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

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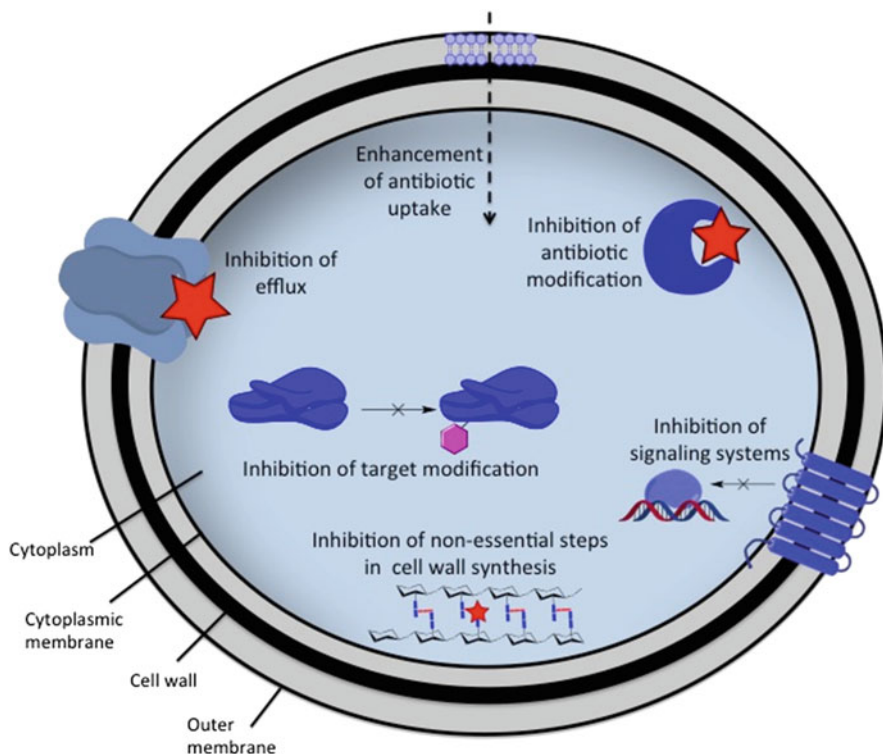
Fig. 1 Targets of small-molecule adjuvants that suppress antibiotic resistance

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 acetyltransferases, 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
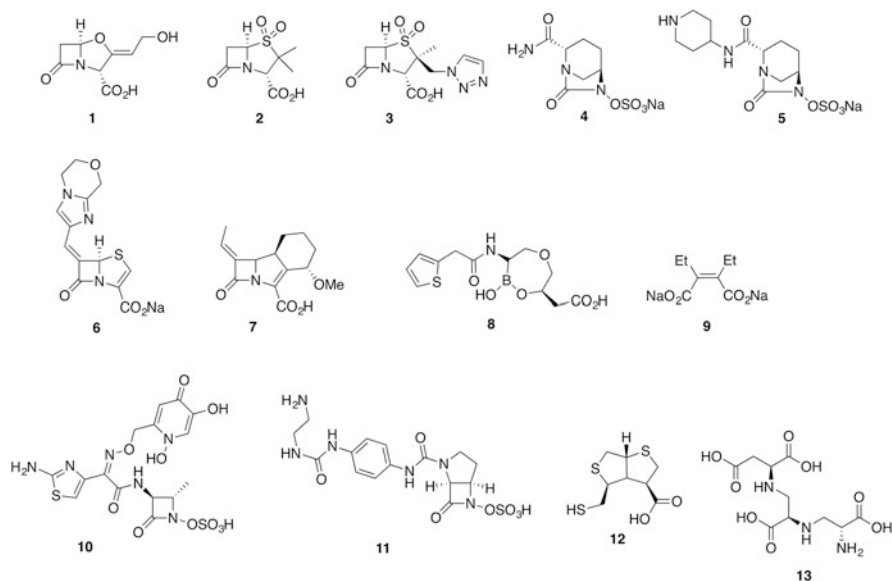

Fig. 2 Adjuvants that inhibit β-lactamases

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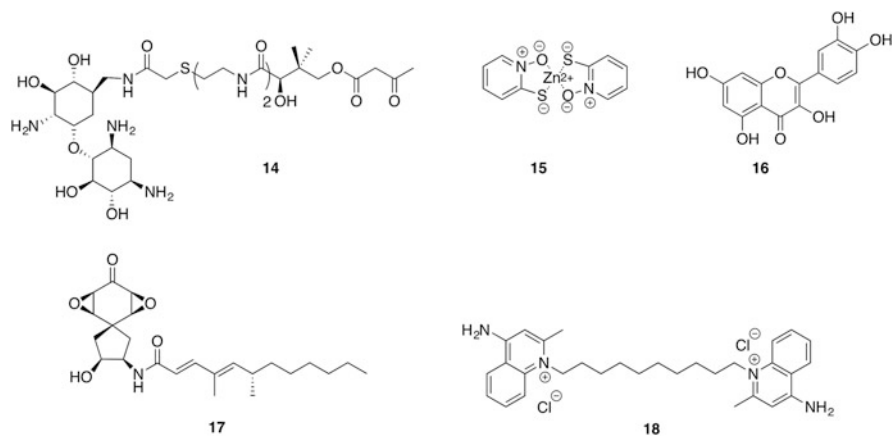
Fig. 3 Adjuvants that inhibit antibiotic-modifying enzymes other than β-lactamases

