBLs), include the imidazole-substituted 6-methylidene-penem compound BLI-489 **6** and the tricyclic carbapenem LK-157 **7** (Fig. 2) [24–26].

The boronic acid class of β-lactamase inhibitors includes RPX7009 **8** (also known as vaborbactam), which inhibits several class A and C β-lactamases including the KPCs. RPX7009 was initially developed for use in combination with biapenem [27]. RPX7009 has also demonstrated activity in combination with meropenem against KPC-producing *Enterobacteriaceae* [28]. The safety, tolerability, and pharmacokinetic profile of RPX7009 in a Phase I study was recently reported, and it is currently undergoing Phase III clinical investigation for the treatment of complicated urinary tract infections, acute pyelonephritis, and serious infections caused by carbapenem-resistant *Enterobacteriaceae* (CRE) [29].

Despite potent activity against serine β-lactamases, neither the early β-lactam-containing inhibitors, the DBO inhibitors such as avibactam, nor the boronic acid-based inhibitors are active against strains producing MBLs [30, 31]. In fact, there are as yet no clinically approved MBL inhibitors. This is partly due to the challenge of overcoming cross-reactivity with human metalloenzymes, and also due to the fact that until recently MBL-mediated β-lactam resistance was not considered a major clinical problem [32]. However, in recent years the emergence and dissemination of Gram-negative bacteria harboring plasmid-encoded MBLs such as the New Delhi metallo-β-lactamase (NDM-1) has increased the clinical importance of this class of β-lactamases [33, 34].

β-Lactamase inhibitors that are active against MBLs include the fumarate derivative ME1071 **9** (Fig. 2), which inhibits the MBLs IMP-1 and VIM-2 and significantly enhances the activity of biapenem against *Pseudomonas aeruginosa* [25]. The triple combination cocktail BAL30367 combines the siderophore

monobactam BAL19764 **10**, the bridged monobactam BAL29880 **11** inhibitor of class C β-lactamases (Fig. 2), and clavulanic acid. BAL30367 has shown good in vitro activity against MBL-producing *Enterobacteriaceae* [35]. More recently, the bisthiazolidine (BTZ) class of compounds (including compound **12**) has been reported to be micromolar inhibitors of several MBLs in vitro and to restore imipenem susceptibility to MBL-producing *Escherichia coli* [36]. In 2014 the Wright lab identified from a screen of natural products the fungal metabolite aspergillomarasmine A (AMA) **13** (Fig. 2) for its ability to inhibit NDM-1 [32]. AMA was previously investigated as an inhibitor of the mammalian metalloenzymes angiotensin-converting enzyme (ACE) and endothelin-converting enzyme and was well tolerated in mice making it a promising lead for the development of adjuvants active against MBL-producing bacteria. AMA selectively inhibited NDM-1 and the related MBL VIM-2 over rabbit lung ACE in vitro. This fact coupled with AMA as well tolerated by mice suggests that potential side effects caused by inhibition of mammalian metalloenzymes might be limited. AMA appears to act via a mechanism involving metal depletion. It was able to fully restore meropenem activity against a panel of clinical isolates of CRE, *Acinetobacter* spp., and *Pseudomonas* spp. harboring NDM-1 or VIM. AMA also demonstrated in vivo activity, restoring meropenem activity in mice infected with NDM-1-expressing *K. pneumoniae*, making AMA a promising potential adjuvant to address the significant clinical challenge of MBL-harboring Gram-negative pathogens [32].

Although the development of adjuvants that inhibit modification of other classes of antibiotics has not received the same degree of attention as the development of β-lactamase inhibitors, there are still several targets that have been investigated, in particular the inhibition of AMEs. AMEs are the major mechanisms of resistance to aminoglycoside antibiotics. Their catalysis of the addition of a functional group to a key site on the aminoglycoside achieves resistance as the added functional group disrupts the interaction of the antibiotic with the rRNA target. AMEs include nucleotidyltranferases, phosphotransferases, and acetyltransferases. AMEs effect modifications at both hydroxyl and amine groups, both on the 2-deoxystreptamine core of the aminoglycoside and on the appended saccharides to the core [11]. Given the prevalence of AMEs and the importance of the aminoglycoside class of antibiotics (particularly for the treatment of infections caused by Gram-negative bacteria), inhibitors of these enzymes are attractive prospective adjuvants. Several classes of AME inhibitors have been reported [11, 37]. We focus here on those that have demonstrated not only in vitro enzyme inhibition but also the ability to suppress aminoglycoside resistance in bacterial cells.

The development of aminoglycoside-coenzyme A conjugates as chemical probes to investigate the catalytic mechanism of aminoglycoside 6-*N*-acetyltransferases (AAC (6)-Ii), which transfer an acetyl group from acetyl-coenzyme A to the amino group at the 6 position of the aminoglycoside [11], led to the identification of the truncated conjugate **14** (Fig. 3). Compound **14** exhibited inhibitory activity against AAC (6)-Ii from *Enterococcus faecium* and was shown to act synergistically with kanamycin A against *E. faecium* harboring *aac-(6)-Ii* [38].

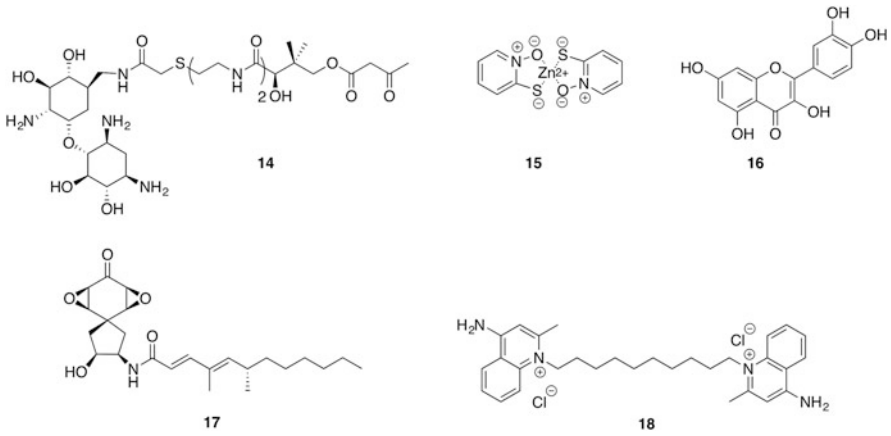

Fig. 3 Adjuvants that inhibit antibiotic-modifying enzymes other than β-lactamases

A screen of metal cations for interference of acetylation of kanamycin and tobramycin by AAC (6)-Ib revealed that Zn(II) is an inhibitor of this enzyme, with $ZnCl_2$ exhibiting an IC_{50} of 15 μM for inhibition of kanamycin acetylation. Suppression of amikacin resistance in *Acinetobacter baumannii* harboring *aac-(6)-Ib* by $ZnCl_2$ was observed only at high $ZnCl_2$ concentrations (800 μM). However, replacement of the chloride counter ions with the ionophore pyrithione to mediate internalization of the cation resulted in suppression of amikacin resistance in *A. baumannii* at concentrations as low as 4 μM, with the complex exhibiting no effect on bacterial growth alone. The zinc pyrithione complex **15** (Fig. 3) also suppressed resistance to amikacin in *E. coli* harboring *aac-(6)-Ib* [39] and was subsequently shown to suppress amikacin and tobramycin resistance in clinical isolates of several Gram-negative bacterial species including *K. pneumoniae* and *Enterobacter cloacae* [40]. Similarly, a copper pyrithione complex was reported to suppress amikacin resistance in *K. pneumoniae* [41].

The extensive attention that has been given to the development of human kinase inhibitors for the treatment of cancer, and the similarities in the 3-D fold structure shared among Ser/Thr/Tyr kinases were exploited by Shakya et al. in the screening of a diverse library of 80 known kinase inhibitors for inhibition of 14 bacterial kinases involved in antibiotic resistance [42]. This screen identified several active inhibitors possessing either broad- or narrow-spectrum inhibition profiles. The flavonol quercetin **16** (Fig. 3) inhibited 12 of the 14 kinases screened, including all of the aminoglycoside phosphotransferases (APHs). Quercetin significantly increased cell death caused by aminoglycoside antibiotics in *E. coli* expressing several APHs [42]. Resistance to arbekacin in methicillin-resistant *Staphylococcus aureus* (MRSA) is thought to be predominantly mediated by the bifunctional enzyme AAC(6′)/APH(2″), which catalyzes both phosphorylation and acetylation of aminoglycosides. The antitumor antibiotic aranorosin **17** has been reported to lower the MIC of arbekacin against resistant MRSA strains at sub-MIC

concentrations by inhibiting AAC(6′)/APH(2″)-catalyzed phosphorylation of the aminoglycoside [43].

Another example of bacterial resistance mediated by modification of the antibiotic is the case of mycothiol (MSH). *Mycobacterium* species use the small-molecule MSH to maintain an intracellular reducing environment and for the detoxification of xenobiotics [44]. MSH has been reported to play a role in the resistance of *Mycobacterium tuberculosis* (*Mtb*) to several clinically important antibiotics including the first-line *Mtb* drug rifampin and the second-line drug streptomycin. Knocking out genes encoding for enzymes involved in MSH biosynthesis leads to increased sensitivity toward several antibiotics, making these enzymes promising targets for overcoming resistance in *Mtb*. It is important to note, however, that MSH also plays a role in the activation of some antibiotics used to combat *Mtb* such as isoniazid, and any inhibitor of MSH biosynthesis or activity would therefore be incompatible with any treatment regimen that uses MSH-dependent antibiotics. Dequalinium **18** (Fig. 3), which had been reported previously as an inhibitor of the MSH biosynthetic enzyme MshC [45], was identified from a high-throughput screen for compounds that enhance the activity of spectinomycin against *Mycobacterium smegmatis*. Dequalinium has potential for the sensitization of *Mtb* to antibiotics that are inactivated by MSH [46].

3 Inhibition of Target Alteration

Analogous to modification of the antibiotic, bacteria may also alter the target of the antibiotic to result in the antibiotic no longer having the required binding affinity to exert its effect. There are few examples of adjuvants successfully targeted at this resistance mechanism. One example is the ErmC inhibitor **19** (Fig. 4). The ErmC methyltransferase class of enzymes catalyzes methylation of an adenine residue of the bacterial 23S rRNA, disrupting binding of the macrolide-lincosamide-streptogramin-B (MLS) classes of antibiotics to the rRNA and thus rendering the bacteria resistant [47, 48]. A high-throughput screening by Clancy et al. identified inhibitors of ErmC including compound **19**, which exhibited synergistic or additive activity with azithromycin against *S. aureus* and *Enterococcus faecalis* [49]. A separate virtual screen for inhibitors of ErmC identified several compounds (including compound **20**, Fig. 4) that decreased erythromycin MICs against an *E. coli* strain constitutively expressing ErmC [50].

4 Inhibition of Efflux

Another major resistance mechanism used by bacteria is efflux, in which membrane-bound efflux proteins pump toxic agents (including virtually all classes of antibiotics) out of the cell. This efflux results in a less-than-efficacious

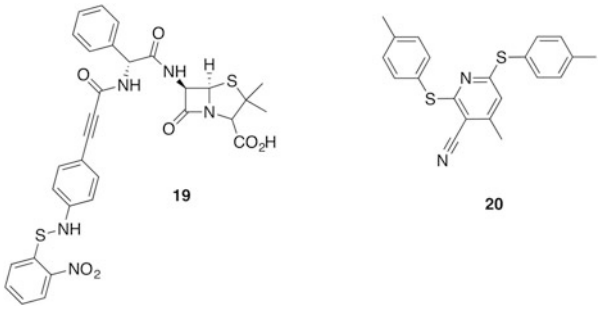

Fig. 4 Compounds that inhibit the modification of antibiotic targets

intracellular concentration of drug. Efflux pumps are ubiquitous in bacteria and present a significant challenge to the development of effective antibiotics. They may be specific for one substrate or one substrate class or may expel multiple unrelated classes of antibiotics, as is the case for the resistance-nodulation-division (RND) superfamily that includes the clinically relevant AcrAB-TolC and Mex pumps that contribute to multidrug resistance [51, 52]. Additionally, efflux pumps can act synergistically with other resistance mechanisms, such as the outer membrane permeability barrier in Gram-negative bacteria, exacerbating resistance [52]. Compounds that inhibit efflux pumps therefore have significant potential to sidestep antibiotic resistance and are attractive as potential adjuvants.

S. aureus possesses more than 15 different efflux pumps, both chromosomally and plasmid encoded, which contribute to resistance against various classes of antibiotics [53]. NorA is the best-studied *S. aureus* efflux pump. It is a multidrug efflux pump that plays a role in resistance to the fluoroquinolone antibiotics and to disinfectants. NorA is thought to be responsible for at least 10% of antibacterial resistance in MRSA strains [54]. The plant alkaloid reserpine **21** (Fig. 5) inhibits NorA-mediated drug efflux and decreases the MIC of the fluoroquinolone norfloxacin against *S. aureus*. Additionally, reserpine increases the bactericidal activity and post-antibiotic effect of ciprofloxacin on *S. aureus* and reduces the emergence of norfloxacin resistance. The activity of reserpine establishes NorA inhibitors as having potential as adjuvants. However, reserpine itself cannot be used in a clinical setting due to its neurotoxicity [55]. In an attempt to identify a more clinically useful potentiator of fluoroquinolone activity, a 9,600-member library was screened for NorA inhibition. Several structurally diverse compounds with increased potency compared to reserpine were identified. The most active compound (**22**) demonstrated synergy with ciprofloxacin against a resistant *S. aureus* strain and considerably reduced the emergence of ciprofloxacin resistance [56].

Several other phytochemicals have also been reported to inhibit bacterial efflux pumps, as reviewed by Abreu et al. [54]. Examples include carnosic acid **23** and carnosol **24**, which inhibit several efflux pumps of *S. aureus* and thus suppress resistance to tetracycline resulting from the TetA efflux pump and to erythromycin resulting from the MsrA efflux pump. Carnosic acid also demonstrated inhibition of ethidium bromide efflux in a NorA-expressing *S. aureus* strain [54]. Other reported inhibitors of NorA that potentiate antibiotic activity include the related abietanes

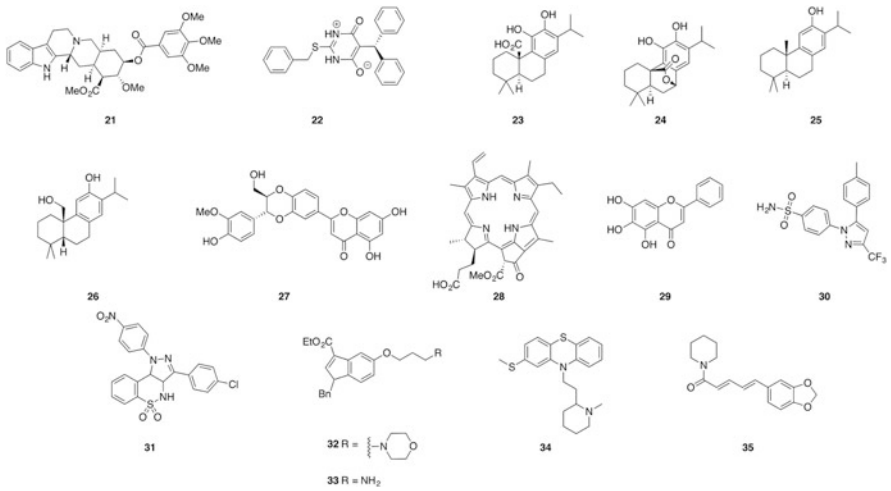

Fig. 5 Inhibitors of *S. aureus* efflux pumps

ferruginol **25** and 5-epipisiferol **26**, the polyphenol hydnocarpin D **26**, the chlorophyll metabolite pheophorbide A **27**, and the flavonoid baicalein **29** (Fig. 5). While baicalein potentiated ciprofloxacin against MRSA and gentamicin against vancomycin-resistant *E. faecium* (VRE) through a mechanism thought to involve inhibition of NorA, it also potentiated the effects of β-lactam antibiotics against MRSA and exhibited synergy with tetracycline against MRSA strains not possessing the TetK efflux pump. Baicalein may therefore act via multiple modes of action [54, 57].

Celecoxib **30** (Fig. 5) is a cyclooxygenase-2 (COX-2) inhibitor that also suppresses drug resistance in cancers by inhibiting the MDR1 efflux pump of the cancer cell. Celecoxib was reported additionally to suppress resistance to multiple antibiotic classes including ampicillin, kanamycin, chloramphenicol, and ciprofloxacin in *S. aureus* [58]. Celecoxib was later confirmed to inhibit NorA. Subsequent virtual screening of a library of 150 celecoxib analogs identified compound **31** as a more potent inhibitor than celecoxib, and as having synergistic activity with ciprofloxacin against a NorA-overexpressing strain of *S. aureus* [59]. The investigation of a series of indole derivatives as NorA inhibitors identified several active compounds including **32** and **33**, which exhibited IC_{50} values of 12.5 μM and potentiated ciprofloxacin against a NorA-overexpressing strain of *S. aureus*, reducing the MIC by eightfold [60]. Phenothiazine antipsychotic drugs such as thioridazine **34** (Fig. 5) possess modest antimicrobial activity and have been reported to potentiate several classes of antibiotics against multiple bacterial species. This class of compounds inhibits both efflux mediated by NorA and by other efflux mechanisms in *S. aureus* and thus reduces norfloxacin MICs in strains overexpressing efflux pumps [61]. Another multidrug efflux pump in *S. aureus* is MdeA, which plays a role in resistance to several antibiotics including novobiocin and mupirocin [53]. The alkaloid piperine **35** (Fig. 5), which has also been reported as an inhibitor

of NorA, markedly reduced the MIC of mupirocin against *S. aureus*, thought to occur by a mechanism likely involving inhibition of MdeA efflux. In an in vivo dermal infection model, mupirocin had increased efficacy at one-quarter the commercially available dose upon combination with piperine, compared to the full dose alone [62].

The inhibition of efflux pumps as a means of potentiating antibiotics in Gram-negative bacteria also has been extensively investigated. Several inhibitors of Gram-negative bacterial efflux pumps have been described. A screening program for inhibitors of *P. aeruginosa* efflux pumps identified the peptidomimetic Phe-Arg-β-naphthylamide (PAβN) **36** (Fig. 6) as an inhibitor of all four of the clinically relevant efflux pumps in this bacterium (MexAB-OprM, MexCD-OprJ, MexEF-OprN, and MexXY-OprM). PAβN decreased resistance to levofloxacin, reducing MICs by eightfold in wild-type strains of *P. aeruginosa*, while MICs were decreased by up to 64-fold in efflux pump-overexpressing strains [63–65]. Further illustrating the intricate role played by efflux pumps in multidrug resistance, the overexpression of MexAB-OprM in *P. aeruginosa* isolates from cystic fibrosis patients was reported to result in both derepression of the cephalosporinase AmpC and decreased membrane permeability. As a result, susceptibility to meropenem is decreased. Addition of PaβN abolishes this decrease [66]. PAβN also inhibits similar pumps in other MDR Gram-negative bacteria, including the clinically important RND family efflux pump system AcrAB-TolC [63]. AcrAB-TolC is the major multidrug resistance efflux pump in *Enterobacteriaceae* and is responsible for the efflux of a variety of structurally diverse compounds, including β-lactams, fluoroquinolones, and tetracyclines. Strains lacking this efflux system are hypersusceptible to several antimicrobials. AcrAB-TolC overexpression in response to antibiotic exposure contributes to multidrug resistance in Gram-negative bacteria [67, 68]. In the *Enterobacteriaceae* AcrAB–TolC production is regulated by the transcriptional activator RamA encoded by *ramA*. Inactivation of

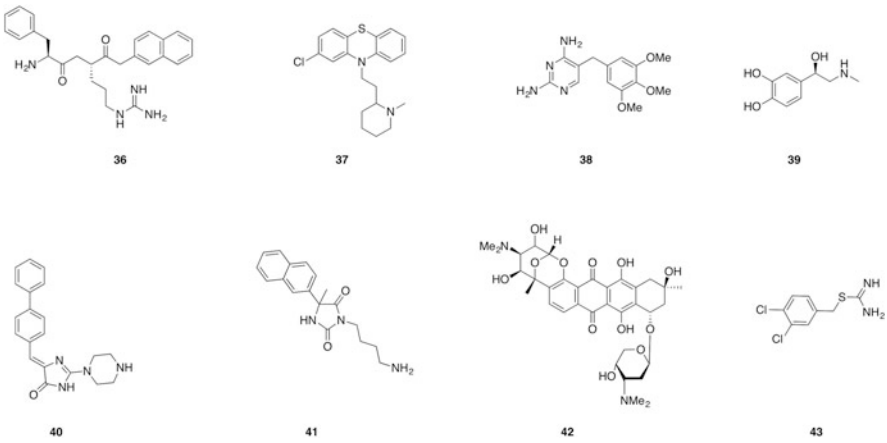

Fig. 6 Adjuvants that inhibit efflux in Gram-negative bacteria

ramA results in decreased *acrB* expression, while high-level but not low-level overexpression of *ramA* leads to increased *acrB* expression [69]. Interestingly, inhibition of efflux by several inhibitors including PaβN results in upregulation of *ramA*, thought to be a response to increased cellular accumulation of internal metabolites [68]. PaβN binds to the bottom of the distal binding pocket of AcrB and interferes with the binding of drug substrates to the upper part of the binding pocket [70]. Counterintuitively, carbapenemase-producing *Enterobacteriaceae* mutants lacking AcrAB-TolC efflux pumps have been reported to exhibit elevated carbapenem resistance levels, a phenotype that was recapitulated by inhibitor-mediated loss of efflux pump function with 72% of clinical isolates tested being more resistant to ertapenem in the presence of PAβN. The increased resistance observed both for the mutants and in the presence of PAβN was attributed to a change in outer membrane porin production [71]. This result highlights the interdependent nature of bacterial resistance mechanisms and the need to evaluate the effect of any new adjuvant on other clinically important antibiotics.

Several other compounds have been reported to inhibit the AcrAB-TolC efflux system, including the previously mentioned NorA inhibitor thioridazine **34** and the related phenothiazine chlorpromazine **37**, both of which effected an increase in *ramA* expression [68]. As mentioned earlier, high-level but not low-level overexpression of *ramA* leads to increased *acrB* expression, and chlorpromazine **37** induced modest overproduction of *ramA*, repressed *acrB*, and increased susceptibility to several antibiotics including nalidixic acid, norfloxacin, ciprofloxacin, chloramphenicol, and tetracycline. These results suggest phenothiazines are not direct efflux pump inhibitors, but suppress resistance by affecting the expression of efflux-related genes [69]. Trimethoprim **38** and epinephrine **39** (Fig. 6) have also been reported to exhibit synergy with ciprofloxacin, tetracycline, and chloramphenicol by inhibiting AcrAB-TolC [72]. The arylpiperazine and arylmorpholine scaffolds constitute two of the most well-studied classes of RND pump inhibitors, and the piperazine arylideneimidazolone **40** was shown to inhibit efflux in an *acrAB*-overexpressing strain of *E. coli* and to increase susceptibility to several antibiotics including levofloxacin, oxacillin, linezolid, and clarithromycin to levels close to those found in an *acrAB*-knockout strain [67]. A series of arylhydantoin derivatives were identified as potentiating the activity of nalidixic acid in strains of *Enterobacter aerogenes* overexpressing the AcrAB-TolC efflux pump [73]. Modulation of this scaffold led to the identification of **41** (Fig. 6) which exhibited synergy with both nalidixic acid and chloramphenicol against an *acrAB*-overexpressing strain of *E. aerogenes* [74].

