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Overcoming Intrinsic Resistance in Gram-negative Bacteria using Small Molecule Adjuvants

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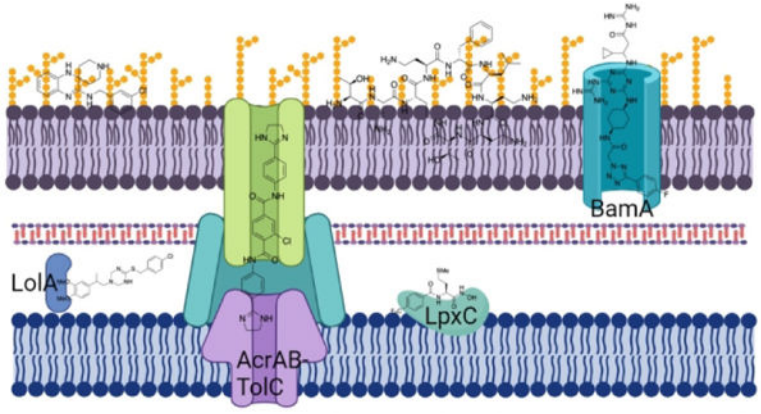
Abstract

Gram-negative bacteria are intrinsically resistant to many classes of antibiotics, predominantly due to the impermeability of the outer membrane and the presence of efflux pumps. Small molecule adjuvants that circumvent these resistance mechanisms have the potential to expand therapeutic options for treating Gram-negative infections to encompass antibiotic classes that are otherwise limited to treating Gram-positive infections. Adjuvants that effect increased antibiotic permeation, either by physical disruption of the outer membrane or through interference with synthesis, transport, or assembly of membrane components, and adjuvants that limit efflux, are discussed as potential avenues to overcoming intrinsic resistance in Gram-negative bacteria.

Graphical abstract



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Introduction.

Infections caused by multidrug-resistant (MDR) bacteria, in some cases resistant to every clinically available antibiotic, are increasing in prevalence and represent a serious health threat.[1] The use of antibiotics as the primary treatment for bacterial infections over the last 70 years has enabled significant medical advances; practices such as: surgery, premature infant care, cancer chemotherapy, and transplantation would not be feasible without effective antibiotics.[2] The World Health Organization (WHO) states that "a post-antibiotic era - in which common infections and minor injuries can kill - is a very real possibility for the 21st century"[3]. If resistance continues to rise at the current rate it is projected that MDR bacterial infections will account for over 10 million deaths, and a global economic cost of 100 trillion USD annually by 2050.[4, 5] The rise in MDR infections has coincided with a decrease in the number of novel antibiotics entering the clinic, due in part to significant divestment of pharmaceutical companies from antibiotic drug discovery.[6, 7]

Infections caused by MDR Gram-negative bacteria are of particular concern. Of the six bacterial species identified as the greatest threat to human health, the ESKAPE pathogens, four are Gram-negative: *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species.[8] The last novel class of antibiotics with activity against Gram-negative bacteria introduced into the clinic were the fluoroquinolones in the 1960's,[9] and even last resort antibiotics such as polymyxins are experiencing increasing levels of resistance.[10] Carbapenem resistant *A. baumannii, P. aeruginosa,* and *K. pneumoniae* were included in the highest category of a list of pathogens released by the WHO in 2017 as resistant to the majority of currently available antibiotics and urgently requiring new therapeutic options.[11]

In this Digest we give a brief background as to why infections caused by Gram-negative bacteria are so challenging to overcome, present an overview of traditional and current treatment approaches, and discuss recent advances in the use of small molecule antibiotic adjuvants to tackle this problem.

Challenges of treating Gram-negative infections.

The fundamental structural differences between the cell envelopes of Gram-positive and Gram-negative bacteria (Figure 1) play a significant role (along with the presence of efflux pump systems) in the response to many antibiotics. The Gram-positive cell envelope consists of a symmetrical phospholipid bilayer, the cytoplasmic membrane (CM), surrounded by a cell wall comprised of many layers of peptidoglycan 30 – 100 nm thick, with a narrow periplasmic space between the two.[12] The Gram-negative cell envelope also contains a CM and cell wall, however the peptidoglycan layer is much thinner, typically only a few nanometers thick.[13] The major difference, and that which plays the most significant role in the differing response to antibiotics, is the presence of an outer membrane (OM). The Gram-negative OM is an asymmetrical lipid bilayer, the inner leaflet of which contains phospholipids, while the outer leaflet is composed of glycolipids, principally lipopolysaccharide (LPS). LPS typically consists of a hydrophobic domain known as lipid A, a core oligosaccharide, and a distal polysaccharide or *O*-antigen,[14]

(in lipooligosaccharide (LOS) *O*-antigen is not present). Most OM proteins are either lipoproteins anchored in the inner leaflet of the OM, or integral transmembrane ß-barrel proteins, known as porins, that mediate the uptake of small molecules across the OM in addition to contributing to membrane stability.[13, 15]

Both LPS and porins play a critical role in the barrier function of the OM, LPS effectively prevents access of hydrophobic molecules, while diffusion of hydrophilic molecules through porins is limited to molecules below ~700 Da.[13] This combination confers intrinsic resistance to many antibiotic classes, including macrolides, glycopeptides, and lipopeptides, despite the fact that the targets of most antibiotics are highly conserved across Gram-positive and Gram-negative species.[16]

Current treatment approaches for Gram-negative infections.