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
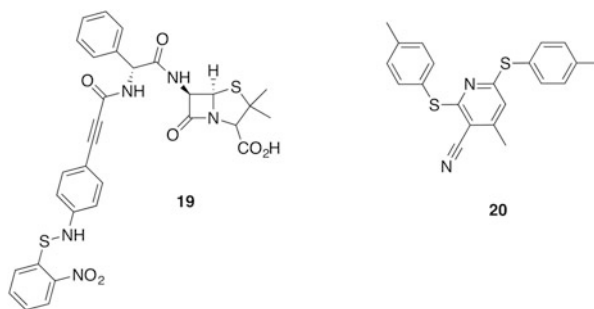
intracellular concentration of drug. Efflux pumps are ubiquitous in bacteria and present a significant challenge to the development of effective antibiotics. They may be specific for one substrate or one substrate class or may expel multiple unrelated classes of antibiotics, as is the case for the resistance-nodulation-division (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
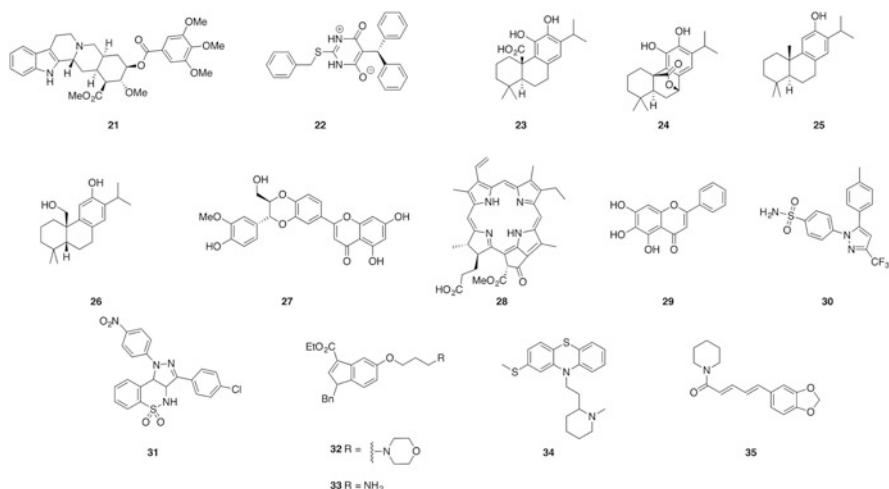
Fig. 5 Inhibitors of *S. aureus* efflux pumps

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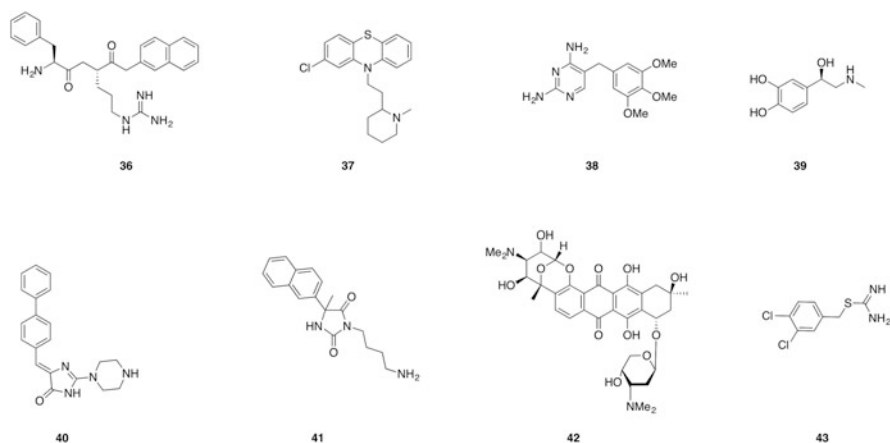
Fig. 6 Adjuvants that inhibit efflux in Gram-negative bacteria