A screen of 1,120 actinomycete fermentation extracts for rifampin potentiation against *E. coli* identified antibiotic 301A1 **42** (Fig. 6). Antibiotic 301A1 does not possess antibiotic activity against Gram-negative bacteria itself, but displays high-level synergy with rifampin against *E. coli*. This compound also displayed synergy with rifampin against *A. baumannii* and with the Gram-positive-selective antibacterial linezolid against both *E. coli* and *A. baumannii* (both Gram-negatives). Inhibition of efflux was postulated to play a role in the adjuvant activity of **42** at least with respect to linezolid potentiation, with **42** competitively inhibiting

extrusion of the AcrAB substrate Nile Red from *E. coli* and exhibiting a loss of activity in an *A. baumannii* strain overexpressing the AcrAB family efflux pump AdeIJK [75].

A screen for compounds that potentiate the Gram-positive-selective aminocoumarin antibiotic novobiocin against *E. coli* identified compound **43** (Fig. 6) that inhibited MreB, a component of the bacterial cytoskeleton with a role in cell division. Alterations in cell shape upon disruption of MreB correlated with decreased efflux and subsequent accumulation of normally extruded antibiotics, making this compound an example of synergy as a result of indirect inhibition of efflux [76].

5 Enhancement of Antibiotic Uptake

Antibiotics with targets that are located within the cytoplasm must cross the bacterial cell envelope in order to exert their effects. This crossing is achieved by several different mechanisms, depending on the antibiotic class and the bacterial species. Reduced permeability of the bacterial cell envelope confers increased antibiotic resistance, and various approaches to circumvent permeability-mediated resistance have been investigated. These studies include destabilization of the bacterial outer membrane and the hijacking of the transport mechanisms used by the bacteria for nutrient uptake [77].

The Gram-positive cell wall is relatively permeable to most antibiotics, and the Gram-positive cytoplasmic membrane is typically crossed by active transport. The additional outer membrane of Gram-negative bacteria poses a much greater barrier. While some hydrophilic antibiotics can traverse the outer membrane by passive diffusion through the pores of the porin proteins, these pores typically exclude larger antibiotics (Mw >800 Da). The cytoplasmic membrane must still be traversed for antibiotics with targets within the cytoplasm. The loss of outer membrane porins, or of active uptake pathways in the cytoplasmic membrane, contributes further to increased antibiotic resistance in Gram-negative bacteria [78, 79]. The development of antibiotics that are active against Gram-negative bacteria is therefore considerably more challenging than the development of Gram-positive antibiotics. Gram-negative bacteria are intrinsically resistant to several classes of antibiotics. Accordingly, breaching the Gram-negative cell envelope has the potential to render antibiotics that are currently only viable for use against Gram-positive bacteria clinically useful for a much broader spectrum of bacteria and is therefore another attractive adjuvant approach.

Several compounds that lack direct antimicrobial activity, but destabilize the Gram-negative outer membrane to an extent that allows antibiotics not normally able to cross the membrane to access the cell, have been investigated for their ability to enhance antibiotic activity [80]. One example is the truncated polymyxin, polymyxin B nonapeptide (PMBN) (**44**, Fig. 7). PMBN lacks the acyl tail and N-terminal aminoacyl residue of polymyxin B. As a result this compound lacks the

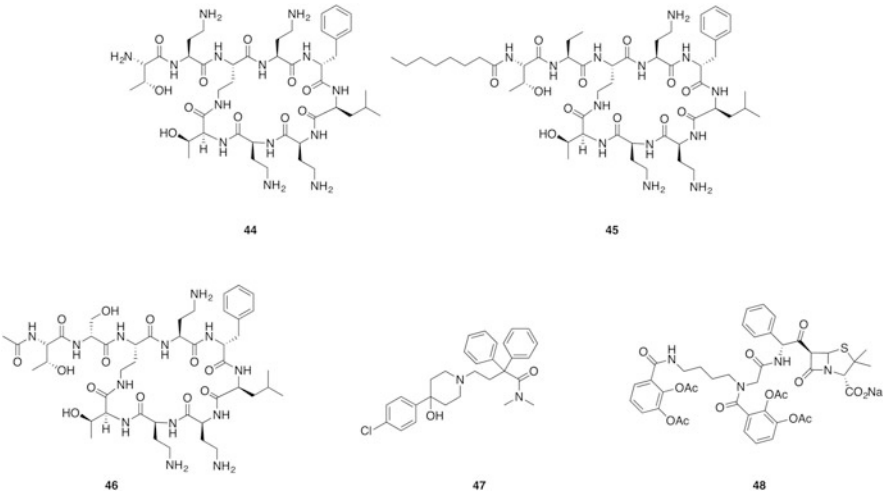

Fig. 7 Adjuvants that enhance the uptake of antibiotics

antibacterial activity exhibited by native polymyxins against Gram-negative bacteria. PMBN increases the susceptibility of several species of Gram-negative bacteria, including *K. pneumoniae* and *P. aeruginosa*, to erythromycin, novobiocin, and fusidic acid. PMBN also exhibits in vivo activity in combination with erythromycin or novobiocin in mice infected with *K. pneumoniae* or *P. aeruginosa* [81, 82]. More recently, PMBN was reported to potentiate ceftazidime and ceftazidime-avibactam against clinical isolates of *E. coli*, *K. pneumoniae*, and *E. aerogenes* [83]. The renal toxicity associated with PMBN prevented its development as a clinically viable adjuvant and led to the creation of second-generation analogs with reduced positive charge (including SPR7061 **45** and SPR741 **46**, Fig. 7) and with potentially reduced renal toxicity [80]. As with PMBN, these analogs lack significant antimicrobial activity against most Gram-negative bacteria, but potentiate the activity of several antibiotics against Gram-negative species including *E. coli*, *K. pneumoniae*, and *A. baumannii*. These second-generation analogs lack, however, activity against *P. aeruginosa*.

A screen for potentiation of the tetracycline minocycline using a library of approved drugs identified the opioid receptor agonist loperamide **47** (Fig. 7). Loperamide potentiated the activity of minocycline (and other tetracycline antibiotics) against several species of Gram-negative bacteria including *P. aeruginosa*, *E. coli*, *A. baumannii*, *K. pneumoniae*, and *Salmonella enterica*. The use of previously approved drugs such as loperamide as potential antibiotic adjuvants has the advantage of identifying adjuvants with well-characterized toxicology and pharmacology. Loperamide **47** exhibited in vivo activity in a murine model of infectious colitis caused by *S. enterica* serovar Typhimurium. Loperamide increased membrane permeability in *E. coli* and *P. aeruginosa* and dissipated the membrane

potential in *E. coli* enhancing the uptake of tetracycline antibiotics, which requires a pH gradient to traverse the Gram-negative outer membrane [84].

As mentioned earlier, the known efflux pump inhibitor PAβN **36** increases the susceptibility of *P. aeruginosa* to fluoroquinolone antibiotics. However, its ability to potentiate the β-lactam class of antibiotics was less studied. The Burrows group showed that while PAβN does indeed potentiate β-lactam antibiotics against *P. aeruginosa*, the mechanism of action is not due solely to efflux inhibition. PAβN additionally acts as a membrane-permeabilizing agent. PAβN enhanced the potency of β-lactam antibiotics against a *P. aeruginosa* strain deficient in all four major RND efflux pumps, effected increased uptake of the fluorescent probe 8-anilino-1-naphthylenesulfonic acid, caused a release of the AmpC β-lactamase from cells, and sensitized the bacterium to vancomycin, which under normal conditions is unable to cross the *P. aeruginosa* outer membrane. All of these effects indicate that membrane permeabilization could be a significant secondary mechanism through which PAβN acts to potentiate antibiotic activity. This property could even expand the scope of antibiotics for which it is effective [85].

Another strategy to increase antibiotic effectiveness by enhancing uptake is to take advantage of native transport systems that are used by bacteria for nutrient uptake. One such system is the iron transport system, which is vital to the ability of bacteria to cause infection, being required for both virulence and survival in the host. Bacteria secrete a variety of high affinity iron chelating small molecules known as siderophores that sequester and solubilize iron and facilitate iron entry into the bacterial cell through siderophore-specific receptors [86]. The ability of these molecules to gain entry into bacterial cells has been exploited to circumvent antibiotic resistance associated with limited uptake by way of a "trojan horse" approach, in which an antibiotic is linked to a siderophore and is transported into the cell via the iron transport system. One example of this approach is the synthesis of siderophore-aminopenicillin conjugates including compound **48** (Fig. 7), wherein the β-lactam ampicillin is conjugated to a biscatecholate moiety. Several conjugates displayed activity against multiple species of Gram-negative bacteria, effecting a >1,000-fold increase in activity compared to ampicillin against *P. aeruginosa* and *Stenotrophomonas maltophilia* laboratory strains and a >100-fold increase in activity against *Enterobacteriaceae* laboratory strains in vitro. The conjugates were active against carbapenem-resistant clinical isolates of *P. aeruginosa* and *S. maltophilia* and exhibited in vivo activity in a murine model of *P. aeruginosa* infection. Importantly **48** and several other conjugates tested were not substrates for the major *P. aeruginosa* efflux pumps MexAB-OprM, MexCD-OprJ, or MexEF-OprN. Although these β-lactam-siderophore conjugates are not typical adjuvants in that they are a single molecular entity, they represent a promising approach to circumventing permeability-mediated antibiotic resistance [77].

6 Interfering with Signaling Systems

An alternative to directly inhibiting the enzyme or protein responsible for imparting resistance to the bacteria, such as occurs with β-lactamase and efflux pump inhibitors, is to interfere with the ability of the bacteria to "switch on" their resistance machinery. Bacteria use various pathways to detect the presence of antibiotics and respond by either activating or upregulating the production of the proteins required for resistance. One such example is the detection of β-lactam antibiotics by the BlaR1 and MecR1 sensor systems of MRSA. Upon sensing a β-lactam antibiotic, BlaR1 and MecR1 are phosphorylated and subsequently initiate a series of events that culminate in the expression of genes encoding for a β-lactamase and penicillin binding protein 2a (PBP2a), respectively, both of which play a role in the resistance of MRSA to β-lactam antibiotics. A recent screen by the Mobashery group of a library of protein kinase inhibitors for their ability to lower the oxacillin MIC against MRSA identified the known mammalian serine/threonine kinase inhibitor **49** (Fig. 8). Compound **49** reduced the extent of phosphorylation of BlaR1 in the presence of a penicillin that otherwise was capable of inducing resistance, which correlated to a lack of induction of the *bla* system, accounting for the reduction in oxacillin resistance. Analog synthesis of compound **49** led to compound **50**, which lowered oxacillin MICs by up to 64-fold at a concentration of just 7 μg/mL [87].

One of the most prominent signaling and regulatory systems used by bacteria to control behaviors in response to external stimuli and stresses are two-component systems (TCS). TCS regulate the expression of genes in response to external stimuli and control a number of bacterial behaviors including sporulation, competence, biofilm formation, pathogenesis, and antibiotic resistance across multiple bacterial species [88, 89]. TCS are activated by a variety of factors such as pH, nutrient level, redox state, osmotic pressure, quorum signals, and the presence of antibiotics. These systems are composed of a histidine kinase and a response regulator. In response to the external stimulus, the histidine kinase undergoes autophosphorylation at a

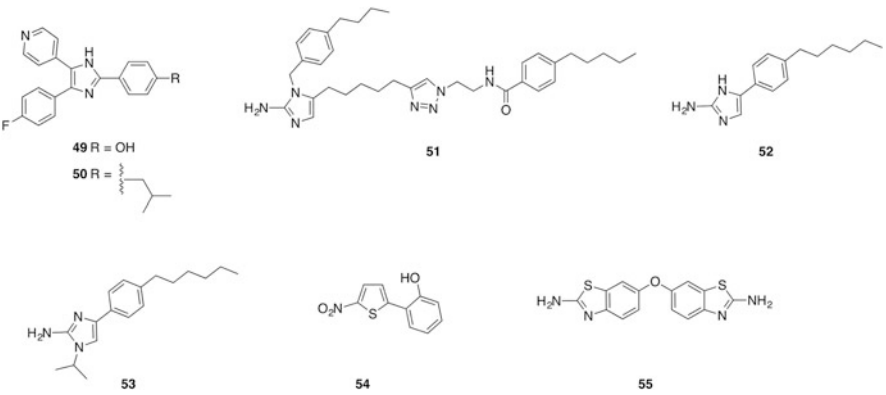

Fig. 8 Inhibitors of bacterial signaling systems involved in antibiotic resistance

conserved histidine residue. This phosphate group is then transferred to a conserved aspartate residue on the response regulator, inducing a conformational rearrangement of the protein and leading to DNA binding of the phosphorylated response regulator and subsequent alteration of gene expression [90]. Many histidine kinases can also act as phosphatases and dephosphorylate the response regulator, thus allowing precise control of gene expression in response to environmental change [88]. TCS are ubiquitous among bacteria and possess common structural motifs not found in higher eukaryotes, potentially allowing selective targeting by small molecules. Furthermore, most TCS are not essential for bacterial growth under normal conditions, and therefore small-molecule targeting of the TCS may place reduced selection pressure on the bacteria to acquire resistance to the action of the small molecule through mutation. The TCS systems are a potentially powerful, and thus far underexploited, antibiotic adjuvant target for small-molecule development [91].

One example of a TCS that plays an important role in antibiotic resistance is the VraRS system in MRSA. Inactivation of *vraRS* decreases methicillin resistance independently of *mecA* expression, supporting the hypothesis that the methicillin-resistant phenotype is influenced by factors other than PBP2a [92]. These factors are potential targets for the potentiation of methicillin and other β-lactams. The VraRS system has been proposed as a "sentinel" system capable of sensing perturbation of cell wall synthesis and coordinating a response that involves the mobilization of genes essential for high-level antibiotic resistance [93]. VraRS is unique among TCS involved with respect to resistance to cell wall-acting antibiotics in that it mediates the response to disruption of both the early and late steps of cell wall biosynthesis. VraRS senses cell wall damage and coordinates a general cell envelope stress response involving numerous genes necessary for cell wall synthesis that are referred to collectively as the cell wall stress stimulon (CWSS). VraRS is induced by the exposure of *S. aureus* to several antibiotics that act upon the cell wall, including β-lactams, glycopeptides, and bacitracin [90]. MRSA mutants that are deficient in *vraRS* are treatable with an oxacillin regimen in vivo, thus validating the potential of targeting this TCS as an antibiotic adjuvant strategy [94]. Recently, a third member of the *vra* operon encoded directly upstream of *vraS* and designated *vraT* was reported to be essential for optimal expression of methicillin resistance [92]. The *vraT* gene encodes a putative membrane protein VraT that has a regulatory role in the aforementioned VraRS-mediated cell wall stress stimulon. Similar to deletion of *vraR* and *vraS*, the deletion of *vraT* improved the outcome of oxacillin therapy in vivo [92]. VraT thus represents an additional target for the potentiation of β-lactam activity against MRSA. In addition to the role played by VraRS, it has been suggested that multiple TCSs might also be responsible for the variation in β-lactam resistance levels observed in clinical strains of MRSA [95]. Several 2-aminoimidazole compounds derived from the marine natural products oroidin and bromoageliferin suppress MRSA resistance to the β-lactams [96–100]. The lead compound from this series, compound **51** (Fig. 8), suppressed resistance in a number of MRSA clinical isolates by up to 512-fold via a mechanism that was dependent on the presence of VraRS [100]. The phenothiazine antipsychotic drug thioridazine **34** mentioned earlier also was reported to potentiate

oxacillin and dicloxacillin against MRSA independently of the PBP2a-mediated resistance mechanisms and to repress transcription of several genes belonging to the *vraRS* regulon [101–103].

TCS also play a role in antibiotic resistance in Gram-negative bacteria. Another 2-aminoimidazole compound **52** (Fig. 8) suppresses resistance to colistin in both *A. baumannii* and *K. pneumoniae* [104]. Colistin resistance in *A. baumannii* is mediated by the PmrAB TCS, which controls the expression of the phosphoethanolamine transferase PmrC that catalyzes modification of the lipid A component of the outer membrane. This modification results in a reduction in the net negative charge of the membrane that subsequently leads to a reduced affinity for colistin and other cationic antimicrobials [105, 106]. Compound **52** downregulates the *pmrCAB* operon in *A. baumannii*. Mass spectrometry-based analysis of the lipid A fraction of bacteria treated with **52** showed a significant reduction in phosphoethanolamine modification, indicating that **52** potentiates colistin activity through a mechanism that involves the PmrAB TCS. Supporting the argument that targeting nonessential pathways may lead to a reduction in evolutionary pressure to develop resistance, it was reported that both colistin-susceptible and colistin-resistant bacteria that were serially passaged in the presence of colistin and **52** were unable to evolve resistance to the combination treatment [104]. The development of a second generation of analogs of compound **52** led to **53**, which exhibits a greater degree of resistance suppression, lower inherent bacterial toxicity, and an expanded spectrum of activity that includes *P. aeruginosa* [107].

A fluorescence polarization displacement assay developed by the Carlson lab has facilitated high-throughput screening for compounds targeting the ATP-binding pocket that is specific to histidine kinases. Three representative histidine kinases were used with the aim of discovering inhibitors capable of targeting multiple histidine kinases. Nine compounds that inhibited at least two of the kinases were identified, including the aminobenzothiazole **54** (Fig. 8) which was subsequently shown to exhibit moderate antibiotic activity against *E. coli* and *Bacillus subtilis* [108]. Although the compounds identified in this screen were not investigated for their adjuvant activity, this high-throughput assay for the identification of broad-spectrum histidine kinases inhibitors is a significant step toward active scaffolds that potentiate antibiotic activity through the TCS. Thiophene **55** is another histidine kinase inhibitor, identified from a virtual screen against the essential *B. subtilis* histidine kinase WalK. It inhibits autophosphorylation of WalK and other histidine kinases in vitro. Compound **55** was selective for histidine kinases, as it lacked activity against the bacterial serine/threonine kinase IreK and exhibited moderate antibiotic activity against several bacterial species in addition to adjuvant activity at sub-MIC levels. Compound **55** potentiated the activity of β-lactam antibiotics against *S. aureus* and *E. coli* and of ofloxacin against one strain of *E. coli* [109].

The SOS DNA repair and mutagenesis pathway plays a role in antibiotic resistance by enabling adaptive resistance mutations and the acquisition of resistance genes and is thought to be induced by bactericidal antibiotics. The SOS pathway involves activation of the recombinase RecA, inactivation of the LexA repressor, and expression of SOS response genes that facilitate antibiotic resistance.

RecA repairs DNA that has been damaged either directly by the antibiotic or by oxidative stress resulting from the action of the antibiotic, resulting in increased antibiotic tolerance. *E. coli* strains lacking *recA* exhibit increased sensitivity to bactericidal antibiotics. Further contributing to the role RecA has in antibiotic resistance, RecA-mediated repair induces a hypermutable state that promotes acquisition of antibiotic resistance. RecA is therefore a promising adjuvant target. A series of phthalocyanine tetrasulfonate (PcTs)-based inhibitors of RecA including iron(III) phthalocyanine-4,4′,4″,4‴-tetrasulfonic acid (Fe-PcTs) have recently been reported that prevent antibiotic-induced activation of the SOS pathway in *E. coli* and potentiate the activity of the bactericidal antibiotics ciprofloxacin, kanamycin, and ampicillin. The PcTs inhibitors also decreased the acquisition of resistance mutations in vitro, and both potentiated ciprofloxacin activity and reduced resistance acquisition in vivo in a neutropenic murine bacterial thigh infection model. Inhibitors of RecA such as Fe-PcTs have the advantage of being able to be combined with a wide range of antibiotics and could potentially offer a general strategy for prolonging the life span of antibiotics [110].

7 Targeting Nonessential Steps in Cell Wall Synthesis

Many cellular functions, including bacterial cell wall synthesis, are carried out not by discreet enzymatic transformations, but by multiple proteins that work together, some of which are interdependent and some of which are functionally redundant. In *S. aureus* deletion of seven of the nine genes that encode enzymes involved in peptidoglycan synthesis had no effect on cell growth or morphology in vitro, but did result in a marked increase in susceptibility to cell wall-acting antibiotics [111]. In practical terms, genes that are nonessential in certain environments can become essential upon changes in environmental conditions (such as the presence of antibiotics) making them an ideal adjuvant target. For example, PBP2 is essential in methicillin-sensitive *S. aureus* (MSSA) but not in MRSA due to the presence of PBP2a, which takes over transpeptidase activity. However, PBP2 becomes essential in MRSA in the presence of β-lactam antibiotics, as cooperation between the transglycosylase domain of PBP2 and the transpeptidase domain of PBP2a is required for survival [111]. The identification of nonessential steps in cell wall biosynthesis, and the effect that inhibiting these steps has upon the potency of cell wall-acting antibiotics, has been the subject of much investigation of late with regard to identifying potential adjuvant targets.

Wall teichoic acid (WTA) is a glycophosphate polymer that is cross-linked to peptidoglycan in the Gram-positive cell wall and has several functions including a role in cell growth and division. Despite this, bacteria lacking WTA are viable, and the genes encoding the proteins involved in the early stages of WTA synthesis are nonessential. Inactivation or alteration of WTA in MRSA results in an increase in susceptibility to β-lactam antibiotics [112, 113], particularly those that exhibit selectivity for PBP2 [114], making WTA a promising potential adjuvant target.

One of the nonessential genes involved in the early stages of WTA synthesis is *tarO*, which encodes the *N*-acetylglucosamine-1-phosphate transferase TarO. Both genetic and pharmacological approaches show that inactivation of TarO, in combination with inactivation of native PBPs (PBPs other than PBP2a) by a β-lactam antibiotic, is synergistic. The natural product tunicamycin **56** (Fig. 9) inhibits the UDP-HexNAc:polyprenol-P HexNAc-1-P family of enzymes that includes TarO and the essential *S. aureus* peptidoglycan synthesis enzyme MraY. Tunicamycin exhibits selectivity for TarO over MraY, allowing it to be used as a probe to demonstrate the potential of TarO as an adjuvant target and to demonstrate synergy with oxacillin at subinhibitory concentrations of oxacillin. Inhibition of TarO by tunicamycin specifically increased susceptibility to β-lactam antibiotics, thought to be a result of mislocalization of PBP2 or PBP2a, and did not sensitize MRSA to other classes of antibiotics including those that inhibit peptidoglycan synthesis by other mechanisms, such as vancomycin [112]. Tunicamycin itself cannot be used clinically as it possesses eukaryotic toxicity as a result of its inhibition of protein glycosylation. Therefore, the identification of selective TarO inhibitors is necessary to realize WTA inhibition as a viable adjuvant strategy for potentiation of MRSA to β-lactam antibiotics. Screening of a library of over 2,000 previously approved drugs for compounds that potentiate the activity of the PBP2-selective β-lactam

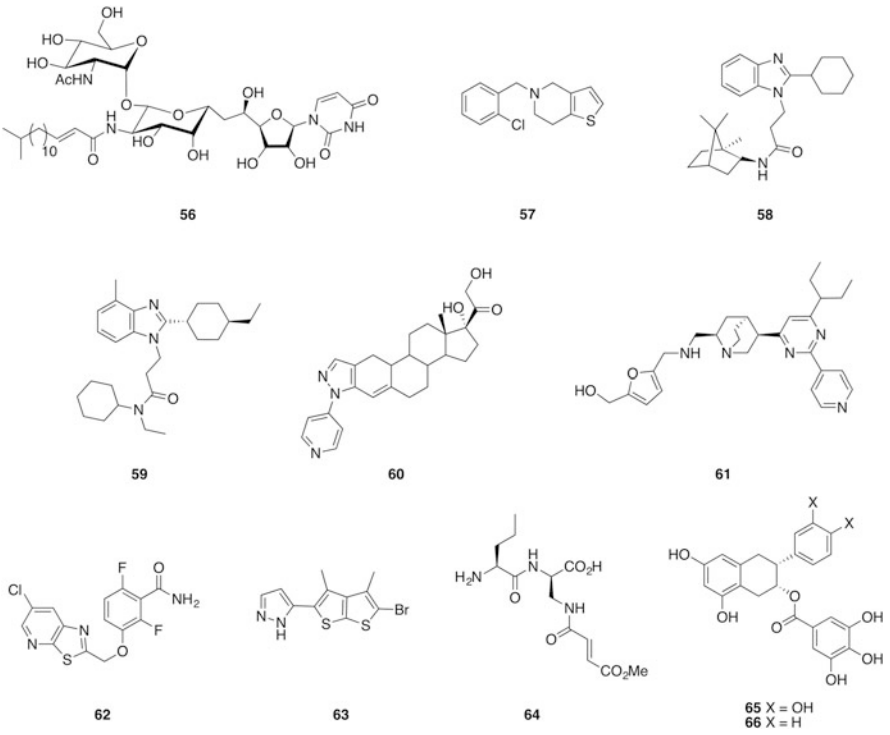

Fig. 9 Adjuvants that target cell wall synthesis

cefuroxime, which is highly sensitized upon deletion of *tarO*, identified the antiplatelet drug ticlopidine (Ticlid) **57** (Fig. 9). Ticlopidine did not exhibit growth inhibition alone but exhibited a strong synergistic effect with cefuroxime against wild-type MRSA, an effect that was abrogated in a *tarO* deletion strain. Ticlopidine **57** potentiated the activity of cefuroxime against a panel of MRSA clinical isolates and also demonstrated activity in vivo in a *Galleria mellonella* model of infection [114]. Another screen to identify inhibitors of TarO identified the benzimidazole tarocin B **58**, which displayed synergy with imipenem against MRSA. Analog synthesis identified compound **59** (Fig. 9), which retained activity across a broad spectrum of clinical isolates of both MRSA and MRSE [115].

Yet another small-molecule library screen for β-lactam potentiation against MRSA identified the steroid-like compound murgocil **60** (Fig. 9) showing synergy with imipenem, acting in a bactericidal manner in combination but exerting only a modest bactericidal effect alone. The β-lactam potentiation activity of murgocil resulted from inhibition of the glycosyltransferase MurG, which catalyzes the final step in peptidoglycan monomer synthesis (conversion of lipid I to lipid II, the substrate for PBPs). Murgocil impaired peptidoglycan synthesis as a result of its inhibition of the biosynthesis of lipid II. Lipid II is required for proper localization of PBP2. Murgocil effected delocalization of PBP2 from the cell division septum, explaining the synergy observed with β-lactam antibiotics [116].

The highly conserved cytoskeletal protein FtsZ is a prime target for antibacterial development due to the essential role it plays in cell division [117]. Its role in the recruitment of PBPs and other downstream components of the divisome means that inhibitors of FtsZ have the potential to enhance the activity of cell wall-acting antibiotics at sub-microbicidal concentrations [118]. For example, the thiazolopyridine PC190723 **61** (Fig. 9), which had been previously developed through a medicinal chemistry program for anti-staphylococcal agents that inhibit FtsZ [119], exhibited synergy with imipenem and other β-lactam antibiotics against MRSA in vitro and in vivo. This synergy was postulated to result from the concomitant delocalization of FtsZ and PBP2 (the respective targets of PC190723 and imipenem) [118]. Another FtsZ inhibitor, the quinuclidine **62**, was identified from a virtual screen and exhibited synergy with methicillin and imipenem against MRSA, in addition to moderate antibacterial activity against several species of bacteria [120]. A loss-of-viability screen identified DNAC-1 **63** as potentiating the activity of oxacillin against MRSA [121]. Similar to PC190723, DNAC-1 elicited the mislocalization of FtsZ and PBP2 (and also PBP4). DNAC-1 **63** exhibited in vivo activity in combination with oxacillin in a murine model of MRSA infection and also exhibited in vitro activity in combination with ceftriaxone against several Gram-negative pathogens including *A. baumannii*, *P. aeruginosa*, *E. coli*, and *K. pneumoniae* [121].

An antisense-interference screen in MRSA to identify auxiliary factors that are involved in resistance to β-lactam antibiotics recently identified several additional genes that were not previously known to play such a role and therefore are potential new targets for adjuvant design. Nva-FMDP **64** (Fig. 9) is an inhibitor of the enzyme encoded by one of these genes, GlmS. GlmS is a glucosamine-6-phosphate

synthase involved in the synthesis of the peptidoglycan precursor [122]. Nva-FMDP had been demonstrated previously to possess antibacterial activity against *B. subtilis* and *S. epidermidis*, but not *S. aureus*. Nva-FMDP strongly potentiated the activity of several β-lactam antibiotics against MRSA [122].

The polyphenol (–)-epicatechin gallate (ECg) **65** (Fig. 9) found in green tea exhibits very little intrinsic antibacterial activity against MRSA but has long been known to suppress resistance to β-lactam antibiotics [123]. ECg does not affect PBP2a production and instead acts by binding to the cytoplasmic membrane and inducing a series of reorganization steps that increase the fluidity of the membrane, increase cell size, thicken the cell wall, elicit changes in production of lysyl phosphatidylglycerol, and culminate in either the delocalization of PBP2 and eventually PBP2a, or the eradication of the cooperation between the two enzymes that is required for cell wall synthesis in the presence of ß-lactam antibiotics [124, 125]. Synthesis of a series of analogs of ECg in which hydroxyl groups were removed in a stepwise manner led to the compound **66**, which lacks two hydroxyl groups from the B ring of ECg. Compound **66** exhibited increased oxacillin potentiation. Removal of additional hydroxyl groups resulted in compounds possessing higher antibacterial activity and reduced resistance-modifying activity [126].

8 Conclusions

New antibiotics are not being developed at a fast enough rate to match increasing bacterial resistance. The development of small-molecule adjuvants offers an additional avenue to combat this significant problem. Several approaches to the identification of adjuvants have been discussed, from the well-known and clinically validated approach of inhibiting β-lactamase enzymes, to more indirect approaches such as inhibiting bacterial signaling and response systems that mediate antibiotic resistance. Also discussed are adjuvants that act by preventing efflux or increasing cellular uptake of antibiotics, adjuvants that inhibit modification of either the antibiotic or its target, and finally the identification of adjuvants that act upon less obvious targets such as nonessential steps in bacterial cell wall synthesis.

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Allosteric Inhibition of Bacterial Targets: An Opportunity for Discovery of Novel Antibacterial Classes

Jayda E. Meisel, Jed F. Fisher, Mayland Chang, and Shahriar Mobashery

Abstract Small molecules that act through an allosteric mechanism to modulate activity of a protein target are abundantly represented in the pharmacopeia. These allosteric modulators are, however, preeminently therapeutics for eukaryotic diseases rather than therapy for prokaryotic infection. Recent examples of the success of biochemical and computational screening methods, paired with protein structural characterization, underscore the promise of allosteric activity modulation as a new approach for antibacterial discovery. In this review, we show how allostery has been leveraged to this objective. In particular, exploitation of an allosteric site on penicillin-binding protein 2a – the resistance enzyme of methicillin-resistant *Staphylococcus aureus* – demonstrates both that allosteric-modulating structures may themselves possess antibiotic activity and additionally may act as synergists within multi-drug combinations. Future discovery strategies against both old and new bacterial targets may exploit the opportunities offered by allosteric checkpoints within critical bacterial pathways.

Keywords β-lactams, Adjuvant, Aminoglycosides, MRSA, PBP2a, Synergy, Virtual screening

Contents

J.E. Meisel, J.F. Fisher (✉), M. Chang, and S. Mobashery (✉)
University of Notre Dame, Notre Dame, IN 46556-5710, USA
e-mail: jfisher1@nd.edu; mobashery@nd.edu

1 Introduction

The first chemical entities in the golden age of antibiotic discovery – the sulfonamides, the β-lactams and the aminoglycosides – were *orthosteric* inhibitors (that is, compounds that act directly against active sites, whether of the ribosome or of enzymes) of critical bacterial pathways. This same characteristic holds for many of the succeeding clinical classes of the antibiotics. After 80 years, however, the luster of the antibiotics as testimony to the power of medicinal chemistry has dimmed [1, 2] in the face of the inexorable and ineluctable spread of time-proven antibiotic-resistance mechanisms [3]. While we have not met the antibiotic apocalypse [4], the realization amongst those in science is universal: our antibiotic pharmacopeia requires immediate and diligent husbandry and expansion [5]. A number of strategies toward these objectives are being explored, and include (beyond the discovery of new chemical matter directed against both validated and new structural targets) the renaissance of old antibiotics, the repurposing of existing drugs, and the pairing of antibiotics for the purpose of mutual synergy [6–11]. The focus of this review is a particular mechanistic intersection of these strategies, that of *allosteric* modulation (antibiotics acting at sites independent of, but functionally linked to the active site) of critical bacterial "checkpoints" to attain direct, or synergistic, antibiotic activity.

The pairing of allosteric mechanism with antibiotic activity implicates interference of critical conformational motion so as to compromise an antibiotic target. The importance of conformational motion in proteins is recognized at every level of study of structural biology. Consider, for example, that under ideal circumstances bacteria replicate on a multiple-minute time scale. While the circumstance of bacterial infection is far from ideal bacterial growth, the compression of this timescale is a forceful reminder that the single-celled bacterium is fully self-contained and has perfected the synchronized replication of the pathways that produce its nucleotides, proteins, lipids, and carbohydrates. A complement to this macroscopic perspective is the further realization that at the microscopic level, many bacterial proteins possess highly dynamic structures (apparently) to enable this synchronization. A list of bacterial proteins that undergo large-scale conformational motion includes in the cytoplasm the ribosome [12, 13], the AmpD amidase [14] and NagZ glycosidase [15] enzymes of peptidoglycan recycling; in the inner membrane the MraY enzyme of peptidoglycan biosynthesis [16]; in the periplasm the BamA protein of the Bam assembly apparatus [17]; and at the periplasm-outer membrane interface the MltF lytic transglycosylase [18] and the OmpA porin [19, 20]. Some of these proteins (such as MraY [21]) are validated antibiotic targets, while others are speculative targets. Notwithstanding the lack of

connectivity among these examples, in each case dramatic conformational change may be conceptualized as relating directly to its function. Although the mechanistic purpose of the conformational change is often speculative, the pairing of dynamics and function intuitively argues for it. For example, in Gram-positive bacteria the biosynthetic pathways of its cell-wall peptidoglycan and of its cell-wall teichoic acid are fully coordinated around the large-scale transmembrane translocation of precursors, and a consequence of this interdependency is opportunity for antibiotic discovery [22–26]. Structure that compromises key conformational motion may be antibacterial. These structural opportunities may abound.

The idealized criteria for the antibacterial structure are well recognized [27]. A potential limitation of exploratory structures that act at orthosteric sites (and as substrate or transition-state mimics, achieve high inhibitory potency in in vitro assay) is resistance development as a result of the bacterium devising a by-pass strategy that circumvents the loss of function of their target. An alternative strategy that may forestall such by-pass resistance is structures that are antibacterial as a result of their binding to a regulatory site of a target protein. These ligands – allosteric modulators – may lack structural resemblance to the substrate, and thus may be less susceptible to by-pass resistance that results from overexpression within the pathway leading to the target, and thus achieving competitive displacement of the orthosteric inhibitor. Indeed, allosteric targeting may be seen as broadening the chemical space available to select for the protein target, and moreover to exploit regions of the target protein that may be less susceptible to resistance pressure [28–30]. While the requirement that the bacterium not be able to bypass the loss-of-function remains for both orthosteric and allosteric inhibition, if modulating an allosteric site on a proven worthy target favorably abets a proven orthosteric inhibitor of the protein, enhanced antibacterial activity may result. In other areas of medicinal chemistry the development of allosteric drugs is a dominant strategy for drug discovery, notably with respect to diseases of the central nervous system [31–33]. The exploitation of allosteric inhibition has value with respect to the breadth of human disease [34].

It is appropriate here that we qualify how the term “allosteric” fits to this purpose. To the biochemist the concept of allostery includes any binding event occurring at a site other than the orthosteric site that modulates the activity (either positively or negatively) of the protein. Allostery can be as simple as a single non-orthosteric ligand-binding event, or it can be as complex as a single ligand-binding event preventing (or promoting) formation of multi-protein complexes to modulate cell–cell signaling [35]. The allosteric site of the protein may be functional, or it may be cryptic [36–41]. Here, we first conceptualize allostery with the use of examples of allosteric targeting in drug discovery in eukaryotic systems. We stress the general absence of the application of this strategy in antimicrobial discovery efforts, and provide examples of new structures that use an allosteric mechanism to elicit antibiotic activity. We highlight this exemplification with a new small-molecule antibiotic class – with demonstrated efficacy in vitro and in vivo – that exploits an allosteric mechanism against penicillin-binding protein 2a, the resistance enzyme of methicillin-resistant *Staphylococcus aureus* (MRSA).

We conclude by drawing attention to other possible proteins that present opportunity for antibacterial discovery by allosteric interference mechanisms.

2 Allostery: Modern Interpretation and Examples in Therapeutic Discovery

Our understanding of the role of allostery in protein function has evolved since the recognition more than 50 years ago that oxygen binding to one subunit of the hemoglobin influences oxygen binding to the other subunits [42, 43]. That a ligand (albeit in the case of hemoglobin, oxygen is also the "substrate") could regulate substrate binding and/or catalysis was then a revelation. Seminal studies over the years also documented that small molecules can exert additional effects on oxygen binding by hemoglobin. These include the effects of the chloride ion, of carbon dioxide (covalent and non-covalent), and of diphosphoglycerate. Classical models of protein allostery – the Monod-Wyman-Changeux (MWC) and the Koshland-Nemethy-Filmer (KNF) models – accepted conformational change (regarded as binary in nature) as the hallmark of allosteric regulation (see, however, [44] for further discussion on this criterion). Whether the conformational change is pre-selected by the ligand (conformational selection) or occurs as a consequence of ligand binding (KNF; induced-fit) is a continuing debate [45]. Kinetic models cannot differentiate between the two. Over the past 20 years the allosteric paradigm has matured into a descriptive model wherein proteins possess a statistical ensemble of conformational states, with each state having a unique location on the free-energy landscape of the protein [46].

The most successful examples of an allostery mechanism for the amelioration of disease are against eukaryotic targets. A recent example is the development of anticancer therapeutics against non-receptor protein-tyrosine phosphatases (PTPs). Notwithstanding both potency ($\leq$0.2 μM) and murine efficacy in a glioma model [47], active-site inhibition strategies against the PTPs have been unsuccessful. The reasons for this failure included poor selectivity as a result of the highly conserved active sites among PTP isoforms, poor oral bioavailability, and poor cell permeability [48, 49]. Recently, researchers at Novartis [50] used a high-throughput screen based on an in vitro fluorescence-based phosphatase assay to identify new chemical matter to stabilize the autoinhibitory (closed and inactive) conformation of SHP2 (see also [51]). SHP2 was the first non-receptor tyrosine phosphatase reported to harbor oncogenic activity [52]. Active-site inhibitors were removed by cross-screening against a truncated protein containing only the active-site domain. Structural analysis of inhibitor-enzyme complexes and thermal stabilization experiments confirmed that the lead inhibitor bound to a previously unidentified allosteric site, which stabilized an inactive state of the enzyme. Further kinetic characterizations and xenograft mouse evaluations support this mechanism for the robust and selective inhibition of SHP2 [53].

A second example is the control of the sodium- and chloride-dependent re-uptake of serotonin into presynaptic neurons by the serotonin transporter (SERT) protein. Deregulation of SERT (and other sodium-dependent neurotransmitters, such as for dopamine and norepinephrine) is a hallmark of neurological and psychiatric disorders [54]. In 2016 crystal structures of human SERT complexed with citalopram (Celexa®) and with paroxetine (Paxil®) demonstrated that these antidepressants bind both to a central cavity and also to a 13-Å distant allosteric site [31]. Their efficacy arises from their ability to prevent serotonin dissociation from the SERT [55]. This example underscores a fundamental difference between allosteric and orthosteric inhibition. The realization that a protein possesses a free-energy landscape expands our thinking of their structure as a "druggable" target. Rather than a binary distribution of active and inactive states, proteins may possess a distribution of states as clearly documented in the case of hemoglobin. Allosteric modulation is a way to turn up or to turn down – not just turn on or off – molecular pathways of therapeutic importance.

3 Examples of Allosteric Targeting in Antimicrobial Discovery

3.1 Aminoglycoside-Modifying Enzymes

Examples of clinically successful drug classes that exploit allosteric targeting are abundant for eukaryotic systems [56] but rare for prokaryotic systems. The aminoglycosides represent one example of clinically successful antibacterials, wherein both their mechanism of action as antibiotics and the mechanism of action of resistance enzymes for the class exemplify allosteric character. The chemistry within the A site of the bacterial ribosome is responsible for high-fidelity translation of the genetic information in mRNA to protein. The A site of the 30S ribosomal subunit serves as the target of aminoglycoside antibiotics [57]. The large-scale conformational changes that transmit molecular information between functional centers of the subunits – often tens of Ångstroms apart – suggest opportunity for allosteric interference. Indeed, the mechanism of aminoglycoside inactivation of this target, wherein the remodeling of the intersubunit bridges of the ribosome assembly that is required for peptide synthesis is blocked, conforms to a medicinal chemistry understanding of an allosteric mechanism [58–60]. The major resistance mechanism against the aminoglycosides is their modification by aminoglycoside-modifying enzymes (AMEs) including acetyltransferase, phosphotransferase, and nucleotidyltransferase activities [61]. In 2005 Kohl et al. presented structural evidence for an allosteric-inhibition mechanism, by an ankyrin repeat protein inhibitor, against a bacterial phosphotransferase [62]. As this bacterial kinase is orthologous to eukaryotic kinases, the important suggestion was made that appropriate in vitro screens might identify inhibitors of this resistance-conferring kinase

[63]. Indeed, new classes of in vitro AME inhibitors emerged from allosteric-site docking and scoring of the ZINC library of compounds against the *E. faecalis* phosphotransferases (APH) 3′-IIIa and 2′-IVa [64]. While the K_i values of the best compounds are too modest (9–85 μM) to have therapeutic value, the data obtained with APH 3′-IIIa – non-competitive inhibition in the presence of the co-substrates ATP and kanamycin – supports the conclusion of an allosteric mechanism [64]. This opportunity is not restricted to the APH class of the AMEs. The demonstration of allosteric communication between the active sites of the homodimeric *E. faecium* acetyltransferase AME, combined with the determination of the structure of this enzyme, would enable structure-based discovery of allosteric inhibitor against this AME target [65, 66].

3.2 *The PBP2a Enzyme of Methicillin-Resistant* Staphylococcus aureus

A powerful example of the successful union of virtual screening to an allosteric mechanism is the serendipitous discovery of allosteric regulation abetting the resistance enzyme penicillin-binding protein 2a (PBP2a) used by *Staphylococcus aureus* against the β-lactam (as exemplified by methicillin) antibiotics. The β-lactams are orthosteric inhibitors of the essential transpeptidase activity of the PBPs used in the biosynthesis of peptidoglycan cell wall. The preferred resistance mechanism used by Gram-positive bacteria (such as *S. aureus*) against the β-lactam antibiotics is target mutation [67]. A prescient deduction early in the study of the β-lactams was their structural mimicry of the -D-Ala-D-Ala structure of the peptide stem that is the substrate for transpeptidation [68]. PBP catalysis of transpeptidation uses an active-site serine of the PBPs to transfer the acyl moiety derived from the penultimate D-Ala (with release of the terminal D-Ala as a leaving group) to an amine of an adjacent peptidoglycan strand [69–71]. This transfer completes transpeptidation by the creation of the essential crosslinks of the peptidoglycan polymer. In contrast, when this same active-site serine is acylated by a β-lactam, the resulting acyl-enzyme is stable ($t_{1/2}$ = 26–77 h) [72] and the enzyme is inactivated [73]. This loss of PBP enzymatic activity is ultimately bactericidal [74]. Most (>60%) clinical strains of *S. aureus* today are β-lactam (methicillin)-resistant [75]. Methicillin-resistant *S. aureus* (MRSA) differs from methicillin-susceptible *S. aureus* (MSSA) due to the presence of an additional PBP (PBP2a) that is intrinsically unreactive to virtually all β-lactam antibiotics [76]. PBP2a performs the critical transpeptidase reaction when the transpeptidase activity of the other PBPs is lost to β-lactam inactivation.

Allosteric Modulation of PBP2a The key question concerning the PBP2a resistance mechanism is the structural basis for endogenous substrate recognition – the -D-Ala-D-Ala dipeptide terminus of stem structure of the peptidoglycan – while selectively discriminating against the β-lactam as a substrate mimic. Comparison

of the apo-enzyme and ceftaroline-acylated PBP2a structures suggested this discrimination occurs both through a disfavored conformation of the active-site serine for reaction with the β-lactam and an active site that is closed preferentially to the β-lactam by gatekeeper residues [77] (see active site conformation in apo-PBP2a in Fig. 1a versus that in ceftaroline-bound PBP2a 1b). Moreover, these structures demonstrated the necessity for dramatic conformational change of PBP2a in order for its active site to accommodate the two distinct stems (one the acyl-donor, and the other the acyl-acceptor) of its peptidoglycan substrates [72]. An allosteric foundation for these events was provided by kinetic studies that revealed significantly increased acylation rates of PBP2a in the presence of synthetic peptidoglycan [78]. Binding of substrate structure – presumably at an allosteric site separate from the active site – would simultaneously open the gatekeeper residues, alter the serine conformation, and increase the accessibility of the active site to its

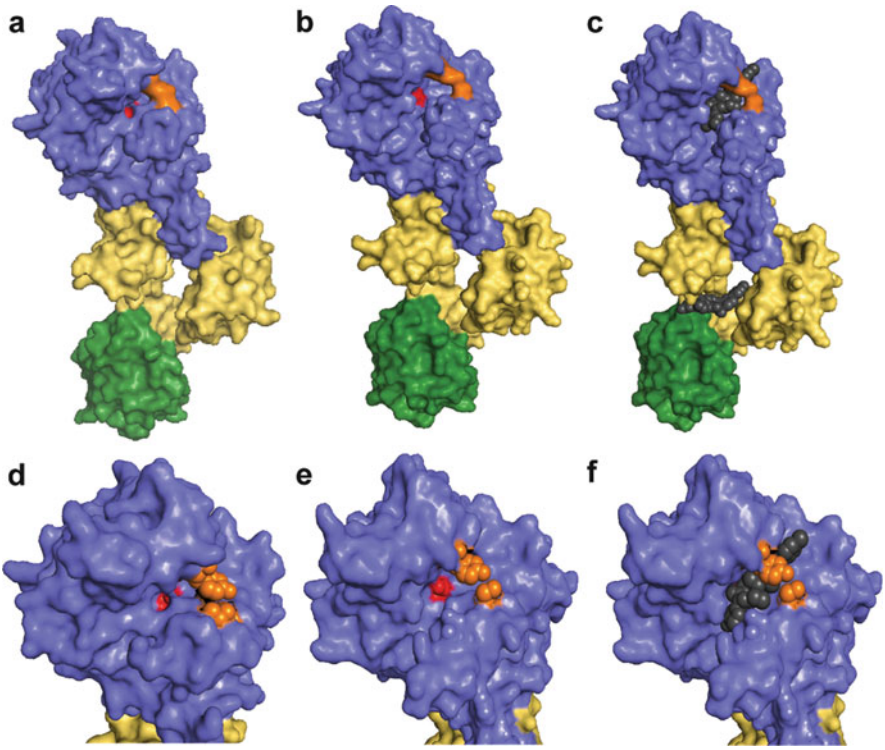

Fig. 1 View of PBP2a as (**a**) apo PBP2a (1VQQ), (**b**) PBP2a with ceftaroline bound at allosteric and active site (3ZG0) with ceftaroline molecules not displayed, and (**c**) 3ZG0 with bound ceftarolines displayed at both active and allosteric sites; transpeptidase domain is highlighted in *blue*, allosteric domain in *yellow*, and the N-terminal domain in *green*; catalytic serine (Ser403) is highlighted in *red*, and gatekeeper residues Tyr446 and Met641 are highlighted in *orange*; closer views of the active site are displayed for 1VQQ in (**d**), 3ZG0 in (**e**) with ceftaroline not shown, and 3ZG0 with ceftaroline shown in (**f**)

peptidoglycan substrates. Computational analysis drew attention to surface-accessible pockets in PBP2a associated with two major grooves [79]. Subsequent crystal structures of PBP2a bound to synthetic peptidoglycan, and acylated at the active-site serine by the β-lactam ceftaroline (Fig. 1b, c), confirmed this presumption [80]. Ceftaroline is a cephalosporin β-lactam with clinical efficacy against MRSA. In these structures the peptidoglycan was bound non-covalently in a site distant (by 60 Å) from the active site and is a site now recognized as enabling allosteric control of PBP2a catalysis. PBP2a crystal structures obtained with ceftaroline showed two bound ceftaroline molecules: one covalently-bound ceftaroline at the active site and one non-covalently-bound ceftaroline at the allosteric site (Fig. 1b, c). The ceftaroline acyl-enzyme shows an increase in the active-site volume from ~500 to ~1,300 $Å^3$ to accommodate the ceftaroline molecule. Comparison of the apo-PBP2a and PBP2a-ligand structures identified a salt-bridge network across the surface of the protein that is allosterically responsive to ligand occupancy of the allosteric site [81]. The differences between the apo-PBP2a structure (1VQQ) and the ceftaroline-bound structure (3ZG0) with respect to these salt pairs are shown in Fig. 2. Mutations in PBP2a that confer clinical resistance to ceftaroline (including the double mutant N146K/E150K and the triple mutant N146K/E150K/H351N) act to prevent ceftaroline from serving as an allosteric trigger to affect alteration of the salt-bridge interactions and thus open the active site [82]. Mutations within the salt-bridge network showed a decreased rate of acylation (and thus decreased susceptibility to inactivation). In two mutants, wild-type acylation rate could not be recapitulated in the presence of synthetic peptidoglycan, suggesting disruption of signal transmission from the allosteric to the active site [80]. Mutations in PBP2a conferring clinical resistance (cyan residues of Fig. 3) to ceftaroline include the double mutant N146K/E150K (moderate resistance), the N146K/E150K/H351N triple mutant (moderate resistance), Y446N (severe resistance), and the double mutant Y446N/E447K (severe resistance). Mutations conferring clinical resistance to a second anti-MRSA cephalosporin, ceftobiprole, include E239K, Y446L, E150K, S649A, F467Y, R589K, E447K (also cyan residues of Fig. 3).