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 amino-coumarin 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
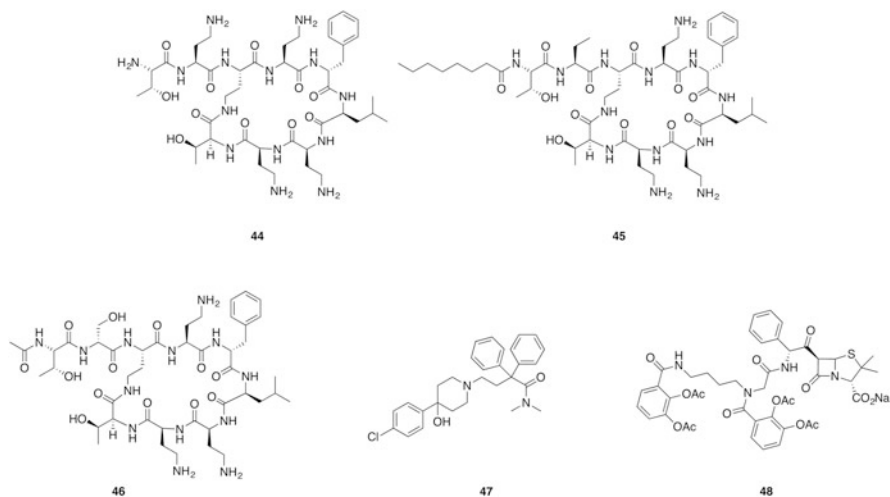
Fig. 7 Adjuvants that enhance the uptake of antibiotics

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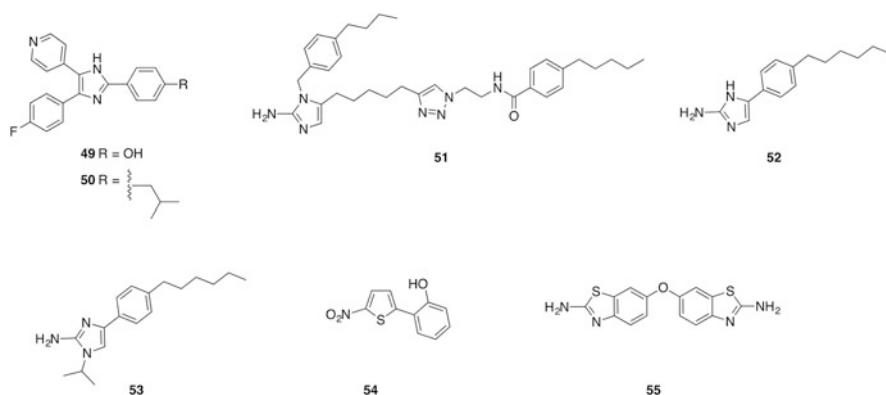
Fig. 8 Inhibitors of bacterial signaling systems involved in antibiotic resistance

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 phospho-ethanolamine 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
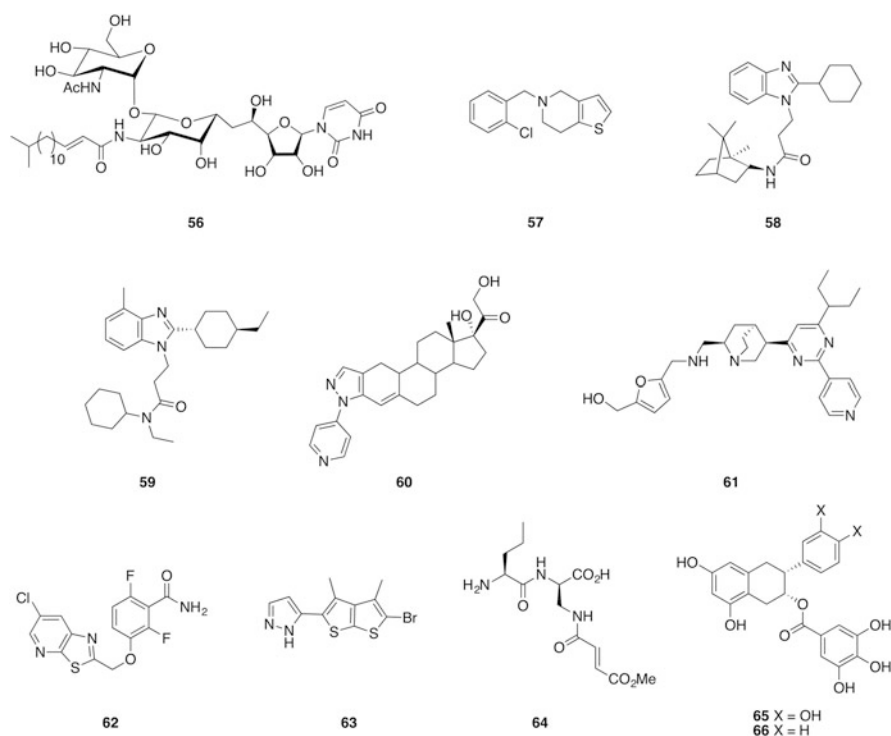
Fig. 9 Adjuvants that target cell wall synthesis

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 thiazo-lopyridine 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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