3.2.1 Antibacterial Quinazolinones Act as Allosteric Modulators of *S. aureus* PBP2a

Notwithstanding the very different amino-acid environment between the active site and allosteric site of PBP2a, both sites recognize both peptidoglycan and β-lactam structure. A virtual screening effort identified a third synonymous structure. The active site of the apo-PBP2a crystal structure was used for the in silico docking-and-scoring of a >1.2 million compound subset of the ZINC database [83]. The top 20% scorers were winnowed by rigorous computational analysis to a sub-set of 2,500 compounds. This set was grouped by structural similarity. Compounds purchased from each structural group were evaluated for antibacterial activity, culminating in the discovery of quinazolinone **1** (Fig. 4). The quinazolinone class

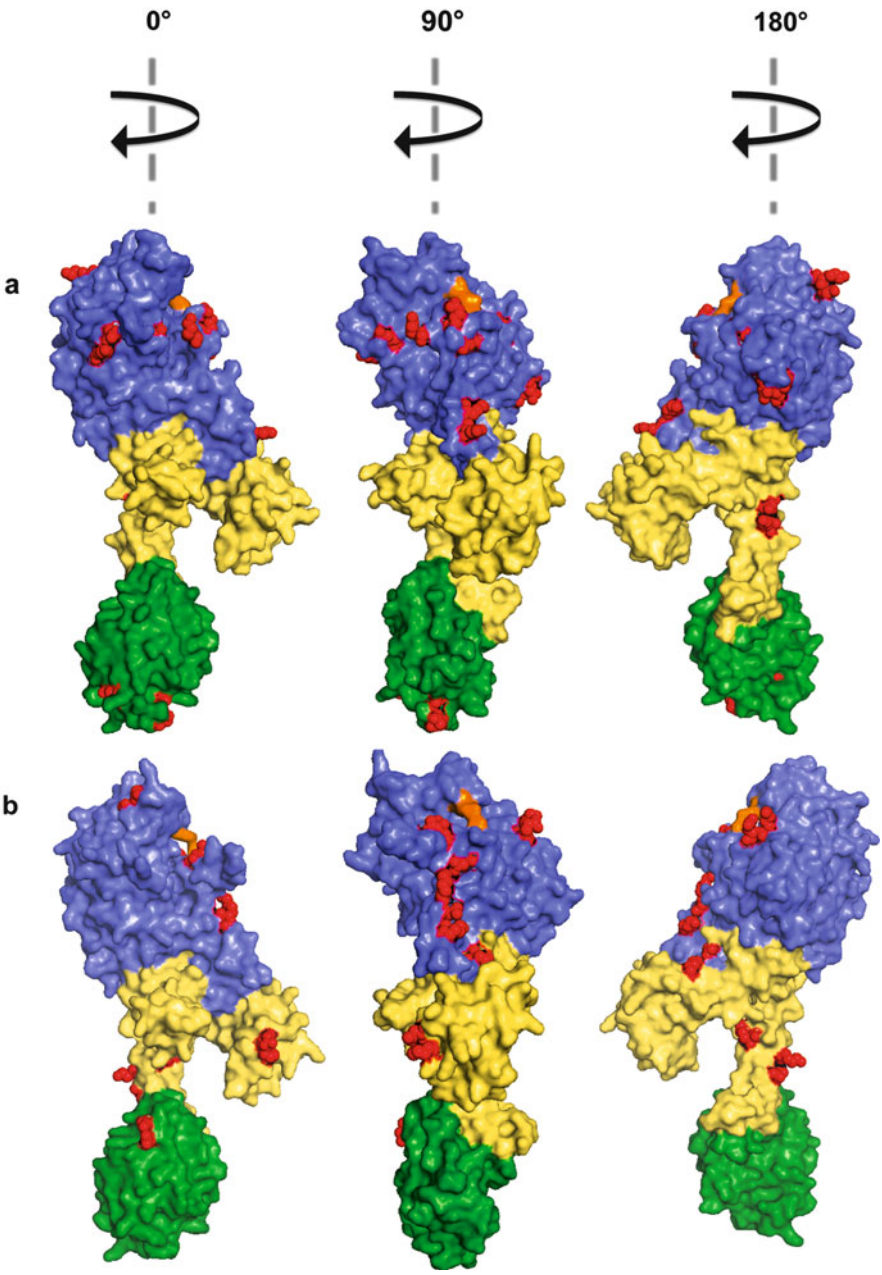

Fig. 2 Unique salt-bridge residues (in *red*) identified in (**a**) apo PBP2a (1VQQ) and (**b**) ceftaroline-complexed PBP2a (3ZG0) with transpeptidase highlighted in *blue*, allosteric domain in *yellow*, and N-terminal domain in *green*; active- and allosteric-site bound ceftaroline are not displayed in structures in (**b**) to allow for clearer comparison between the two panels; gatekeeper Tyr446 and Met641 are highlighted in *orange* and obscure view of active site Ser401; in each panel, structures from left to right are 90° rotations of the left-most structure

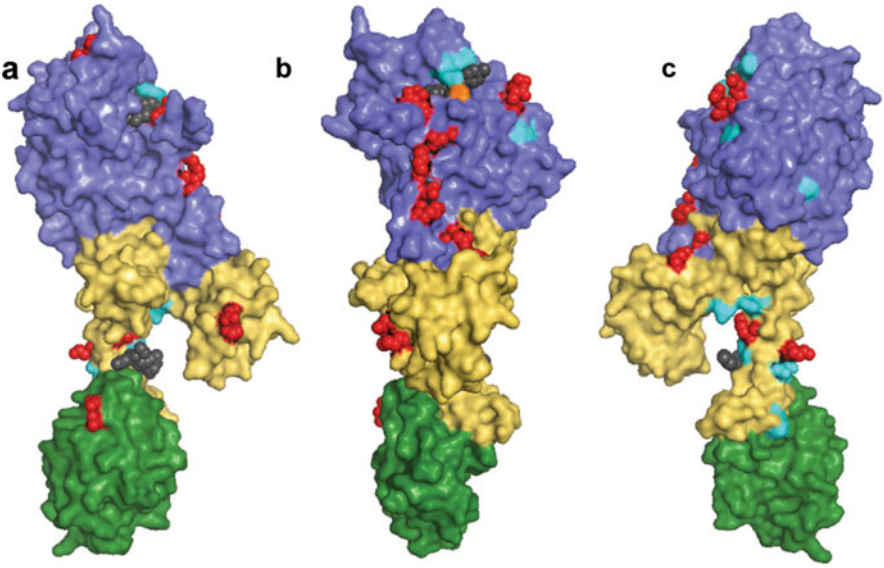

Fig. 3 Clinically reported mutations in MRSA PBP2a (3ZG0) that confer ceftaroline and/or ceftobiprole resistance (*cyan*); salt-bridge residues (*red-sphere* representation) are identical as those displayed in Fig. 2b; bound ceftaroline molecules are displayed in *black*; gatekeeper residue Met641 is displayed in *orange*. In each panel, structures from *left* (**a**) *to right* (**b** and **c**) are 90° rotations of the preceding structure. Two mutations F467Y and R589K that confer ceftobiprole resistance are buried in the transpeptidase domain and are not visible

represented by **1** has potent antibacterial efficacy against methicillin-resistant *S. aureus* strains [84, 85]. The most active analogs were evaluated for toxicity, protein binding, metabolic stability, pharmacokinetic properties in mice, and efficacy in the murine peritonitis and murine neutropenic thigh models of infection. Proof that this class inhibited PBP2a was obtained from the ability of compound **2** to competitively suppress the acylation of PBP2a (as well as this reaction of a second PBP of *S. aureus*) by a fluorescent β-lactam, and from its competitive suppression of the incorporation of a radiolabeled precursor into the bacterial peptidoglycan [84].

The 1.95-Å resolution crystal structure of compound **2** bound to PBP2a (Fig. 5) revealed that **2** was bound to the allosteric site, and not to the active site. The unoccupied and open active site seen in this structure displayed significant conformational change as compared to the structure of the apo-enzyme, reflecting the occupancy of **2** at the allosteric site. Intrinsic fluorescence experiments with **2** and recombinant PBP2a confirmed allosteric-site binding ($K_d = 7 \pm 2$ μg/mL). Compound **2** was evaluated with several PBP2a variants with mutations at the active site and at putative salt-bridge connecting sites. The single mutant Y446N and the double mutant Y446N/E447K showed the largest loss in affinity for **2** at the active site [85]. This outcome is notable because these residues are close to, but not part of the active site. Because these mutations attenuate significantly the inhibition of

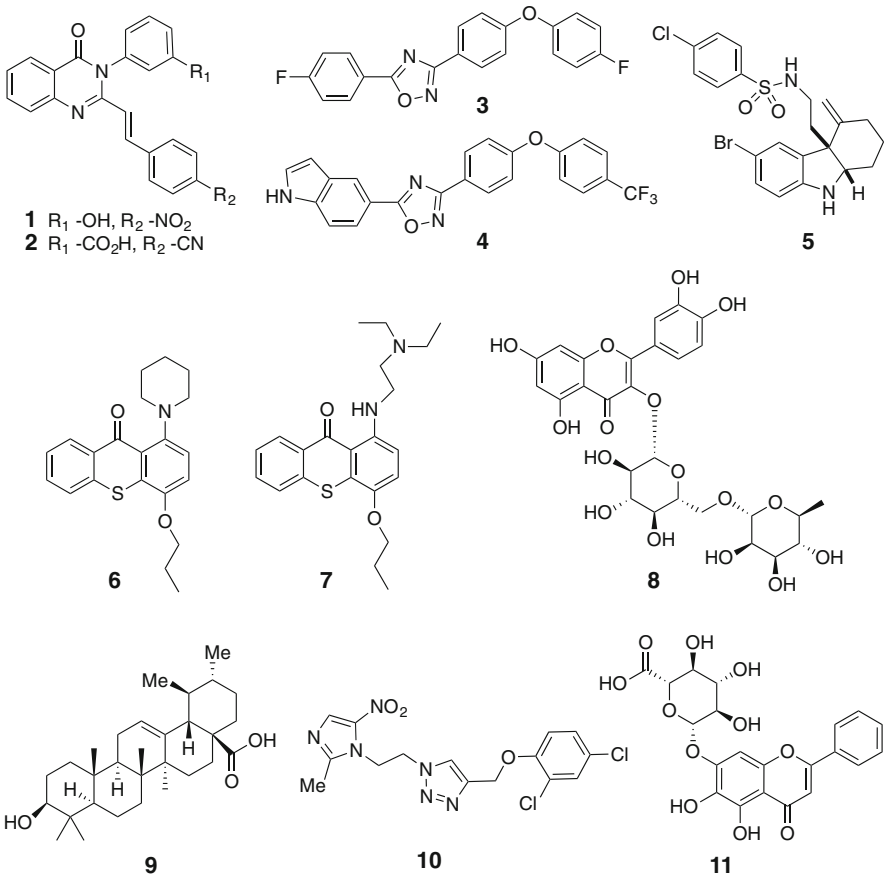

Fig. 4 **1**–**2** Lead compounds of the quinazolinone class [81, 82, 84, 85]; **3**, **4** Oxadiazole leads with activity against MRSA [86, 87]. Compound **4** displays synergy with oxacillin in vivo [88]; **5** Indoline alkaloid lead from Podoll et al. [89]; **6**, **7** Thioxanthone leads from Bessa et al. [90]; **8** Quercetin 3-rutinoside; **9** Ursolic acid; **10** Metronidazole-triazole derivative lead identified from docking and scoring to PBP2a active site from Negi et al. [91]; **11** Baicalin

PBP2a by compound **2**, quinazolinone binding to the active site is implied. Mutants Y105A/Y297A and E239K also affect active-site binding. The Y105A/Y297A mutations also affect ligand binding at the allosteric site significantly ($K_d = 31 \pm 12$ μg/mL). An understanding of the relationship between PBP2a allosteric-site affinity and the salt-bridge network as a means of communication between the active and allosteric sites is an ongoing effort [81].

The possibility that the quinazolinones would synergize with β-lactams was evaluated. Ceftaroline itself synergizes in vitro with imipenem and meropenem against three strains of MRSA, suggesting the possibility of combination β-lactam therapy for MRSA infection [92]. In particular the triple β-lactam combination of

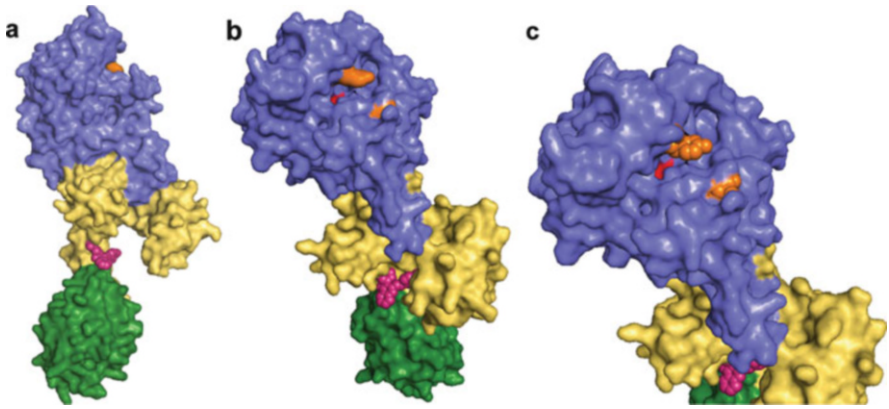

Fig. 5 (**a**) Crystal structure (4CJN) of quinazolinone **2** (*pink*) bound to the allosteric site of PBP2a; (**b**) top view of the active site with Ser403 highlighted in *red* and gatekeeper residues highlighted in *orange*; (**c**) a closer view of the active site with gatekeeper residues represented in *sphere* form [84, 85]

meropenem/piperacillin/tazobactam was efficacious in a neutropenic mouse peritonitis model of MRSA infection [93]. While synergy was observed only for the binary combinations of meropenem/piperacillin (FICI = 0.44) and piperacillin/tazobactam (FICI = 0.22), a 3D isobologram of the triple β-lactam combination suggested synergy optimally coinciding with the 1:1:1 combination (2 μg/mL of each antibiotic). The control experiment using a sensitive (MSSA) *S. aureus* strain (lacking PBP2a) indicated this combination here was additive, and not synergistic. Xylose-inducible anti-sense RNA experiments confirmed additional targeting of the triple combination against PBP1 and PBP2. These observations are consistent with an ability of the meropenem (K_d = 270 μM) of the meropenem/piperacillin/tazobactam combination to affect allosteric triggering. Comparison with mean plasma concentrations suggests that meropenem secures the allosteric triggering in vivo. More generally, any small molecule (β-lactam or otherwise) that demonstrates significant affinity to the allosteric site of PBP2a could function as an allosteric trigger in a dual or triple antibiotic combination.

The realization that the β-lactam and quinazolinones are synonymous structures with affinity for two separated sites of the PBP2a enzyme precludes a simple answer as to whether the antibacterial activity of the quinazolinones is the result of orthosteric inhibition or allosteric modulation. Rather, the data decisively indicate allosteric communication between the sites, and the added value provided by the in vitro and in vivo assays designed upon this communication. Future drug design within the quinazolinone class will benefit from the understandings from these assays, and from the understandings of the complexities of the β-lactam resistance mechanism used by MRSA. A second PBP of *S. aureus*, PBP4, is now recognized to contribute [94–98]. The involvement of PBP4 in MRSA resistance establishes a new medicinal chemistry objective (dual PBP2a- and PBP4-inhibition).

3.2.2 Other Structures as Possible PBP2a Allosteric Modulators

The identification of synergistic antibacterial combinations is an active area of research [11]. We summarize recent observations on this topic using MRSA as the therapeutic focus. In contrast to the quinazolinones (for which there is extensive experimental support) the mechanistic basis for the synergy observed with these structures is speculative.

Oxadiazoles The oxadiazole class of Gram-positive antibacterials inhibits peptidoglycan synthesis in MRSA, is bactericidal [86, 87], and is believed to target PBP2a. The class (exemplified by compound **3**, Fig. 4) was discovered (like the quinazolinones) by the iterative docking-and-scoring of structures from the ZINC database against the active site of PBP2a [86, 99]. Oxadiazole binding to PBP2a was demonstrated using a chromogenic β-lactam assay. Efforts to secure an X-ray structure of an oxadiazole-PBP2a were unsuccessful. In vitro studies of the lead oxadiazole structure **4** in combination with β-lactams show synergy, and **4** synergizes with oxacillin in a mouse model of infection. Compound **4** is statistically equivalent to, or better than, the standard of care linezolid [88].

Indoline Alkaloids A bio-inspired library of indolines (120 compounds) was screened to identify potentiators of β-lactam activity against the multi-drug resistant BAA-44 MRSA strain [89]. Although none of the compounds tested displayed antibacterial activity alone (MIC >128 μg/mL), nine compounds sensitized BAA-44 to methicillin. One of the nine (compound **5**, Fig. 4) sensitized BAA-44 to all β-lactams tested with varying efficacy, but did not potentiate methicillin, amoxicillin/clavulanic acid, and cefazolin in a methicillin-sensitive strain (MSSA). These observations are consistent with the likely targeting of PBP2a. The structure-activity relationship for this class, and rigorous evaluation of its mechanism of action, is being explored [100–102]. However, as of yet there have been no in vivo efficacy studies performed with this class. Other indole-based structures synergize with the quinolone antibacterials (non-β-lactam structures) in multi-drug resistant *S. aureus*, although here likely as a result of inhibiting the NorA drug-efflux pump NorA [103]. Future studies with this class (and other indole-based potentiators) have promise.

Thioxanthones A screen of 40 thioxanthone derivatives identified nine compounds that displayed modest antibacterial activity against both susceptible and drug-resistant *S. aureus*. Two derivatives (**6** and **7**, Fig. 4) strongly synergized with the β-lactams oxacillin (FICI = 0.1–0.2) and ampicillin (FICI = 0.3) against the B1 MRSA strain [90]. Growth curves with **6** and oxacillin (128/8 μg/mL), and **7** with oxacillin (4/8 μg/mL) indicate a bacteriostatic mechanism. Compounds **6** and **7** alone (both at 8 μg/mL) are also bacteriostatic. Docking and scoring studies suggest that **6** and **7** bind to the allosteric site of PBP2a, and act by allosteric potentiation of oxacillin [90].

Quercetins An in silico screen of a quercetin derivative predicted binding at both the PBP2a active and allosteric sites. Quercetin 3-*O*-rutinoside (**8**, Fig. 4) was the most favorable binding partner with PBP2a. It was predicted to bind at the allosteric site of PBP2a in a manner that is similar to ceftaroline and ceftobiprole [104, 105]. However, in vitro data do not support this conclusion. For example, quercetin 3-rutinoside suppressed MRSA growth better compared to in combination with the β-lactam cefixime (although the magnitude of the inhibition is modest) [105]. In a separate report, synergy of unmodified quercetin with the β-lactam cefotaxime was not observed [106]. In addition a 3–4 $\log_{10}$ reduction was reported for unmodified quercetin combined with oxacillin and ampicillin, along with possible synergy with other non-β-lactam antibiotics (the aminoglycoside gentamicin, the macrolide erythromycin, and fluoroquinolones) [107].

Ursolic Acid Ursolic acid (**9**, Fig. 4) has very modest antibacterial activity (MIC = 16 μg/mL for MSSA, 64 μg/mL for MRSA) and synergizes with both ampicillin and tetracycline against both methicillin-sensitive and resistant strains of *S. aureus* [108]. This observation suggests a non-PBP2a mechanism. This conclusion is supported further by reports that other pentacyclic triterpenoids show synergy with vancomycin (again, both methicillin-sensitive and resistant strains) and also with the β-lactams methicillin, ampicillin, and oxacillin [109, 110].

Metronidazole-Triazoles A series of metronidazole-triazoles showed anti-MRSA activity (MIC 4 to >128 μg/mL) against 30 MRSA strains. The 1:1 combination with the β-lactam oxacillin displayed synergy (MIC 1–8 μg/mL, depending on the strain) [91]. This study also reported docking/scoring studies with the PBP2a active site, and interpreted the results in terms of the most active compound (**10**, Fig. 4) having a similar affinity for the PBP2 active site as oxacillin. Computational evaluation of this hypothesis was not undertaken. Comparison with methicillin-sensitive strains likewise has not been performed. Since metronidazole itself disrupts DNA synthesis [111] these comparisons are critical to the support a PBP2a-mediated mechanism.

Flavones The flavone derivative **11** (Fig. 4) from *Scutellaria baicalensis* displayed synergy (FICI <0.5) with the β-lactam cefazolin in four out of the five MRSA strains tested (SA01–SA05) [112].

4 Allosteric Discovery Opportunities

PBP2a exemplifies the discovery of antibacterial structure that culminated in the discovery of an allosteric (or synergistic) inhibitory mechanism. In key respects, however, PBP2a is exceptional. The importance of PBP2a as a resistance mechanism and its three-dimensional structure were both known. The discovery of its allosteric mechanism was a consequence of the structure arising from, rather than the instigation of, the virtual-screening effort. For the vast majority of critical

proteins at the nexus of key bacterial pathways, neither their identity (as checkpoints) nor their respective structures would be known. Furthermore, the knowledge of whether a given protein might be regulated by allostery can presently be gleaned from detailed mechanistic understanding of the function of the protein. Such knowledge is lacking for the vast majority of the bacterial proteins. Accordingly, we have parsed our further discussion into concise perspectives on discovery based on proteins of known structure surmised as checkpoints, and on methods for the discovery of targets for allosteric intervention. The following examples represent inchoate (to different degrees) efforts to implement allosteric antibacterial discovery.

Undecaprenyl Pyrophosphate Synthase The key lipid intermediate undecaprenyl diphosphate (UPP) unquestionably is a decisive molecular entity at the intersection of peptidoglycan and teichoic acid biosynthesis in Gram-positive bacteria [113], and peptidoglycan and lipopolysaccharide biosynthesis in Gram-negative bacteria [114, 115]. UPP is biosynthesized by the UppS synthase enzyme. UppS is subject to allosteric control, possibly involving a cryptic binding site [116, 117]. UppS catalyzes the successive elongation of its substrate farnesyl pyrophosphate (FPP, a 15-carbon-containing substrate) to form undecaprenyl pyrophosphate (UPP, a 55-carbon-containing product). During substrate binding and elongation the protein adopts a "closed" conformation in which a flexible loop covers the entrance to a β-sheet core. It is thought that as elongation progresses toward UPP synthesis two structural changes occur: (1) the flexible loop moves away from the substrate-binding site into an "open" conformation and (2) the β-sheet core (also termed the "hydrophobic cleft") expands. The amino acids at the base of the β-sheet cleft control substrate elongation, and the mobile loop at the top of the barrel may control substrate binding. UppS is the focus of several independent drug discovery efforts. These efforts include both virtual screening [118] and high-throughput library screening [113, 119–122]. Each of these library screens identified nanomolar-potency, lead orthosteric inhibitors. Concha et al. reported a new class of bacterial UppS inhibitors that bind at the base of the β-sheet cleft [123]. This class is suggested to impede substrate elongation within the hydrophobic β-sheet cleft. This proposed mechanism is consistent with the overall hydrophobicity of the inhibitors (clogP >5), a value high enough to suggest undesirable pharmacokinetics and as a consequence, diminished in vivo efficacy [123]. A review of UppS inhibition emphasizes the medicinal chemistry transition to leads that target the protein structure of the active site, and at the bottom of the hydrophobic pocket [121].

MraY Translocase The integral-membrane protein phospho-MurNAc-pentapeptide translocase (MraY) performs the first reaction in the lipid cycle of bacterial peptidoglycan biosynthesis by conjugating UDP-MurNAc-L-Ala-γ-D-Glu-*m*-DAP-D-Ala-D-Ala with undecaprenyl phosphate (UPP) to form undecaprenyl-diphospho-MurNAc-pentapeptide (lipid I) [21]. Its phosphotransfer reaction is critical to peptidoglycan synthesis, and its function is validated by numerous antibacterial natural products, including a caprazamycin derivative (CPZEN-45) that is currently in clinical trials as a therapeutic against *Mycobacterium tuberculosis* infection.

Substantial progress has been made over the past 25 years toward the purification and assay of the activity of this enzyme. Two crystal structures of MraY (both from *Aquifex aeolicus*) show apo $MraY_{AA}$ as a homodimeric protein, and with the interface of the ten transmembrane helices of each monomer forming a hydrophobic tunnel [124]. A catalytically requisite Mg^{2+} ion interacts with one of the three essential aspartic acids (Asp267). A putative binding site for UPP contained the second of the three aspartates (Asp117) and a U-shaped groove interfaced with the active site. A second structure of MraY inhibited by the natural product muraymycin D2 ($K_d \sim 20$ nM) by the same group revealed significant conformational change to the active site [16]. Muraymycin D2 occupies the active-site cleft, a newly formed nucleoside-binding pocket and also a newly formed peptide-binding pocket. Muraymycin D2 does not interact with the Asp residues at the active site or with the Mg^{2+} ion. Rather, muraymycin D2 imparts specificity to $MraY_{AA}$ through interactions with three highly conserved residues (Asp193, Phe262, and Gln305). Tunicamycin and muraymycin D2 are both competitive nucleoside natural product inhibitors of MraY, and their binding modes to $MraY_{AA}$ and MraY from *C. bolteae* [125] are similar in that the conformations of the active sites are similar. However, differences were observed in that tunicamycin interacts with the catalytic aspartate triad residues while muraymycin D2 does not. These studies reveal important structural information about how MraY may interact with its natural substrate, and underscore the significant conformational rearrangement of the active site that is requisite for substrate (and competitive inhibitor) access to the active site. Identification of key residues that regulate substrate access by preventing active-site rearrangement would represent discovery opportunities for novel inhibitors (presumably bactericidal) that employ an allosteric mechanism.

Dihydrodipicolinate Synthase An allosterically controlled antibacterial target that does not display discernible conformational change is dihydrodipicolinate synthase (DHDPS). DHDPS catalyzes, in both Gram-negative and Gram-positive bacteria, the first step in the lysine biosynthetic pathway, the transformation of pyruvate to aspartate semialdehyde. Because lysine is a precursor to peptidoglycan, DHDPS is an attractive target for the discovery of novel antibacterials. The DHDPS enzyme is either dimeric or tetrameric. It is allosterically inhibited through a lysine-binding site (as a negative feedback loop) in each monomer [126]. The inhibition is cooperative. In the case of DHDPS from *Campylobacter jejuni*, bis-lysine ($K_i \sim 200$ nM) is significantly more potent inhibitor than L-lysine ($K_i = 65$ μM) [127]. While the inhibition mechanism was cooperative, only subtle conformational changes were seen upon examination of the inhibitor-DHDPS complex. These crystallographic studies further indicate that substrate turnover may be mass-transport limited, as the substrate pyruvate must diffuse through a channel in the protein to access the active site. Deuterium exchange kinetics from hydrogen-deuterium exchange (HDX) mass spectrometry indicated that the most dynamic regions of the protein (most solvent accessible) are adjacent to the substrate-access channel. When lysine (or bis-lysine) is bound, this same region of the protein becomes less dynamic [128]. The authors hypothesize that substrate access to the

active site is unfavorable due to rigidity of the region near the access channel. DHDPS remains an example wherein allosteric inhibition might coincide with subtle conformational change.

Dihydropteroate Synthase This enzyme of the folate biosynthetic pathway is validated as the target of the sulfonamide antibacterials, a class once used extensively as single agent therapy (but now as a result of resistance require combination for antibacterial efficacy) [129]. A fragment screen for inhibitors and ensuing structural study of the screening actives, using the homodimeric enzyme from *Bacillus anthracis*, identified a cryptic site at the dimer interface [130]. Occupancy of this site by the allosteric inhibitors identified by the screen resulted in inhibited catalytic turnover. Regrettably, the modest micromolar-level potency and poor solubility of the most active structure precluded more extensive studies. Nonetheless, this study is a key exemplification of the potential opportunity of the allosteric antibacterial.

FtsZ Modulation to Disrupt the Bacterial Cytoskeleton FtsZ is a key structural component of the cytokinetic Z-ring that forms at mid-cell as the bacterial cell divides. FtsZ assembles into a protofilament that oligomerizes (5–10 filaments) into a single-layered bundle, in a GTP-dependent process, underneath the bacterial inner membrane. This assembly process explicitly requires extensive protein–protein contact, and disruption of this contact is the focus of extensive medicinal chemistry exploration [131, 132]. The most promising of these inhibitors is the "benzamide" class, first exemplified by compound PC190723, which acts to block a "disassembly switch" through an allosteric interaction to stabilize the bundle [133, 134]. The concern for facile resistance mutation to abolish the allosteric site (notwithstanding the highly conserved "tubulin" structure of the FtsZ) has abated [135]. Structure-activity alteration of the benzamides to block metabolism and the use of a prodrug delivery strategy give benzamide structures having acceptable pharmacokinetics and showing efficacy in both MSSA and MRSA infection models [136]. Combination of these new benzamides with a β-lactam was both synergistic and resistance-suppressing across a spectrum of drug-resistant *S. aureus* strains [137]. All of these observations establish FtsZ as sensitive to allosteric inhibition, as well as validating the use of modified inhibitors as reporters in the assay of the assembly of the bacterial cytoskeleton [138]. This ability in turn creates opportunity for the discovery of yet additional allosteric targets among the conserved proteins comprising the cytoskeletal edifice [139].

Virulence Antagonism Prokaryotes live no less regulated lives than eukaryotes. They must be responsive for example to friend or foe, and to nutrient or antibacterial. The adaptability of prokaryotes is impressive. Notwithstanding this adaptability, the possibility of manipulating pathways so as to abet antibiotic or immune function is an important frontier in antibacterial research. This frontier covers a wide breadth of medicinal-chemistry initiatives, often described with the term virulence suppression [6, 140–142]. While the transcriptional regulation of bacterial virulence pathways is not typically thought of as an allosteric

phenomenon, the derepression (or repression) of these pathways certainly involves deliberate conformational adjustment between protein and DNA as a result of small-molecule interaction [143], and accordingly fall within the medicinal chemist's purview of allostery. Notwithstanding the challenge of the transcription factor as a protein target for the medicinal chemist [144], notable progress has been made with respect to antagonizing key pathways directly connected to bacterial virulence. The best-studied bacterial pathway is that of quorum sensing through extracellular signaling molecules, called autoinducers [145, 146]. While the expression of bacterial virulence through quorum sensing involves a confluence of pathways, selective design of agonist/antagonist structure to dissect the roles of these pathways is now possible [147–149], with explicit recognition that the mechanism of some of these structures is allosteric [150]. A detailed perspective on the creation of "pathoblocker" suppressors of virulence is presented in a companion chapter of this volume [151].

5 Allosteric Discovery Methods

5.1 Virtual Screening

While the determination of protein structure and the use of that structure for virtual screening to identify structural starting points [30, 152, 153] are increasingly successful efforts – this statement is made with no intention to minimize either the effort required for both accomplishments – virtual screening presents two uncertainties. The first is whether the allosteric site is recognizable. Although the location of an allosteric site may be surmised from inspection, experimental verification is necessary. The second uncertainty is differentiating agonist and antagonist structure. These challenges are exceedingly well recognized in ligand-receptor study [154, 155]. New computational methods for allosteric-site discovery modify the familiar docking/scoring strategy by incorporation of "ensembles" of structures corresponding to different protein conformations [156]. A recent report highlights that protein conformations with an accessible cryptic (non-orthosteric) binding pocket do not necessarily correspond to energy minima, with multiple fragment-based docking and scoring algorithms used to weed out false positives [41]. Several free programs dock ligands (AlloScore) [157] and screen for cryptic sites (CryptoSite [40], Allosite [158], and AlloPred [159] and ExProSE [160]). The likelihood of success will improve with the increasing ability of computational resources to accommodate the full conformational space available to both ligand and protein [161–165]. The output structure from the virtual screen must match necessarily to available structure, and here the well-recognized mismatch between the criteria for drug likeness of antibacterials compared to other drug classes (and commercial compounds) also represents a potentially serious limitation in moving structure forward [166]. Virtual screening of hydrophobic pockets may result in

leads with undesirable hydrophobicity, while pockets with multiple ionizable groups may give leads with decreased cellular permeability. The challenge of reverse-engineering chemical properties to improve cell permeability or solubility while retaining activity cannot be underestimated [167].

5.2 Assay Implementation

A basic challenge to the discovery of the allosteric antibiotic is the implementation of the assays to guide the medicinal-chemistry effort. The hit-to-lead progression by the medicinal chemist requires typically robust and high-capacity assays to determine potency and selectivity against the target, robust and high-capacity assays to assess biological strengths and weaknesses at the cellular level, and reliable assays for the disease pharmacology. While the allosteric drug fairs no differently in the latter two assays in this progression, the direct kinetic assessment of many antibacterial targets to determine allosteric modulation is not possible. The evaluation of the allosteric network for PBP2a, for example, was accomplished exclusively by means of structural biology assays given the complete absence (due to scarcity of substrate, and the implicit complexity of the membrane-centered, likely multi-enzyme and multi-protein assembly of which PBP2a is a part) of a kinetic assay for this enzyme. Although the MIC determination is robust, high-capacity and meaningful (and in the exceptional case of PBP2a, the comparison of the MICs for MSSA and MRSA strains gives an implicit assessment of the relationship between the antibacterial structure and PBP2a), the value of cell-based assays to the medicinal chemist for SAR development is decidedly less than the guidance provided by direct measurement of target affinity. Accordingly, the relationship of assay to the discovery of allosteric antibacterials will be that of structure-based design, empirical small-molecule structure discovery using surrogate assays to identify synergy or potentiation with antibacterials having a known target, or empirical small-molecule structure discovery using surrogate assays for pathways to identify new targets for structure-based drug design. The availability now to the medicinal chemist of surrogate assays for the SEDS (shape, elongation, division, sporulation) bacterial pathways [168] deserves particular mention. The SEDS pathways encompass the confluence of the pathways for cytoskeletal assembly and cell-wall assembly. Both pathways are populated with proven antibacterial targets. The confluence of these pathways is expected to have allosteric control. Although the micrometer dimensions of the bacteria were historically at the limit of fluorescent interrogation, modern high-resolution fluorescent microscopy now permits the direct determination of protein expression and localization, and the functional profiling of exploratory antibacterials [169–172]. Moreover, a consequence of SEDS pathway disruption is often altered bacterial shape, a phenotypic property that can be directly correlated by flow cytometry to a specific gene (enzyme) function [173–176]. While these assays may not yet have the robustness of an MIC determination, the power of their ability to directly link antibacterial structure to the

interconnection of gene, protein, pathway, and phenotype will unquestionably influence the future of antibacterial – whether orthosteric or allosteric structure – discovery.

6 Conclusion

Allosteric targeting has had great success as a therapeutic strategy in eukaryotic systems. The limited structural and pathway data for prokaryotic systems has limited its application to antibacterial discovery. However, PBP2a is one bacterial protein target with a recently discovered allosteric pocket, which was leveraged for therapeutic discovery as exemplified by the novel quinazolinone class. A second class, the oxadiazoles, might also target PBP2a as the molecular class acts as potentiator of the β-lactam antibacterials in vitro and in vivo. The oxadiazoles and quinazolinones harbor anti-MRSA activity of their own, and the example of PBP2a illustrates the feasibility of leveraging allosteric mechanisms as targeting strategies alone, or in concert with existing antibacterials.

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The Clinical Development of Antibacterial Drugs: A Guide for the Discovery Scientist

David M. Shlaes

Abstract Every decision a drug discovery scientist makes along the way will impact the ultimate product to emerge from the long and arduous discovery and development process. To meet this challenge, an innovator must have a basic understanding of those steps in this process that demand far more than knowledge of basic bench science. Perhaps the most difficult of these steps involves an understanding of regulatory and clinical development issues that only become relevant years after the potential product has overcome its initial scientific hurdles. This chapter provides a review of currently available clinical development paradigms for antibacterial drugs including non-inferiority trials and various approaches to superiority trials. The thorny problem of how pathogen-specific antibiotics can be developed is explored. The goal of this chapter is simply to familiarize the bench scientist with the challenges ahead for any project and to provide a framework for assessing risk in that context.

Keywords Antibiotics, Antimicrobial resistance, Bacterial infection, Clinical development, Combination therapy, Drug target, Enhancers, Infectious diseases, Nonclinical development, PK/PD, β-lactamase

Contents

D.M. Shlaes (✉)
Retired from Anti-infectives Consulting, LLC., 219 Montauk Ave, Stonington, CT 06378, USA
e-mail: Shlaes.david@earthlink.net

1 Introduction

The scientist working on drug discovery at the laboratory bench is frequently in a world unto one's self. Corporate strategy, or even academic laboratory strategy, may seem distant or even irrelevant. This distance is a paradox that can lead to eventual frustration, conflict, and wasted energy and resources. It is critical, therefore, for the discovery scientist to develop a level of understanding of the world they seek to change by their innovative work. To achieve this success, it is key for the researcher to have clearly in mind at the outset the key characteristics of the ultimate product that is the goal of the research. These characteristics usually take the form of a Target Product Profile. An example of a target product profile for a new β-lactamase inhibitor – a current objective of several pharmaceutical companies for the purpose of restoring the antibacterial spectrum of a proven β-lactam antibacterial against multidrug-resistant Gram-negative bacteria – is shown in Scheme 1.

The objective of this chapter is to provide to the bench-level innovators key insights into how their products will – or will not – progress to achieve the benefit they seek to provide ultimately to patients.

2 Novel Targets?

The first consideration is target selection. This consideration is foremost since the choice of target directly influences the likelihood that the ultimate product will be successful. This topic is the subject of the chapter by Sutterlin and colleagues [1]. The advantages in pursuing inhibitors of targets that have never led to a marketed antibacterial product are many. It is likely that the inhibitor of a novel target will belong to a new chemical class, and thus will not demonstrate cross-resistance with antibacterials belonging to known classes. Any new class of antibacterial may offer the potential for novel antibacterial combinations that may have additional advantages over currently used combinations. Finally, there is intrinsic commercial value to a novel class, as demonstrated by the fact that every new antibacterial wants to be known as defining a new class, whether this designation is accurate or not. This raises the question: how do we define a novel target? Could it be new binding sites on the ribosome? What if those binding sites are adjacent to those used by marketed antibiotics? Or does "novel" have to imply a target that has never led to a marketed product? In considering these questions, we must understand that

Target Product Profile – Novel β-Lactamase Inhibitor

- β-Lactamase spectrum
  - Activity against AmpC is mandatory
  - Activity against Class A ESBL may not be mandatory, depending on the β-lactam partner
  - Activity against Class D OXA ESBLs (OXA-2 and OXA-10/13 variants)
  - Activity against Class D OXA carbapenemases (OXA-48, -51, -23, -24, -58)
- Improved cell penetration
- Acceptably low resistance frequency
- IV only is acceptable.
  - Improved bioavailability for hospital iv/po switch
  - With an *Enterobacteriaceae* spectrum (NXL104-like)
  - Targeting β-lactamase producing *P. aeruginosa* and *A. baumannii*
- For community (UTI ?)
  - Narrow β-lactamase spectrum acceptable – oral required.

Scheme 1 Target product profile – novel β-lactamase inhibitor

pursuing a non-validated (by achieving market authorization) antibacterial target is a high-risk effort.

The results of decades of efforts to address novel targets have not resulted in a single marketed novel antibacterial for systemic use. On the other hand, at least one novel class of antibacterial has been discovered via brute force screening that turned out to have a novel target – daptomycin, a lipopeptide [2]. Given that the antibiotic wars between microorganisms have been occurring within various ecological niches for billions of years, it may be that the targets that we already know are those targets most likely to yield efficient and safe inhibitors.

The chemical libraries in pharmaceutical companies are probably biased against antibacterial compounds [3, 4], and these libraries leave significant portions of chemical space uncovered. A second issue is safety. It is important to remember the enormity of the task upon which we embark. We are looking for a poison for a living bacterial cell that will not be a poison for our own cells, even though we are related albeit distantly. Most antibiotics fail because of safety either in nonclinical

studies or during early clinical development [5]. While we know the safety risks of known classes of antibiotics, the safety risks of new classes are unknown. New classes directed at new targets may present a greater safety risk than new classes directed at known targets.

Novel targets have not borne fruit during the genomics era of the 1990s. A total of 67 high-throughput screens were undertaken at GlaxoSmithKline during the period of 1995 and 2001 against essential gene targets in bacteria [6]. The compound libraries used varied between 260,000 and 530,000 structures. Some screens were run a second time using a different analytical format. Only 16 of these screens identified hits. Of these 16 screens, only five resulted in lead compounds. Empiric screening of a 500,000 compound library against wild-type *Staphylococcus aureus* and wild-type *Escherichia coli* was also carried out. The *E. coli* screen yielded no hits at all. The *S. aureus* screen yielded a large number of hits, almost all of which turned out to be nuisance compounds and nonspecific membrane-active agents. This experience remains typical of that encountered by antibacterial researchers across both industry and academia even today.

The caveats noted above should be viewed as just that: caveats. There is nothing wrong with having novel targets as part of an overall strategy, or even as the main strategy, as long as one is cognizant of the risks involved and is prepared to address these risks early in the discovery process. A careful review of previous experience is required in order to avoid the pitfalls that are now well known to the "old hands" of antibiotic hunters. All efforts to discover antibiotics inhibiting novel targets should be balanced with lower risk approaches.

3 Not "Novel" Targets

Two alternatives to novel antibacterial targets include identifying novel inhibitors of known targets or modifying known inhibitors of known targets to improve one or more aspects of their profile [7]. A current example of the former approach is avibactam, a novel β-lactamase inhibitor targeting the same active sites of the same β-lactamases targeted by marketed compounds [8–10]. The great advantage of avibactam (and its congener structures) is its ability to increase the spectrum of activity against key β-lactamases like the KPC and OXA-48 carbapenemases, and the class C "cephalosporinases." Examples of medicinal chemistry efforts towards this latter objective abound and include the advances in cephalosporin structures from the first-generation compounds like cephalothin and cefazolin through the fourth-generation structure, cefepime. Additional examples from other antibacterial classes include tigecycline, eravacycline, telithromycin, and solithromycin.

4 Nonclinical Development

The translation of scientific findings during the early preclinical phase of discovery science into a potential product that is ready to begin its first trials in man is an important process and one that cannot be adequately dealt with in this chapter. Most antibiotics, if they are to fail, will do so during these translational activities or during Phase I trials to establish pharmacokinetics and safety. Three key issues must be clearly resolved during translation studies.

- Using a variety of approaches, it must be clear that the new product is unlikely to directly select for mutational resistance among targeted pathogens during therapy. There are several approaches to this problem. The discussion presented by Singh et al. [11] provides a reasonable roadmap. Several strains should be tested in this regard since resistance rates are occasionally strain specific, probably related to other genetic characteristics. A no-go decision for a compound should be considered when, in a single step, at a frequency of 10^{-7}–10^{-8} assessed at 2–4 times the minimum inhibitory concentration (MIC), the resulting MIC jumps to a level above the pharmacokinetic exposure likely to be achieved in humans.

  An example of what happens when a compound is studied in the clinic without attention to in vitro data occurred with GSK2251052, a leucyl t-RNA synthase inhibitor. During Phase II studies in complicated urinary-tract infections, highly resistant mutants emerged within 1–2 days of therapy causing recurrent bacteremia [12]. In vitro data had already predicted this possibility. This outcome is not the one you want for your clinical trials.
- Pharmacokinetic and pharmacodynamic studies of the new compound in animal models are essential to the further development of antibacterial products [13]. These studies set potential efficacious dose levels for the animal models that can be extrapolated to humans [14]. In the nonclinical setting, this is accomplished first by understanding the MIC of the product required to inhibit 90% of key pathogens (MIC_{90}). Next, one must identify the pharmacokinetic parameter that most correlates with in vivo activity. This can be C_{max}, area under the concentration curve or time above the MIC. Once this is established, using the appropriate animal model and Monte Carlo simulations, the pharmacokinetic exposure required to inhibit infections caused by 90% of pathogens can be established. This dose can then be extrapolated to the human (in most but not all cases) and be used to estimate an efficacious dose.
- Nonclinical safety studies carried out under Good Laboratory Practice conditions must establish safe doses of the new compound in animal models [15]. These safe doses can be directly compared to the efficacious dose as determined by the PK/PD studies noted above. Once a safe dose is established in animals, one can calculate the starting dose for the first-in-man trials. The determination of the starting dose for Phase I trials will be a critical result of the safety studies. Clearly, if a safe dose has not been established, or if the ratio of the safe dose to the efficacious dose is 2 or less, it may not be practical to continue on to human studies.

5 Clinical Development Considerations

Notwithstanding the importance of the safety and efficacy activities comprising "pre-clinical development," recent experiences in antibacterial development emphasize the critical importance of correctly defining the objectives of the clinical development strategy. Here I provide a brief statement of the range of clinical development designs corresponding to a range of exploratory antibacterial mechanisms. These designs include:

- A standard antibiotic undergoing non-inferiority type trials versus a marketed comparator
- A fixed-dose combination study. For example, the pairing of a marketed antibacterial (such as a β-lactam) with an enhancer (such as a β-lactamase inhibitor), using modified non-inferiority trials
- A new "enhancer" that could be combined with a number of marketed antibacterials to increase efficacy
- A new antibiotic optimized for activity only against a single bacterial species, such as *Pseudomonas aeruginosa* or *Acinetobacter baumannii*

5.1 Non-inferiority Trials

Non-inferiority trials have remained the accepted design for testing new antibiotics since the 1950s [16]. These trials are the most risk-free route to secure the regulatory approval necessary to enter the marketplace. The reason that non-inferiority trials are the standard design is the recognition – with good reason! – that it is unethical to withhold efficacious therapy from patients with serious bacterial infections. A design comparing a new antibiotic to a placebo would fall under this "unethical" umbrella. The non-inferiority trial targets a clinical indication such as urinary-tract infection, skin and skin-structure infection, intra-abdominal infection, or pneumonia. In this trial design, the bacterial pathogen itself is a secondary consideration. The selection of the comparator antibacterial is paramount. The comparator should be a generally accepted (clinically) standard, or first-line, antibacterial for the clinical condition being studied. The comparator should have clinical approval in most (or all of the) countries where the trials will be conducted.

Non-inferiority trials are designed to provide a statistical margin that demonstrates that the new antibacterial is not inferior in efficacy to the marketed comparator antibacterial [16, 17]. This margin, or M2 in statistical parlance, derives from two design criteria. The first criterion is the estimated activity of the comparator compared to placebo (M1). The second criterion is the number of patients that it is feasible to enroll in a trial within a reasonable time period. The comparator must have a treatment effect that is greater than placebo. For most infections, the treatment effect is estimated by comparing data from the pre-antibiotic era to data from clinical trials of antibacterials in the modern era. The treatment effect has varied

from about 20% to 70% [18, 19]. The M2 margin generally should not be more than one-half of the treatment effect. In reality, it is rarely more than 10% for the US FDA and 12.5% for the European regulators (see reference [16] for the reasoning behind these values). The M2 margin value is a critical consideration because it is the primary determinant of the number of patients who must be enrolled in the clinical trial. Given this margin value, and with consideration to the various other factors (such as the evaluation of the patients enrolled), the typical non-inferiority trial requires approximately 800 patients. The cost of this trial will be roughly $25–$50 million. Trials in nosocomial pneumonia tend to require greater expense. Two such trials (plus the subjects studied in the Phase I and Phase II trials) define a safety database of close to 2,000 individuals. A safety database of 1,500–2,000 individuals traditionally has been considered by the regulatory agencies as adequate for antibacterial development [18, 19].

5.2 *Fixed-Dose Combination Antibacterials*

There is a long history of the use of this approach. These clinical trials have all been studied in the context of proof of non-inferiority. Examples include sulfamethoxazole-trimethoprim, quinuprisitin–dalfopristin, and all marketed β-lactam–β-lactamase inhibitor combinations. The non-inferiority approach to the fixed-dose combination is unique for antibacterials. In contrast, antiviral and oncology combination drugs are generally studied in the context of superiority trials, where the combination is thought to be more efficacious than either of its components, or than other combinations already marketed. The unique considerations of antibiotic therapy, in which placebos are not allowed and most existing therapies are already highly efficacious, preclude the use of a superiority criterion. The one area where there is opportunity to look for superiority is among patients infected with resistant pathogens. However, enrolling such patients into the clinical trial is very difficult unless the majority of such infections are already due to the resistant pathogen. The best example of this situation is the global pandemic of methicillin-resistant *S. aureus* infection where in many countries (including the USA), where up to 70% of strains were resistant. In this situation, enrolling patients with resistant infections is relatively straightforward. But who wants to wait for a global pandemic of resistance to enable as possible this approach to antibacterial clinical design?

Fixed-dose combination study in the future might correspond to an already marketed antibiotic plus a compound that enhances its activity. Such a combination can be studied in traditional non-inferiority trials. If both compounds are safe and non-inferior to a reasonable comparator, ready approval may follow. A fixed-dose combination that includes two experimental agents is more challenging and requires much more preclinical (as well as additional clinical) effort [20]. But the approach is not impossible, and such a combination could still be studied ultimately in standard non-inferiority trials.

This assessment brings us to consideration of the clinical approach to β-lactam–β-lactamase inhibitor combinations. These fixed-dose combination drugs target a very specific resistance mechanism – the mechanism arising due to the presence of a bacterial β-lactamase. Here, non-inferiority trials must produce some minimum number of patients infected with infections caused by pathogens resistant to the β-lactam drug in the combination, but susceptible to the drug combined with the inhibitor in the combination. In a certain way of thinking, this circumstance allows confirmation of the superiority of the combination against resistant strains, without the necessity of carrying out a superiority trial solely consisting of resistant infection. This approach leans heavily on a partner antibacterial that has been previously marketed and has well-understood characteristics, corresponding to a clear regulatory label. This approach has worked well for the development of all currently approved β-lactam–β-lactamase inhibitor combinations. The approach becomes more challenging when the target organisms are encountered less frequently in the clinic. Good examples are the carbapenem plus β-lactamase inhibitors currently still in development (imipenem–cilastatin–relebactam and meropenem–vaborbactam) that target pathogens resistant to the carbapenem alone, as a result of the presence of carbapenemase enzymes. The recruitment of even small numbers of patients infected with these pathogens into traditional non-inferiority trials has proven difficult. Nevertheless, regulators seem ready to accept in vitro, animal model and pharmacokinetic/pharmacodynamics data in support of these combinations instead of the clinical data they required previously [21]. This demonstratres the importance of preclinical data.

5.3 Development of an "Enhancer" Compound as a Stand-Alone Agent

This possibility is likely neither feasible nor commercially desirable. The enhancer in this case is a compound simply added to whatever cocktail of antibiotics is thought to be the best available therapy to treat an infection, such as urinary-tract infection. The cocktail plus enhancer is compared to the cocktail alone in a randomized active-control superiority trial. Because the control cocktail (or single antibiotic, such as a carbapenem) in general is so effective in these infections, achieving superiority will require such a large number of patients as to render the study infeasible. Of course, if resistance to the antibiotics in the cocktail was common and if the enhancer allowed these drugs to overcome that resistance, such a trial might be feasible. Nonetheless, the enrollment of sufficient patients with resistance into a clinical trial will be exceedingly difficult. There are two primary reasons for this difficulty. Many patients will present a severe underlying illness that will exclude them from the trial. Many (if not most) patients will have been treated previously with other antibiotics and would also be excluded from participation (see the section on pathogen-specific antibiotics and superiority trials below).

Even if such a study could be conducted, how would such a drug be used in the clinic? Useful instruction is provided by the attempt by Pfizer to market the β-lactamase inhibitor sulbactam as a single agent for physicians to add to whichever β-lactam partner they desired in the treatment of various infections [22]. This marketing effort (with sulbactam marketed as "Combactam") was undertaken in Germany and Austria. The problem was that this approach required physicians to understand which β-lactamases might be present in the infection they were treating, and what the appropriate dosage of Combactam was required for combination with their selection of a β-lactam. These requirements were too much for the majority of practicing physicians. Combactam sales suffered. The attempt was an abortive one. Would an enhancer drug fare better in the marketplace?

5.4 Pathogen-Specific Antibiotics

I exclude from this discussion a consideration of compounds targeting *Clostridium difficile* and *Mycobacterium tuberculosis*, given the exceptional circumstances of these infections. While antibiotics active against specific genera or species of bacteria are seen by many as being highly desirable from the point of view of antimicrobial stewardship and sparing the microbiome, such products are difficult to discover and even more challenging to develop. Most antibiotics that are discovered, regardless of the screening program used, are active against a fairly broad spectrum of bacterial pathogens. Most hits are broadly active against Gram-positive bacteria. Some compounds – but these compounds are rare – are restricted to activity against Gram-negative pathogens only. A more likely scenario is the discovery of a compound with broad activity that has additionally a particular advantage against a specific genera or species. Examples might include some tetracyclines like minocycline, tigecycline, and eravacycline that have enhanced activity against *Acinetobacter* spp. compared to their activity against other Gram-negative species. Another example might be the carbapenem–β-lactamase inhibitor combinations noted earlier, having broad-spectrum activity attributed to the carbapenem but with activity targeted towards certain carbapenem-resistant strains attributable to the β-lactamase inhibitor. As noted above, such compounds or combinations are much more straightforward to develop and bring to market more than something that targets only a specific genera or species.

The problem for truly pathogen-specific antibiotics remains the clinical trial design. To carry out the clinical trial, a sufficient number of enrollable patients with serious infections caused by the pathogen in question must be identified. A very instructive hypothetical case example was examined at a recent FDA workshop [21]. This case example involved a fictitious antibiotic (called X-1) exquisitely but specifically active against only *P. aeruginosa*. In attempting to design a clinical trial for X-1, previous trials that enrolled at least some patients with *Pseudomonas* infections were examined. In nosocomial pneumonia, about 15% of patients enrolled were infected with *Pseudomonas*. For urinary-tract infection and intra-abdominal infection, the numbers were around 3% and 7%, respectively. One can see the challenge already.

To get sufficient numbers of patients for a non-inferiority trial, enrollment of thousands of infected patients would be required to secure a sufficient number of evaluable patients with actual *Pseudomonas* infections. If one assumes that 200–300 patients with such infections are required, given the statistical requirements discussed above, a total enrollment of 3,000–5,000 patients would be necessary (depending on the exclusion criteria used). The largest antibacterial trial of this sort that was undertaken using this approach compared linezolid against vancomycin for nosocomial pneumonia [23]. That trial included around 1,184 patients and took 5 years to complete. It demonstrated statistical superiority of linezolid in the context of a non-inferiority trial. The problem is that outside non-inferiority trials for antibacterial drugs, there is almost no other such clinical experience. Accordingly, a clear and feasible pathway to regulatory approval using a non-inferiority design approach is lacking.

The FDA workshop, in the context of the fictional X-1, hypothesized a novel diagnostic test that could help by enriching the trial for those patients actually infected with *P. aeruginosa* – the target of X-1. Not only does this test not exist but such a test is not even on the near-term horizon. Such a test would almost certainly have to be a bedside or point-of-care test. That means it would have to be waived from the Clinical Laboratory Improvements Act (CLIA) that requires most diagnostic tests to be conducted in certified medical laboratories [24]. In order to achieve this status, the test would need to be simple such that untrained personnel would be able to carry out the test reliably, and that the specificity and sensitivity of the test would remain the same across operators with widely varying training and skill sets. The reason that such a test would be required has to do with the time required to enroll patients in trials. A test that is sent to the laboratory will require hours to complete and report back to the physicians, just given hospital logistics (transport, lab protocols, and so forth). But for serious infections, delays in antibiotic therapy can be deleterious.

New guidelines that may be forthcoming from FDA may help to ameliorate this situation, though. The Clinical Trials Transformation Initiative has proposed streamlining these trials by pre-enrolling patients at risk of serious infections such as those caused by *P. aeruginosa* [25]. Such pre-enrollment and prior evidence of colonization by *Pseudomonas* would eliminate the need for an enriching rapid diagnostic test. Such an approach, if adopted by the FDA (which I believe is likely), might be a major step forward for the study of pathogen-specific antibacterials in nosocomial pneumonia. At this point, I will note that trials for drugs against even less frequent pathogens like *A. baumannii* will be even more challenging.

At the X-1 workshop, I suggested a potential trial design based on superiority (for a review of the superiority trial approach to bacterial infections, see references [26, 27]). The basis of my suggestion involved including external or historical controls. The reason for this inclusion is that since all (or the vast majority of) patients are treated with the experimental therapy, you only have to enroll about half the number of patients compared to the number that would be required if half were treated with a comparator or standard of care cocktail.

The video presentation by Ellenberg [28] given at an NIH conference on trial designs for emerging infectious diseases is highly recommended with respect to the consideration of external controls in clinical design. This presentation is very informative.

In designing trials to address rare infections, rare pathogens, and pathogen-specific indications, patient numbers may not support a randomized design. We might not even be able to achieve statistical inference with an externally controlled design. Nonetheless, in my opinion this design is where we will have to go. According to Byar [29] and later Elllenberg [28], an externally controlled trial design can be justified if the conditions listed below can be met.

- A randomized trial is infeasible because of the rarity of the condition under study.
- Sufficient experience exists to ensure that patients not receiving therapy will have a uniformly poor prognosis.
- The therapy must not be expected to have substantial side effects.
- There must be a justifiable expectation that the potential benefit to the patient will be sufficiently large to make interpretation of the results of a non-randomized trial unambiguous.
- The scientific rationale for the treatment must be sufficiently strong that a positive result would be widely expected.

I would argue that a new antibiotic expected to be active against resistant pathogens would meet these criteria, assuming that it was shown to be safe in a sufficient number of volunteers/patients. The data supporting a lack of efficacy of antibiotics where the exposure obtained is insufficient to meet the pharmacodynamic target required for the pathogen are clear and overwhelming. While the statistical problems to this approach are numerous, they hinge on a single assumption: that the distribution of patients with good versus poor prognoses will be the same in the experimental and control groups. This assumption is a key basis for preferring a randomized trial but may be subject to quantitative interrogation.

Most of the failures of externally controlled trials to provide reliable results have resulted from inadequate controls:

- Controls had been derived from a different time such that control therapy had changed by the time the actual trial was conducted.
- Or supportive care had changed altering prognosis for controls.
- Or the effect size in controls had simply been underestimated for other reasons.

How can we overcome these obstacles for antibacterial drugs? The key features that will contribute to future successful antibacterial clinical include:

- Providing the resources needed for comprehensive PK/PD studies.
- Having clearly and adequately designed PK/PD targets.
- Making certain that adequate PK is achieved in the patient population to be studied (possibly including the study of the PK of the new antibacterial as an add-on to the standard-of-care control in the patient population to be studied for efficacy later).
- Consider a small, open label Phase II study to help convince physicians and regulators that a new antibiotic will indeed benefit patients as expected based on PK/PD considerations. This study will also bolster related PK/PD arguments and will provide at least some data on efficacy.

- Define inclusion/exclusion criteria early. I would advise being expansive rather than constrictive with respect to these criteria. One does not want a lot of amendments in the middle of a pivotal trial, as this trial is not non-inferiority.
- Carry out a *retrospective* (within the previous year or two) observational study of the key patient population treated with standard-of-care or with comparator drug to define control level of response. This retrospective study should utilize the same inclusion and exclusion criteria to be used for the trial and should be done in centers likely to participate in the trial, so as to remove as much as possible center-to-center bias.
- Early in the trial, carry out a *prospective* study of standard-of-care or comparator to validate the assumptions you have made about controls during your retrospective standard-of-care. Obviously this study must be done in the centers actually participating (and contributing patients to) the ongoing trial.
- Alternatively, randomize patients in a 4:1 ratio of experimental therapy versus standard of care, simply to validate the external controls you are using in the trial.

The design I proposed at the workshop involves using external controls. Patients would be those with either nosocomial pneumonia or complicated urinary-tract infection caused by *P. aeruginosa*. Controls would come from a retrospective study of such patients treated with a carbapenem antibiotic, with or without the addition of an aminoglycoside antibiotic. The retrospective study would focus on outcome (clinical cure in my view) in those patients found to have infections with carbapenem-resistant pathogens. The literature supports the expectation of roughly a 50% clinical cure rate under these circumstances [30]. Carbapenem-resistant *Pseudomonas*, in general, already comprises about 15%–20% of strains in most hospitals. If our experimental therapy gave us an 80% cure rate, with a 30% absolute difference we might be able to see an important trend towards superiority in as few as 30 treated patients compared to about 100 external control patients. To obtain 30 patients infected with carbapenem-resistant *Pseudomonas*, we might expect to enroll 300 patients total. Most of these patients would not have *Pseudomonas* infections, or their *Pseudomonas* would not be resistant to carbapenem antibiotics. This number would be more than adequate to establish a safety database under the FDA guidance on antibacterials for unmet needs [31], especially given patients who would have been exposed to the drug in Phase I and possibly in Phase II trials as well. If the assumptions regarding efficacy of our new therapy and control (carbapenem) are correct, in such a trial we should be able to prove superiority 90% of the time at the $P = 0.05$ level (caveat: I am not a statistician).

As noted in the workshop, two companies, Achaogen and The Medicines Company, are conducting superiority trials for their new products (plazomicin and meropenem-vaborbactam, respectively). Both companies have also carried out non-inferiority trials in complicated urinary-tract infection as well. Their second superiority trial will lean heavily on their non-inferiority trial results to support both efficacy and safety. Both superiority trials have been problematic. Achaogen recently announced top-line results for their trial comparing plazomicin to colistin in the treatment of nosocomial pneumonia caused by carbapenem-resistant pathogens [32]. Notwithstanding the small patient

numbers in their trial, the trial data themselves are informative: Day 28 all-cause mortality or significant disease related complications (primary endpoint); 4/17 (23.5%) for plazomicin versus 10/20 (50.0%) for colistin, corresponding to a difference of 26.5% (90% CI: 0.7, 51.2%); Day 28 all-cause mortality 2/17 (11.8%) for plazomicin versus 8/20 (40.0%) for colistin, corresponding to a difference 28.2% (90% CI: 0.7, 52.5%). As is evident, these results do not reach statistical significance at the $P = 0.05$ level. Nevertheless, they are indicative of the results the FDA may expect from pathogen-specific trials targeting resistant infections.

As it stands as of this writing, no such trial has ever been carried out as a stand-alone pivotal trial for approval of an antibacterial drug. As noted at the beginning, there is no established regulatory pathway for approval of a pathogen-specific antibacterial drug. In addition, the regulatory landscape for antibacterials is changing rapidly. There are clearly efforts within the FDA to look at how to obtain and use real-world data [33]. These efforts could have an impact on the selection and use of external controls in future trials. Such efforts might help lead to the regulatory pathway we need. In addition, the recent passage of the 21st Century Cures Act will further spur the agency to develop these needed pathways [34].

6 Conclusions

Everything the discovery scientist does from the outset will ultimately influence the risks that will be encountered on the way from the laboratory bench to regulatory approval and the marketplace. A clearly delineated Target Product Profile giving the desired and the acceptable characteristics of the compound to be discovered, even in a preliminary way, will help the team keep the goal in focus and keep the research on track. Accepting targets that are novel or that guarantee a pathogen-specific focus will increase the risk, but should not necessarily be discounted based on the increased risk. The risks must simply be balanced within the context of an overall program. The regulatory landscape is changing quickly. It will behoove the scientist to be aware of this evolution as it proceeds. In all cases, the burden of preclinical testing will surely increase as pharmacokinetic and pharmacodynamic data assume greater importance for future regulatory filings. A strategy to identify enhancer compounds that could be combined with any of a number of potential partners and to develop the enhancers as stand-alone products is especially risky given the requirement for superiority trials, and given the difficulty in marketing such compounds. By contrast, enhancers or resistance inhibitors (such as β-lactamase inhibitors) that can be partnered with a single compound and then developed as a fixed-dose combination minimize risk. Pathogen-specific antibacterials do not fit well with traditional development pathways such as non-inferiority trials, and as such will probably have to be studied using superiority designs. These superiority designs for antibacterials have not been clearly delineated by regulatory authorities and remain untested. Strong PK/PD data will be required to justify such trials. But I am optimistic that such pathways will be available soon given the recent FDA workshop on unmet needs for antibacterial drugs, FDA's

efforts at defining how to examine real-world data and the passage of the 21st Century Cures Act.

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Four Ways to Skin a Cat: Inhibition of Bacterial Topoisomerases Leading to the Clinic

Gregory S. Basarab

Abstract Four classes of antibacterial agents that operate by inhibition of the Type II topoisomerases, DNA gyrase and Topoisomerase IV, have progressed at least through Phase 2 clinical trials. Compounds from each of the four classes are not cross-resistant to one another as determined by analyses with laboratory and clinical resistant bacterial strains. Hence, they are defined herein as sharing a mode of action, in that they inhibit the same targets, but differing in mode of inhibition, in that they obstruct enzyme activity via divergent binding modes. Two of the classes, fluoroquinolones and aminocoumarins, were long ago approved for clinical use, though the use of the latter has been limited. Two newer classes, spiropyrimidinetriones and quinolines, are represented by the advanced drug candidates zoliflodacin and gepotidacin, each featuring a novel scaffold and a distinct binding motif. X-ray crystallography has shown fluoroquinolone and spiropyrimidinetrione binding at DNA cleavage sites of the topoisomerases. However, the two differ by their dependence on [Mg^{2+}] for binding serving in part to explain the lack of cross-resistance. Quinolines bind to DNA-topoisomerase complexes offset from the cleavage sites as ascertained by X-ray crystallography. Novobiocin, the only aminocoumarin to receive regulatory approval, competes with ATP binding at a site quite remote from the DNA-binding domain. As novobiocin has been withdrawn from the clinic, considerable drug discovery efforts have focused on alternative ATP site binders (ATPase inhibitors). With widespread use of fluoroquinolones leading to resistance, the importance of developing novel antibiotics that would not be cross-resistant is clear. Reviewed herein are the current understandings of the respective mechanisms of inhibition and the respective topoisomerase binding modes for the four classes of antibacterials now with clinical proof of concept.

G.S. Basarab (✉)
Department of Chemistry, Drug Discovery and Development Center (H3D), University of Cape Town, Rondebosch 7700, South Africa
e-mail: greg.basarab@uct.ac.za

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Contents

1 Introduction

As the proverb goes that there is more than one way to skin a cat, there is also more than one way to target a target. This becomes particularly relevant for the discovery and development of antibacterial drugs toward combatting resistance and therefore establishing both scientific rationale and public policy for selecting where best to place a research dollar. The immense concern for public health due to resistance has been contradicted by the downsizing of antibacterial discovery research at larger pharmaceutical companies due to diminishing economic incentives [1–3]. Drug classes can be classified according to their biological target or the chemical core of the compound. Over the last few decades, antibacterial discovery efforts that embraced a genomics approach to find new targets and inhibitors thereof have not successfully delivered new drugs to the clinic [4–7]. By contrast, newer generations of established antibacterial drugs continue to be brought to the marketplace quite successfully despite concerns of cross-resistance. A third avenue to bring novel therapies to the clinic is to identify novel scaffolds that inhibit validated targets differently. If this circumvents cross-resistance, it would then be categorized as a novel mode of inhibition against that target. Considerations of mode of inhibition therefore emerge for dealing with bacterial resistance: If a new compound is not cross-resistant with existing antibacterial drugs, would it be tantamount to having a novel mode of action? There have been a number of recent calls to increase research funding directed at overcoming antimicrobial resistance (AMR) via innovative means [8–10]. In the coming years, the variety of ways to inhibit Type II bacterial topoisomerases promises to be a fruitful area of research to address the serious consequences of AMR.

Two bacterial Type II topoisomerases, namely, DNA gyrase (gyrase) and Topoisomerase IV (TopoIV), have been clinically validated by two different classes of antibacterial agents, fluoroquinolones (FQs) and aminocoumarins. Extraordinary progress has been made in the identification of multiple additional classes of topoisomerase inhibiting antibacterials, two of which are currently progressing through

clinical trials in patients. Gyrase and TopoIV are highly homologous enzymes carrying out the similar reaction, whereby one strand of double-stranded DNA (dsDNA) is sequentially cleaved, translocated, and reannealed. The result is an altered dsDNA topology [11, 12]. The precise mechanism of both enzymes is cleavage of the leading strand of the dsDNA, pushing the lagging strand of DNA through the break and religating the cleaved strand. These events introduce a coil in the dsDNA. The primary function of gyrase is to create an energetically uphill negative supercoil in DNA before the DNA replication fork, thus relieving torsional strain during replication. The primary function of TopoIV is to unknot and decatenate interlocked DNA beyond the replication fork enabling independent migration of the replicated DNA. Each enzyme is independently required for bacterial viability. Each enzyme exists as a tetrameric A_2B_2 homodimer. The respective protein subunits are designated GyrA and GyrB for gyrase and ParC and ParE for TopoIV. Compounds that inhibit one of the enzymes oftentimes inhibit the other, albeit to varying degrees. However, the relative importance of each as an antibacterial target is determined by the nature of the bacterium. For example, the primary target for the FQ ciprofloxacin is TopoIV in Gram-positive *Staphylococcus aureus* and gyrase in Gram-negative *Escherichia coli* as determined by characterization of primary mutations in clinical resistant strains [13]. Care must be taken not to equate relative inhibitor potencies against isolated enzymes in what are necessarily artificial assays that do not account for the complexities and variability of cellular expression levels, metabolite concentrations, enzyme velocity, and regulatory mechanisms [14]. Additionally, it is also known that structural variations can shift the mutation profile complicating the nature of resistance even within a chemical class [15].

Because clinically useful drugs that inhibit gyrase and TopoIV are widely used to treat infections, there is some hesitancy to develop additional ones operating against the same targets. This hesitancy is due to perceptions that resistance to the established drugs crosses over to new ones by virtue targeting the same enzyme and to worries that target- or nontarget-based toxicity would similarly cross over. There are other recent reviews that more broadly describe advancements in the identification and characterization of gyrase/TopoIV inhibitors [16–20]. Despite the great success in identifying what has become a diverse range of such inhibitors, progression to the clinic has proven to be exceedingly arduous due to the difficulty in surmounting pharmacokinetic and toxicology issues that compromise utility in humans. This review will focus on the four classes of gyrase/TopoIV inhibitors that have advanced into clinical trials: FQs, spiropyrimidinetriones (SPTs), quinolines, and ATP-binding site (ATPase) inhibitors. Discussed will be the current understandings around mode of inhibition, mutation events, and structure. Notably these four classes operate by orthogonal modes of inhibition in that clinical or laboratory-generated resistance to any one does not cross over to any of the other three. The definition of "resistance" herein will refer to a significant decrease in bacterial drug susceptibility. For investigational drugs, such resistance is identified by the isolation of laboratory mutants that may or may not yield clinical resistance. For example, laboratory resistant frequencies and resistant genotypes can be dependent on the growth medium, which, in turn, may not be representative of the in vivo situation [21, 22]. Genetic

mutations that lead to clinical resistance have therefore only been well characterized with clinically used FQs [13, 23, 24]. In this case, larger increases in MIC values (eightfold or more) need to be achieved to exceed a susceptibility breakpoint to result in treatment failures, which have generally arisen from multiple mutations and modes of resistance [25].

2 Fluoroquinolones

FQs are the preeminent class of gyrase/TopoIV inhibiting drugs with an enormous clinical impact for treating infections and saving human lives. Nearly 30 FQ antibacterials have received regulatory approval for human use (others for animal use) and are differentiated with respect to potency, spectrum of action, pharmacokinetic attributes, and safety considerations (Fig. 1) [26]. However, since widespread use of FQs has led to considerable resistance, a more potent FQ that might maintain activity against resistant strains brings worries that the benefit would be marginal in a clinical setting due to altered pharmacokinetics and continued development of resistance [27–29]. In another concern, the FDA recently mandated that all FQs bear black box warnings on their label for spontaneous tendon ruptures and other side effects associated with muscles, joints, and nerves [30, 31]. First-generation FQs, including the first member of the class, nalidixic acid, are indicated for urinary tract infections caused by Enterobacteriaceae; rapid plasma clearance has mitigated

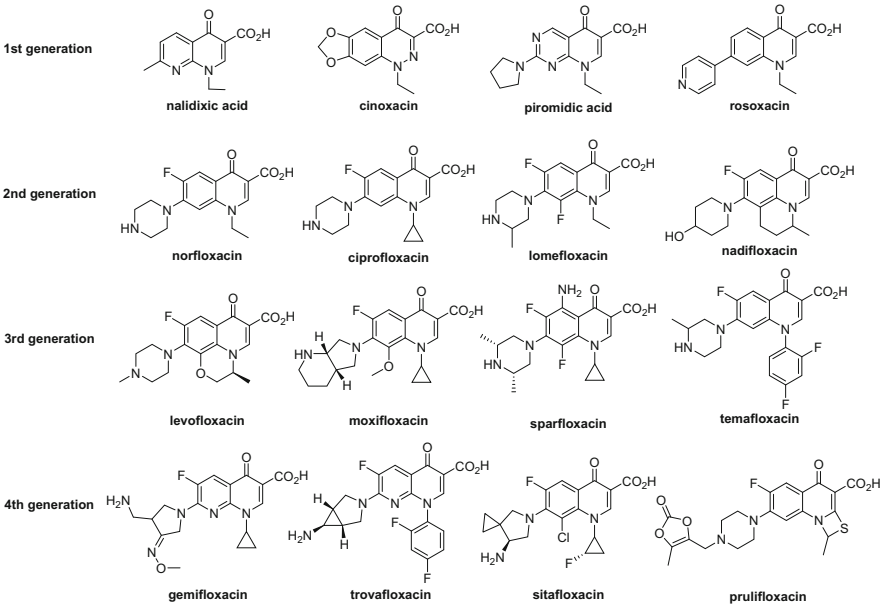

Fig. 1 Representatives for the four generations of fluoroquinolones

use for systemic infections. Second-generation FQs, including ciprofloxacin and norfloxacin, incorporate a fluorine atom on the quinolone core and demonstrate expanded and clinically useful Gram-negative and Gram-positive antibacterial activity as well as improved systemic exposure supporting treatment of skin and skin structure infections (SSSIs), intra-abdominal infections (IAIs), sexually transmitted diseases (STDs), respiratory tract infections (RTIs), and bone and joint infections. Levofloxacin and moxifloxacin are representative of third-generation FQs that demonstrate expanded Gram-positive (*Streptococcus pneumoniae*), mycobacterial, and atypical bacterial activity enabling treatment of expanded RTI indications and tuberculosis. Fourth-generation FQs, represented by gemifloxacin and trovafloxacin, have received FDA regulatory approval for systemic infections, though the latter was withdrawn from the market due to hepatotoxicity. Sitafloxacin and the prodrug prulifloxacin, approved for use in Japan, are undergoing trials for submission to the FDA. In addition, four new FQ drugs (Fig. 2) are in various stages of patient clinical trials [6, 32].

FQs have been shown to bind to a state of the topoisomerases in which both strands of DNA form covalent bonds to catalytic tyrosines from each of the GyrA or ParC subunits, thus classifying the drugs as topoisomerase poisons [33]. A consequence to the stabilization of this cleaved DNA complex is rapid killing kinetics attributed to the induction of the cellular SOS response wherein bacteria upregulate a number of genes to repair DNA, arrest the cell cycle, and thereby impede proliferation [34–36]. The covalent trapping of DNA by FQs was further supported by X-ray crystallography in 2009 when a structure of moxifloxacin (4 Å resolution) was determined in a quaternary complex with a 55-kDa DNA-binding-breakage-reunion domain of ParC, a 30-kD TOPRIM (*to*poisomerase *prim*ase) domain of ParE (*S. pneumoniae*) responsible for DNA binding, and a 34-base pair dsDNA [37]. DNA in the complex was covalently bound to the each of the catalytic ParC Tyr118 residues. Two molecules of moxifloxacin were present, each intercalated between the +1 and −1 nucleotides relative to the DNA breaks and associated each of the ParC/ParE interfaces of the A_2B_2 complex. Four Watson-Crick base pairs separated the two intercalating moxifloxacin molecules and the DNA strand breaks. Subsequently in 2010, the first gyrase-FQ X-ray structure (3.35 Å) was disclosed, in this case having ciprofloxacin bound to a truncated GyrA/truncated GyrB fusion construct from *S. aureus* and a 34-base pair double nicked dsDNA [38]. The GyrA/GyrB construct encompassed the DNA-binding domain (GyrB27-GyrA56; Gkdel(Δ544-579)) and included a GyrA Tyr123Phe mutation (equivalent to *S. pneumoniae* Tyr118 of ParC) to disable DNA strand cleavage. Since these

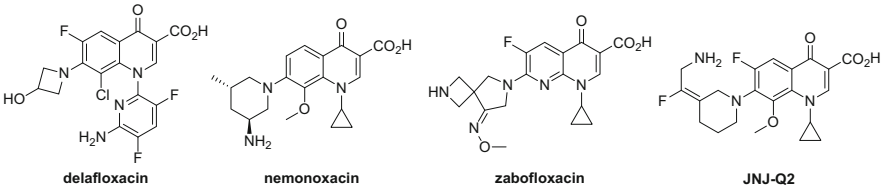

Fig. 2 Fluoroquinolones currently in clinical development

disclosures, a higher-resolution (2.95 Å) crystal structure (Fig. 3a) was published using the complex of moxifloxacin, dsDNA without the double nick, and the *S. aureus* gyrase fusion construct with Tyr123 intact. As with the ParC/ParE structures, the DNA was captured in the doubly cleaved state with covalent phosphate bonds to the Tyr123 hydroxyls of each GyrA subunit [39]. Through these structures, the binding model that has emerged is that the key FQ ketone and acid pharmacophores chelate a non-catalytic Mg^{2+} that is associated through a bridging water molecule to carboxylate of Glu88 on GyrA. The hydroxyl of Ser84 was positioned for an H-bond to the moxifloxacin carboxylate and a bridge to the Mg^{2+} through a water molecule. Over 90% of target-based clinical resistance to FQs disrupt these two critical residues [40]; higher levels of resistance are seen through multiple secondary mutations on either or both of GyrA and ParC. The considerable resistance to FQs in the clinic has been seen across all indications and accentuates the need for new drugs with alternate modes of action and alternate modes of topoisomerase inhibition. Perhaps the resistance to FQ is best illustrated by the example of *Neisseria gonorrhoeae* where FQs, once the primary treatment option, have been removed from the formulary for gonorrhea [41].

3 Spiropyrimidinetriones (SPTs)

SPTs represent the most recent class of Type II topoisomerase inhibitor with a representative compound having entered human clinical trials [42]. The first reported member of the class, QPT-1, was discovered by Pfizer via antibacterial phenotypic screening and has the structurally distinctive spirocyclic architecture on a tetrahydroquinoline scaffold [43]. Notably, QPT-1 had sufficiently good anti-*S. aureus* activity and pharmacokinetic properties to demonstrate efficacy in a mouse infection model on oral dosing, unusual for a screening hit. The compound was separated into

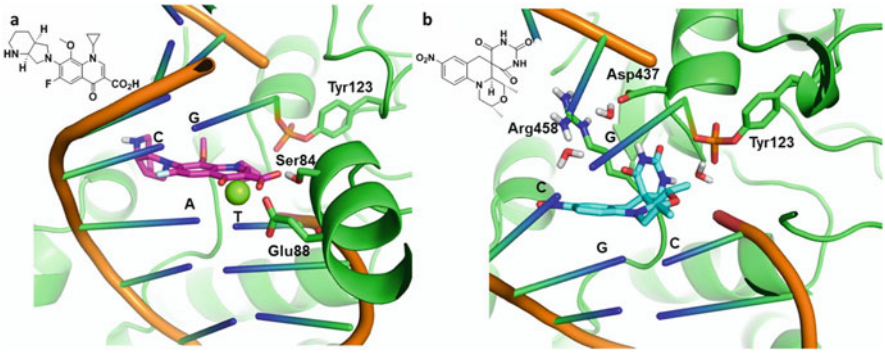

Fig. 3 Crystal structures of inhibitors bound to *S. aureus* DNA gyrase and cleaved DNA; (**a**) moxifloxacin (*magenta*); Mg^{2+} (*green sphere*), covalent phosphate bridge between Tyr123 and DNA guanosine (PDB 5CDQ). (**b**) QTP-1 (*cyan*) covalent phosphate bridge between Tyr123 and DNA guanosine (PDB 5CDM)

the optical antipodes with activity residing in the (−)-isomer and the absolute configuration as shown in Fig. 3b. The unprecedented spirocyclic architecture gave reason to suspect a novel mode of action, which was determined to be inhibition of DNA gyrase and was differentiated from FQs by the lack of cross-resistance. Three Pfizer patent applications delineated a robust analogue program [44–46]; two analogues **1** and **2** (Fig. 4) exemplified by larger-scale syntheses might be interpreted to have generated higher interest. However, Pfizer closed down its antibacterial discovery program in 2011 and has not reported progression of any QPT-1 analogue. Zoetis and AstraZeneca filed patent applications that maintained the tetrahydroquinoline scaffold with scopes covering SPT analogues **3** and **4**, respectively [47, 48]. Tetrahydronaphthyridine **5** is an isostere reported to show high antibacterial activity [49]. Finally, AstraZeneca disclosed compounds with a benzisoxazole scaffold (e.g., zoliflodacin and compounds **6**, **7**) that maintained the spirocyclic and morpholine architectures and incorporated a diverse array of benzisoxazole substituents [42, 50, 51]. Zoliflodacin (also known as AZD0914 and ETX0914) has been the only analogue to progress to the clinic, completing a Phase 2 trial in which it was described as safe and effective for the treatment of uncomplicated gonorrhea [52].

SPTs bind to the complex of gyrase (and TopoIV) with doubly cleaved DNA in both Gram-positive (*S. aureus*) and Gram-negative organisms (*E. coli*, *N. gonorrhoeae*) making the class, like FQs, a Type II topoisomerase poison [34, 42, 51]. SPTs similarly show fast killing kinetics associated with the SOS response. A differentiating factor between the two classes is that FQ activity against both gyrase and TopoIV was dependent on [Mg^{2+}], while SPT activity was [Mg^{2+}] independent in *S. aureus* and *E. coli*. This conclusion was determined by monitoring religation of DNA in the inhibitor-cleaved complexes on titrating with EDTA. In *N. gonorrhoeae*, the [Mg^{2+}] independence was seen for gyrase but not for TopoIV [34]. Researchers at GSK

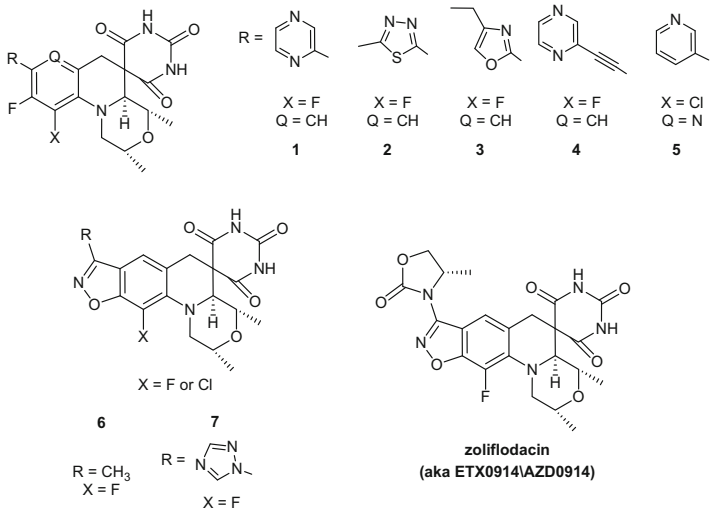

Fig. 4 Structures of various spiropyrimidinetriones (SPTs)

reported an X-ray crystal structure of QPT-1 (Fig. 3b) in a ternary complex with the *S. aureus* GyrA/GyrB fusion construct (intact Tyr123) and dsDNA without the double nick [39]. As with the capture of the cleaved complex by FQs, the structure showed cleavage of dsDNA via a covalent phosphate bond to each Tyr123 of each GyrA subunit. For QPT-1, the tetrahydroquinoline scaffold fusion with the morpholine formed a flat surface that intercalated base pairs of double-stranded DNA. The morpholine adopted a chair conformation with both methyl groups in equatorial orientations, which is important for preserving the flat surface and accounted for the preferred stereochemistry for activity [49, 51]. The −1 Watson-Crick pair (GC) differed from that optimized for FQs (AT) and used for the moxifloxacin crystallizations resulting in a modified intercalation environment [53]. A study of the specificity associated with SPT binding to cleaved DNA in the gyrase complex has not been reported as has been done with FQs [54]. The pyrimidinetrione pharmacophore has been described as unalterable for activity and radiated toward GyrB residues beyond the intercalating DNA framework [39, 42]. The distal pyrimidinetrione carbonyl formed an H-bond to the Asp437 backbone NH from GyrB. The two pyrimidinetrione NH moieties donated an H-bond each to a respective crystallographic water molecule: one water molecule was seen in an H-bond network with the carboxylate of GyrB Asp437. The other water was in an H-bond network at the DNA cleavage site with the covalently bonded DNA phosphate on one side of the cleavage and the released 3′-ribose hydroxyl on the other. There was considerable overlap between the positions of QPT-1 and moxifloxacin seen in the similarly derived co-crystal structures, this despite there being no cross-resistance (see below).

A broad scope was associated with the R-substituent on SPT benzisoxazoles (Fig. 4) toward improving DNA gyrase inhibitory potency as well as antibacterial activity [42, 50, 51]. Modeling of the zoliflodacin oxazolidinone R-substituent from the QPT-1-gyrase structure showed it to lie in a solvent-accessible area outside of the intercalating DNA framework [39]. A wide variety of aromatic rings, non-aromatic rings, and aliphatic groups were tolerated for activity. It followed that the R-substituents influenced other parameters important for drug optimization including solubility, lipophilicity, pharmacokinetic properties, and toxicity. In particular for toxicity, the proper selection of an oxazolidinone R-substituent eliminated issues of genotoxicity and bone marrow toxicity seen with other substituents, as best exemplified by the triazole of compound **7** [42, 50]. The antibacterial spectrum of zoliflodacin encompasses an array of Gram-positive pathogens including *S. aureus*, *Streptococcus pyogenes*, *S. pneumoniae*, and *Streptococcus agalactiae*, as well as fastidious Gram-negative pathogens including *Moraxella catarrhalis*, *Haemophilus influenzae*, and *N. gonorrhoeae* [55, 56]. This spectrum combined with favorable oral and parental pharmacokinetic properties supports treatment of skin and skin structure infections, respiratory tract infections, and infections caused by sexually transmitted diseases. The zoliflodacin development program has only targeted *N. gonorrhoeae* and urogenital gonorrhea as an expedient approach to assess safety and efficacy in the patient population with such a first-in-kind scaffold. SPTs have shown markedly lower antibacterial activity (upward of 100-fold) than FQs against *E. coli* and other Gram-negative pathogens despite being equipotent against gyrase, the primary target

[34, 50]. This result is likely due to differential bacterial permeability, which, in addition to target potency and secondary effects (as with the SOS response), contributes to the antibacterial activity of a drug. For example, permeation experiments with FQs suggested that chelation of lipopolysaccharide-associated divalent cations enhances passage through the bacterial outer membrane via a self-promoted uptake mechanism [57] that would not be expected with SPTs. Considerable interest continues to build toward understanding mechanisms of compound bacterial permeability and retention within the cell toward improving activity as part of drug design [7, 9, 58].

It proved difficult to generate robust lab mutants to SPTs in *S. aureus*, *S. pneumoniae*, and *N. gonorrhoeae* with relatively small MIC shifts being seen. Four first-pass target-resistant determinants that have been isolated were all localized on GyrB and did not impart cross-resistant to FQs [21, 55, 56]. No TopoIV mutations were observed indicating that, though zoliflodacin inhibited both topoisomerases, the primary mode of action is gyrase for all three organisms. As mentioned, the frequency of resistance and its relevance to the clinical setting cannot be well assessed from such in vitro experiments, and the fitness cost of mutations was not evaluated. Nonetheless, mapping the mutations that reduce bacterial susceptibility can offer insights into binding interactions. One resistant determinant to zoliflodacin with *S. aureus* seen at GyrB Asp437 (homologous to an Asp429 mutation seen with *N. gonorrhoeae*) bordered the QPT-1-binding region where the carboxylate interacts with a bridging crystallographic water molecule. A second mutation at GyrB Lys450 (*N. gonorrhoeae*, equivalent to Arg458 in *S. aureus*) had the aliphatic portion of the side chain positioned alongside the benzylic and aromatic C-H functionalities of QPT-1. A third mutation at Ser442 (*S. aureus*) was located well away from the binding pocket along an α-helix that extended to the inhibitor binding pocket. A fourth mutation at Ala439 was identified by plating QPT-1 in *S. aureus* culture; it formed part of same α-helix as Ser442 lying well away from the binding pocket [43]. The mutable sites to SPTs are conserved across species and have not been reported in FQ-resistant strains. Furthermore, the zoliflodacin mutant strains were fully susceptible, if not more susceptible, to ciprofloxacin. Surveillance studies with zoliflodacin across a variety of pathogens have not shown cross-resistance to FQs or any other antibacterial agent [59–62]. As mentioned, clinical FQ resistance due to mutations of GyrA Ser84 and Glu88 (*S. aureus* numbering) involves disruption of interactions with Mg^{2+}. Gyrase inhibition by zoliflodacin being independent of [Mg^{2+}] likely accounts for the difference in cross-resistance profiles between FQs ad SPTs. The caution is understood that the crystallographic models and artificial constructs generated for QPT-1 and FQs bound to gyrase may not precisely reflect the inhibitory states or pertinent enzyme conformations.

Other topoisomerase inhibiting antibacterial agents have been reported that are structurally related to FQs, but, like SPTs, they are not cross-resistant. Among these are aminoquinazolinediones (AQTs: see PD0305970, Fig. 5) [63, 64], isothiazoloquinolones (ITQs, ACH-702) [65] and oxazoloquinolinones (OQs, REDX04139) [66, 67]. A structure of PD0305970 and its complex with the *S. pneumoniae* ParC-ParE DNA-binding domain and dsDNA were determined by X-ray crystallography

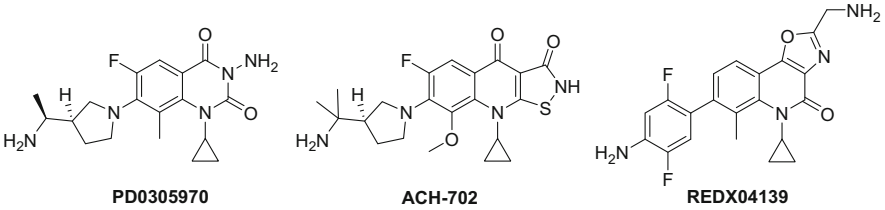

Fig. 5 Topoisomerase inhibitors with FQ structural motifs

showing the compound in a similar position to levofloxacin [68]. As with FQs and SPTs, the structure showed DNA in the cleaved state. However, PD0305970 lacks the Mg^{2+} chelating keto acid pharmacophore of FQs, again accounting for the lack of FQ cross-resistance associated with mutations at ParC (Ser79 and Asp83, equivalent to *S. aureus* Ser84 and Glu88) or the equivalent sites on GyrA. Mutable sites to PD0305970 have been isolated with difficulty by plating in *S. pneumoniae* culture and include Asp435, Arg456, Glu474, and Glu475 from GyrB [69], residues that abut the pyrrolidine substituent as seen in the co-crystal structure [64]. A ParE Arg453 laboratory mutation to REDX04139 has been isolated in *S. aureus* [66]. None of these mutations conferred reduced susceptibility to FQs, but the two arginine residues map to the zoliflodacin Lys450 and Arg458 resistant determinants seen in *N. gonorrhoeae* and in *S. aureus*, respectively. Asp435 maps to the conserved zoliflodacin Asp437 resistant determinant seen in *S. aureus*. These results suggest that these compounds are closer in their mode of inhibition to that of the SPTs. Overall, it appears that compounds binding in some fashion at the FQ binding site, but serving as poor ligands for Mg^{2+}, are immune to target-based modifications that lead to FQ resistance [70]. As of yet, no reports have emerged for an AQT, ITQ, or OQ structure progressing to the clinic.

4 Quinolines and Related Analogues

Gepotidacin (also known as GSK2140944) is the most advanced member of a very large and diverse class of gyrase/TopoIV inhibiting antibacterial agents showing a distinct mode of inhibition relative to FQs (Fig. 6) [66, 71–73]. Initial analogues in the class have the quinoline (not to be confused with quinolone) nucleus and were derived from *d*-quinotoxine. In 1853, Pasteur described the acid-catalyzed isomerization of quinine to *d*-quinotoxine [74, 75], the structure of which was established by Rabe in 1910 (Fig. 7) [76]. *d*-Quinotoxine was described as being intensely poisonous [75] but was shown to have medicinally useful vasodilation and neuronal serotonin reuptake inhibitory activities at lower doses [77, 78]. Modifications of *d*-quinotoxine led to a series of patents describing utility for treating cardiovascular disorders including arrhythmia and hypertension and depressive pathologies. In 1994, a patent from the Indian CSIR highlighted compound **8** as being useful against

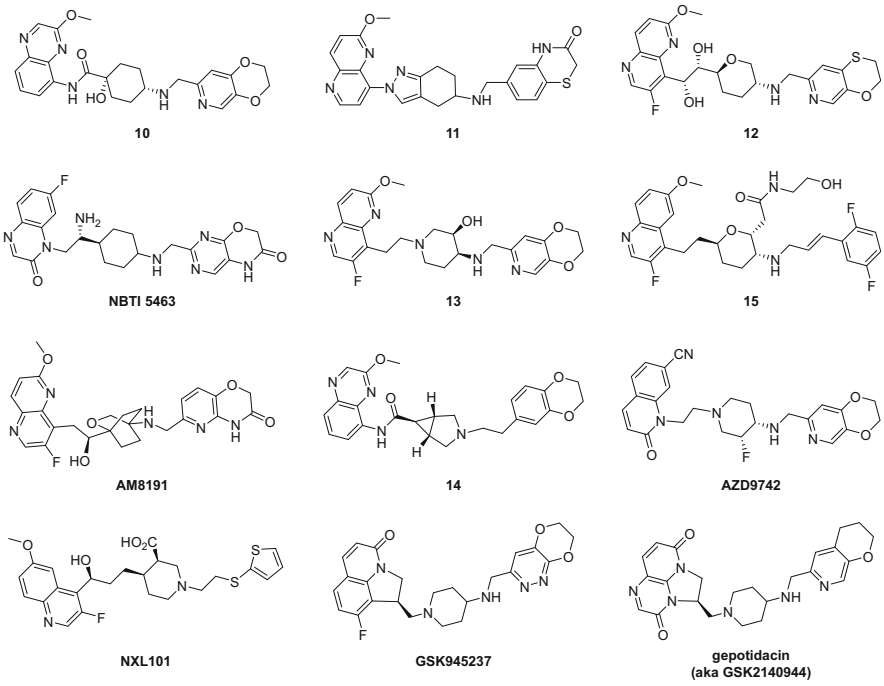

Fig. 6 Diverse structural array of quinolines from patents and literature

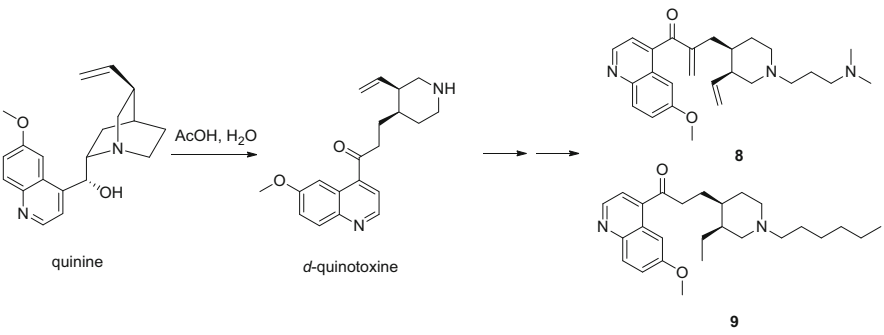

Fig. 7 Origin of first disclosed quinoline compounds

sexually transmitted diseases including syphilis and gonorrhea [79]. In 1999, GSK reported derivatives such as **9** with antibacterial activity heralding the start of extensive analogue work and investigations on the compound class [80]. In 2007, the mode of action of compound **11** (Fig. 6) was disclosed as inhibition of Type II topoisomerases [81]. Through time, it was recognized that the "left-hand side" quinoline could be replaced by many other fused heterocycles including naphthyridines,

naphthyridinones, quinazolinones, quinoxalines, oxoquinolines, pyridopyrazinediones, and others. The term "novel bacterial Type II topoisomerase inhibitor" (NBTI) was coined in 2010 [38], but as time and extensive investigations will necessarily dampen the "novel" label, the class will continue to be denoted by the original quinoline designation in this review. The quinoline and similar fused bicyclic replacements are generally linked by an eight-atom bridge to a second "right-hand side" heterocycle, most often a pyridine fused to a nonaromatic ring (Fig. 6). However, the length of the bridge can vary as can the nature of the right-hand side group. The bridge between the two heterocycles, though generally quite flexible, incorporates one or more rings reducing the number of rotatable bonds. Upwards of 80 patent applications have been filed in the class with about 30% by GSK. Other companies with significant filings (five or more) include Actelion, AstraZeneca, Taisho, and Toyoma. Compounds are reported with broad-spectrum activity against both Gram-negative and Gram-positive pathogens as well as mycobacterial and mycoplasma species.

An issue described in a number of quinoline publications has been toxicity associated with hERG binding and QT prolongation [82]. This toxicity may be predictable since initial quinolines are structurally related to compounds that induce arrhythmias and incorporate features associated with a hERG binding pharmacophore including a basic center spaced 6–7 Å from a hydrophobic aromatic moiety [83–85]. Hence, many of the compounds of Fig. 6 have features designed to decrease hERG binding including the incorporation of a carboxylic acid (NXL101) [86] as well as the reduction of amine basicity and compound polarity with strategic incorporation of electronegative substituents (compound **13** and AZD9742) [87–89]. NXL101 and AZD9742 (also called AZ6142 [90]) showed sufficiently high margins to hERG binding in vitro and QT prolongation in vivo during preclinical evaluations to progress to Phase 1 clinical trials; however, neither was progressed further due to QT aberrations in man [91]. Another method to mitigate hERG binding would be to rigidify compounds toward steering away from a hERG binding conformation as might be exemplified by compounds **11** and **14**, GSK945237, and gepotidacin, where two rings bridge the left- and right-hand side heterocycles. GSK945237 completed Phase 1 clinical trials in humans without issues of QT prolongation [92], but progression of the compound was stopped in favor of a more promising compound, presumably gepotidacin [93]. The IC_{50} for hERG binding with gepotidacin was quite high, 1.4 mM [94], considerably higher than values registered by other quinolines. Phase 1 evaluations with gepotidacin showed no evidence of EKG abnormalities [95]. A separate Phase 1 study to evaluate cardiac conductance has been completed [96]. Gepotidacin shows antibacterial activity (MIC_{90}'s ≤ 1) against an array of Gram-positive bacteria including *S. aureus*, *S. pneumoniae*, and *S. pyogenes* and fastidious Gram-negative bacteria including *N. gonorrhoeae*, *M. catarrhalis*, and *H. influenza* [73]. A Phase 2 clinical trial for the treatment of acute Gram-positive skin and skin structure showed clinically significant utility [97]; another Phase 2 trial for uncomplicated urogenital gonorrhea has been completed [96]. Other quinolines such as NBTI 5463 demonstrated broader spectrum activity including serious Gram-negative pathogens such as *E. coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* and efficacy in an *E. coli* animal model of infection [98].

In gel-based supercoiling and decatenation assays, quinolines bind to nicked DNA or to intact DNA-enzyme complexes, depending on the isozyme investigated. However, accumulation of the signature FQ and SPT double-strand cleavages was not seen, and quinolines are classified as catalytic inhibitors rather than topoisomerase poisons [18]. With NXL101, relaxed dsDNA accumulated for both gyrase and TopoIV from *S. aureus*. Similarly, NXL101 inhibition of gyrase from *E. coli* accumulated dsDNA; with *E. coli* TopoIV, singly nicked DNA was also seen [86]. NBTI 5463 showed accumulation of singly nicked DNA with *E. coli* DNA gyrase [98]. In 2010, GSK reported the crystal structure of quinoline GSK299423 in a ternary complex with gyrase from *S. aureus* with the fusion GyrA/GyrB Tyr123-mutated construct and a 20-base pair dsDNA (Fig. 8a) [38]. The quinoline of GSK299423 was intercalated between two Watson-Crick paired bases of the DNA that lie between the two tyrosine cleavage sites where FQs and SPTs were shown to bind. The inhibitor reached across a solvent-accessible region of the complex with the aminopiperidine linker projecting the pyridyl ring fusion into a hydrophobic region of the GyrA-GyrA dimer interface, where there was a measure of symmetry among the surrounding residues. The pyridine π-face was flanked on either side by the side chain of Met121 from each of the GyrA monomers, and the acyclic amine on the linker formed a salt bridge with the carboxylate of Asp83. Other than the salt bridge to the amino group, there was no specific binding interaction along the bridge linking the inhibitor left- and right-hand sides, in line with the variations that have been operable for activity. Selection for laboratory resistance in *S. aureus* to NXL101 showed mutations in the aforementioned Met121 and Asp83 residues. Two other mutable sites were identified using NXL101, namely, His81 that is positioned in a second shell removed from the GSK299423 binding site and Arg92 that is more distally located [86]. Resistant determinants generated with AZD9742 in *S. aureus* include GyrA Met121, and a number of other more distal residues (GyrA: Pro36, Val45, Gln85, Gly171; GyrB: Lys417) scattered around the DNA-binding pocket [90]. Overall, the mutant analyses and crystallography work fully accounted for the lack of cross-resistance between quinolines and FQs [73, 86, 98].

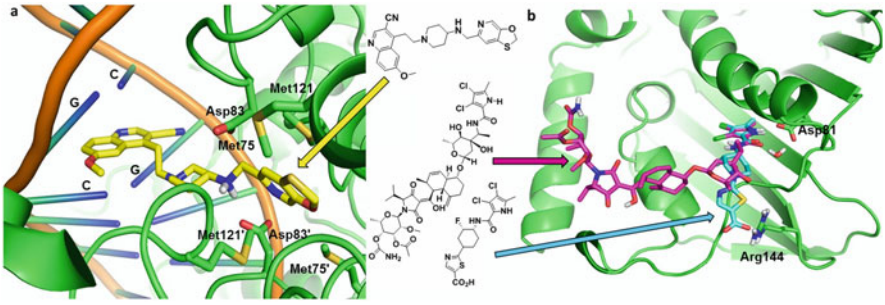

Fig. 8 Crystal structures of inhibitors bound to *S. aureus* DNA gyrase; (**a**) GSK2992423 (*yellow*); DNA-binding region (PDB 2XCS). (**b**) Kibdelomycin (*magenta*), GyrB ATP-binding site (PDB 4URM); overlay of a pyrrolamide (*cyan*) in GyrB ATP-binding site (PDB 3TTZ)

5 ATPase Inhibitors

Novobiocin is an aminocoumarin natural product antibiotic discovered in 1955 well before its mode of action was determined to be inhibition of gyrase/TopoIV by competing with ATP and disrupting the energetics required for the catalytic machinery. It alone has received regulatory approval among a wide structural variety of ATPase inhibitors delineated in patents and publications. The primary utility of novobiocin has been for the parental treatment of Gram-positive infections associated with skin infections; it has also been used orally for respiratory tract infections. However, the antibiotic has not been widely used and was withdrawn from the market in 2007 for reasons ascribed to the existence of more effective drugs with better safety profiles [99]. Though resistance to novobiocin was problematic when it was in clinical use [100], resistance today does not seem to be a serious issue, and the ATP-binding site continues to be a target for drug design. Figure 9 shows a sampling of structures operating as ATPase inhibitors; a thorough rendering of the much wider variety of structural types was recently quite comprehensively reviewed [17].

Beyond clinical validation with novobiocin, work with the ATP-binding site itself has a number of attributes that are enticing for drug design. First, though most assessments of topoisomerase inhibitor potency are based on low-throughput gel-based assays, monitoring of ATP hydrolysis with either gyrase or TopoIV can be carried out in high-throughput mode to interrogate compound libraries [101, 102]. Second, the structure of the ATP-binding site encompassed in the GyrB/ParE 24 and 43-kDa N-terminal domains has been determined with a variety of isozymes and a diversity of ATP-site inhibitors [17]. The use of the 24- and 43-kDa ATPase domains preceded more recently solved structures of GyrA/GyrB and ParC-ParE DNA-binding domains. Finally, the ATP-binding site ranked high as a druggable target with a compact concavity and appropriate nonpolar and total surface areas associated with complementary binding of compounds that fall within Lipinski and other physical property parameters [103, 104]. Consequently, numerous crystal structures with bound inhibitors have

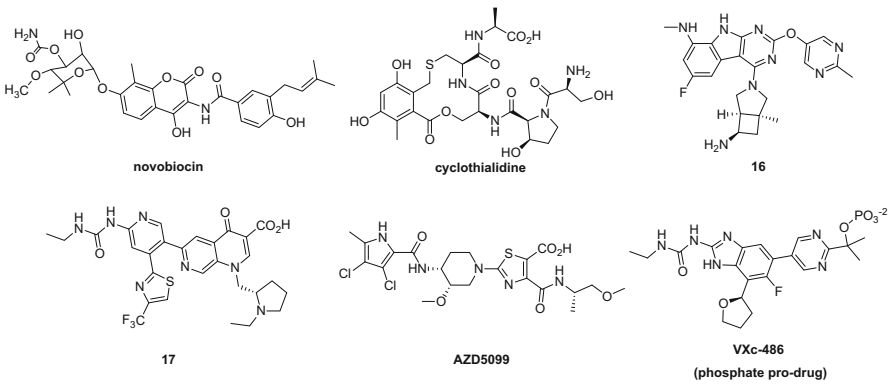

Fig. 9 Representative ATPase inhibitors

emerged over the years with the GyrB or ParE ATPase domains from a variety of bacterial organisms. The structural work has shown a consensus binding motif in which a conserved active site aspartate carboxylate (Asp81 in *S. aureus* GyrB) receives a hydrogen bond from an inhibitor H-bond donor and bridges to an inhibitor H-bond acceptor via a crystallographic water molecule. This push-pull H-bond motif constituted a recognition element seen for adenine of ATP seen in a co-crystal structure with non-hydrolyzable AMPPNP [105]. The conformation of ATP in the crystal structure turned through a region surrounding the ribose ring to a polar region where the triphosphates are localized. Crystal structures with novobiocin showed it to extend more linearly into a region not occupied by ATP where it engaged the guanidinium of a conserved arginine (Arg144 in *S. aureus* GyrB) via a hydrogen bond to the coumarin carbonyl [106, 107]. Other noteworthy interactions seen with novobiocin were an Arg-Glu salt bridge ceiling to the binding pocket forming a π-cation interaction over the coumarin ring and an Asn54 carboxamide in an H-bond array with the novobiose hydroxyl group. These residues in addition to a number of hydrophobic interactions line the binding regions of nearly all more potent (low nanomolar) ATPase inhibitors. *S. pneumoniae* Thr172 and Lys143 from GyrB (equivalent to Thr173 and Arg144 in *S. aureus*) are two binding site resistant determinants to novobiocin that have been shown to be cross-resistant to other ATPase inhibitors; as would be expected, the resistance did not cross over to FQs, SPTs, or quinolones [42, 101, 108]. The extensive structural work around the ATP-binding site of gyrase/TopoIV has made it a prototype for validating novel fragment-based, analytical, and computational tools for working with enzymes [109].

Co-crystallizations with the recently isolated natural product kibdelomycin (Fig. 8b) and the N-terminal domains of GyrB (24 kDa) and ParE (43 kDa) from *S. aureus* showed a notable deviation from the novobiocin reach to Arg144. Kibdelomycin was discovered by screening culture extracts against a collection of antisense-repressed *S. aureus* strains and shown to have potent Gram-positive antibacterial activity. The dichloropyrrolecarboxamide pharmacophore in the crystal structures occupied the adenine pocket overlaying almost exactly the dichloropyrrolecarboxamide of pyrrolamides such as AZD5099 (Figs. 8b and 9). AZD5099, the only ATPase inhibitor to have reached the clinic in recent years, was derived in an optimization program that followed the NMR identification of a fragment pyrrolecarboxylic ester binding at the adenine pocket [108]. Kibdelomycin represents a quite unusual juxtaposition wherein a discovery from nature (the dichloropyrrolecarboxamide pharmacophore) followed what was designed in an iterative drug discovery program. Structural work showed a U-shaped conformation for bound kibdelomycin, following a trajectory from the adenine pocket beyond the ribose of ATP but bending before the trajectories toward Arg144 seen with novobiocin and other ATPase inhibitors [107].

Only three compounds besides novobiocin have progressed to clinical trials. Two of these three are the aminocoumarins coumermycin A1, a natural product, and the semisynthetic BL-C43. Both compounds entered Phase 1 clinical trials in the 1960s but were withdrawn from development due to safety liabilities [17]. The third compound, AZD5099 (Fig. 9), was evaluated in Phase 1 but was suspended from further development due in part from nonclinical toxicology issues and in part from redirection

of the AstraZeneca portfolio [110]. The antibacterial spectrum of AZD5099 includes Gram-positive and fastidious Gram-negative bacteria, similar to the spectrum of novobiocin and most other GyrB/ParE inhibitors [108]. Other ATPase inhibitors have broadened activity to include serious Gram-negative pathogens such as *E. coli*, *K. pneumoniae*, and *P. aeruginosa*. Among those are fused tricyclic pyrimidoindoles (such as **16**) from Trius (now belonging to Merck) [111] as well as pyridylurea inhibitors from Biota [112] and AstraZeneca (such as **17**) [17, 113]. The urea benzimidazole VXc-486 from Vertex showed potent Gram-positive and fastidious Gram-negative activity [114]. Recently, the compound was licensed to Spero Therapeutics. Spero has developed a membrane permeabilizing polymyxin-derived potentiator SPR741 [115] and investigated combinations with VXc-486 to address infections caused by serious Gram-negative bacteria [116].

6 Summary

Mutations that reduce susceptibility to any single of the four classes of gyrase/TopoIV inhibiting drugs do not do so for the other three. Clearly, it would be imperative that new clinical compounds are not compromised by existing FQ resistance, which has been borne out for zoliflodacin and gepotidacin in surveillance studies. The primary FQ-resistant mutations seen commonly in the clinic include residues associated with a non-catalytic Mg^{2+} that bridges the FQ keto acid pharmacophore to GyrA/ParC. Clinical strains with multiple target mutations remain susceptible to novobiocin, gepotidacin, and zoliflodacin. The relatively rare plasmid-borne Qnr mode of resistance has been shown to confer reduced susceptibility to FQs by blocking access to topoisomerase DNA-binding/cleavage domain. SPTs and other compounds that bind to the DNA-binding domain have been shown to be cross-resistant, while ATPase inhibitors have not [117]. This mode of resistance may cross over to quinolines based on their proximal binding site. If zoliflodacin and gepotidacin or analogues progress to commercialization, more will be learned about the propensity for clinical cross-resistance with other modes of resistance including those associated with efflux (e.g., OqxAB and QepA), FQ-modifying enzymes (e.g., aac(6′)-lb.-cr and AAC(6′)-Ib-cr), and porin (e.g., OmpF) alteration [13].

The molecular mode of action of the four classes of inhibitors can be divided into those that are topoisomerase poisons (FQs and SPTs) and those that are catalytic inhibitors (quinolines and ATPase inhibitors). The topoisomerase poisons stabilize cleaved DNA-topoisomerase complexes inducing the bacterial SOS response contributing to cell death and faster killing kinetics at least relative to novobiocin and other ATPase inhibitors and to the quinoline NBTI 5463 [34, 55, 98]. The further correlation follows that catalytic inhibitors need to attain higher inhibitory potencies than topoisomerase poisons to show antibacterial activity. Hence, the ratio between gel-based gyrase/TopoIV IC_{50}s (*E. coli* isozymes) and MIC values against *tolC*$^{-}$ *E. coli* (used to diminish differences in bacterial permeability) was lower for catalytic inhibitors than topoisomerase poisons [50, 98, 118]. FQs and STPs mode-

of-inhibition differentiation became clear from biochemical Mg^{2+} sequestration studies where the former are dependent on [Mg^{2+}] and the latter are not. Crystallography has shown the relative positioning of FQs, SPTs, quinolines, and ATPase inhibitors (Fig. 10) and illustrates the considerable overlap between the FQs and SPTs. Both FQs and QPT-1 intercalated Watson-Crick pairs at the DNA cleavage site, while quinolines intercalated DNA between the cleavage sites. Binding of FQs is driven by chelation to Mg^{2+} that otherwise engages water solvates that bridge to the enzyme (Fig. 3a). Binding of SPTs showed the key pyrimidinetrione pharmacophore interacting with a water molecule bridging cleaved DNA and with a conserved aspartate residue from GyrB (Fig. 3b).

As a class, FQs have become the most widely prescribed antibacterials. Newer-generation analogues have improved antibacterial activity as well as broadened and differentiated the spectrum of activity. To this day, additional members of the class continue to progress in clinical trials. If zoliflodacin and gepotidacin advance to the marketplace, confidence in their tolerability and efficacy will grow, and second- and third-generation analogues will follow. Zoliflodacin has a pathogen-specific (*N. gonorrhoeae*) development path, in line with a diminished remit for broad-spectrum agents and a greater emphases on rapid diagnostics toward better stewardship of antibacterials and management of resistance [119]. Neither zoliflodacin nor gepotidacin addresses infections caused by serious Gram-negative bacteria, which would certainly be an important objective. Members of the quinoline class in particular have shown broad-spectrum (Gram-negative and Gram-positive) antibacterial activity, forming the basis for optimism that compounds appropriate for clinical use will be identified. The issue of cardiac toxicity that has held back progression of the class will continue to be addressed in future analogue programs, as it is clear that such toxicity is not topoisomerase mechanism based. Similarly, the wide range of structural types

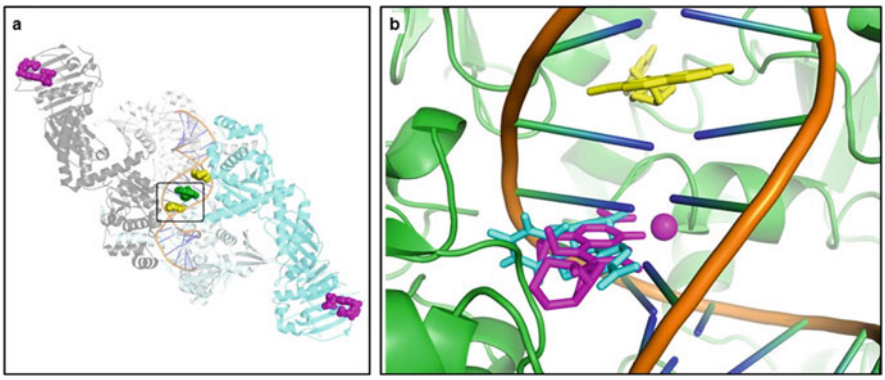

Fig. 10 Relative locations of four classes of DNA gyrase inhibitors. (**a**) "Overhead" view of composite DNA gyrase/TopoIV structure (*S. aureus* GyrB, 4URO; *S. pneumoniae* TopoIV, 4I3H), binding sites for ciprofloxacin (two molecules in *yellow*), GSK2992423K (*green*), and novobiocin (two molecules in *magenta*) published in *Scientific Reports* [42]. (**b**) DNA-binding domain of *S. aureus* gyrase (PDB 2XCS) showing GSK2992423 (*yellow*) overlaid with the orientations seen for moxifloxacin and the associated Mg^{2+} (*magenta*) (PDB 5CDQ) and QPT-1 (*cyan*) (5CDQ)

associated with ATPase inhibitors is quite impressive offering optimism that novobiocin will not be the only compound in the class to reach the marketplace. ATPase inhibitors have also achieved Gram-positive and both fastidious and serious Gram-negative antibacterial activity, and the expectation is that continuing research will address the problems that have held the area back. Structures of the N-terminal ATPase domains for gyrase/TopoIV have significantly contributed to the design of ATPase inhibitors, but the DNA-binding domain structures have not yet been exploited for FQ, SPT, and quinoline analogue design. The payoff from these more recent structures can be expected in time. Overall, the emergence of newer medicines will extend beyond their use as single agents to include combination therapy toward achieving therapeutic synergy, improved pathogen coverage for empiric therapy, and management of resistance. The prospects are encouraging that newer medicines operating by gyrase/TopoIV inhibition will be added to the arsenal that physicians can draw from to fight infectious diseases and deal with the increasing problem of antimicrobial resistance.

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