Treatment options for infections caused by Gram-negative bacteria have typically consisted of small broad-spectrum hydrophilic antibiotics such as ß-lactams, tetracyclines, and fluoroquinolones that enter the cell through porins.[17] Other antibiotics used to treat infections caused by Gram-negative bacteria gain access via a self-promoted uptake pathway that involves the displacement of divalent cations and subsequent destabilization of the LPS; such antibiotics are usually cationic and include polymyxins, aminoglycosides, and the later generation macrolide azithromycin. Some compounds enter through a combination of both pathways.[18, 19]

Most antibiotics used to treat infections caused by Gram-negative bacteria (with the exception of polymyxins) are broad-spectrum, and acquired resistance is becoming widespread.[20] This is in part due to the fact that many resistance determinants reside on mobile genetic elements and thus can disseminate rapidly, and multiple resistance genes are often co-harbored on the same genetic elements.[21] As the pool of effective antibiotics for the treatment of Gram-negative infections is inherently smaller than that for infections caused by Gram-positive bacteria, increasing levels and prevalence of resistance to established therapeutic options represents a formidable predicament.

While attempts to identify novel antibiotic classes with activity against Gram-negative bacteria have proven fruitless in recent decades, synthetic modification of scaffolds within known classes has seen some success in (at least temporarily) overcoming acquired resistance. ß-Lactam antibiotics such as the later generation cephalosporins and newer carbapenems display increased stability toward various classes of ß-lactamases.[22,23] Similarly, the novel ß-lactamase inhibitors avibactam and vaborbactam show increased inhibition of class A and C ß-lactamases compared to ß-lactam containing inhibitors.[24, 25] Semi-synthetic aminoglycosides such as arbekacin and plazomicin are not as readily modified by aminoglycoside modifying enzymes,[26] while the glycylcycline tigecycline circumvents many of the resistance mechanisms that befall the tetracyclines[27].

Structural modifications to known antibiotic classes that overcome intrinsic resistance in Gram-negative bacteria, i.e., that convert a Gram-positive only antibiotic to a broad-spectrum antibiotic have been exploited for decades. The addition of an amine moiety

to penicillin G brought about the first Gram-negative acting ß-lactam, ampicillin,[28] while ring expansion of the erythromycin macrocycle with a basic nitrogen to form azithromycin results in improved activity against some Gram-negative species.[29, 30] Richter and Hergenrother have developed a series of guiding principles for compound accumulation in *Escherichia coli* that they term "eNTRy Rules"[19,31], which should aid in future structural modification approaches to expanding the spectrum of Gram-positive acting antibiotics to encompass Gram-negative species.

Combinations of two or more antibiotics have a long-term precedent in the management of infectious diseases,[32] and this approach has been utilized to overcome the ineffectiveness of many antibiotics in monotherapy for treating Gram-negative infections.[20] Polymyxins in particular have been utilized in combination with antibiotics from several other classes, including ß-lactams, tigecycline, fluoroquinolones, aminoglycosides, and rifampin.[32] One mechanism by which polymyxins synergize with other antimicrobials is through a permeabilizing effect on the Gram-negative OM that facilitates entry of partner antibiotics.[33] This has relevance both for antibiotics for which acquired resistance is a result of decreased uptake, and antibiotics to which Gram-negative bacteria are intrinsically resistant.[32]

Antibiotic adjuvants.

The combination of an antibiotic with a non-microbicidal adjuvant that potentiates antibiotic activity, termed an adjuvant,[34] has long been successfully utilized clinically to overcome acquired resistance in the case of ß-lactamase inhibitors.[35] The adjuvant approach also holds promise for intrinsic resistance in Gram-negative bacteria, and is receiving increasing attention. An adjuvant that circumvents intrinsic resistance to one or more of the several classes of antibiotics that are clinically employed to treat infections caused by Gram-positive bacteria would have significant implications for the treatment of infections caused by MDR Gram-negative bacteria, for which there are in some cases no available treatment options and very few likely to materialize in the near future.

OM disruptors.

Gram negative bacteria with a permeabilized OM exhibit increased susceptibility to otherwise Gram-positive selective antibiotics.[19] The use of an adjuvant that permeabilizes the OM is therefore an attractive approach to sensitizing Gram-negative bacteria to Gram-positive selective antibiotics; however, indiscriminate membrane disruptors often exhibit mammalian toxicity and therefore require structural optimization for *in vivo* application.

As mentioned, polymyxin antibiotics are used clinically in combination with several other classes of antibiotics, and synergize through permeabilization of the OM to effect increased entry of the partner antibiotic to the cell.[33] Polymyxin antibiotics have several distinct interactions beyond the initial encounter with LPS that play a role in the mechanism of bacterial killing.[36] This phenomenon has been exploited to develop analogues that retain permeabilization but lack bactericidal activity. Polymyxin B nonapeptide (PMBN) **1** (Figure 2), generated by the enzymatic removal of the acyl group and *N*-terminal 2,4-diaminobutyryl (Dab) residue from polymyxin B (PMB), retains the OM permeabilizing

activity of PMB but lacks microbicidal activity.[37] PMBN increases the susceptibility of *E. coli* to the hydrophobic antibiotics erythromycin, clindamycin, rifampin, fusidic acid, novobiocin, and cloxacillin.[37] However, as nephrotoxicity is a common adverse effect of polymyxin antibiotics, and PMBN retains this nephrotoxicity, the further development of polymyxin analogues to both improve potency and reduce toxicity has been explored.

Deacylation of polymyxin B or colistin to generate deacylpolymyxin B (DAPB) **2** and deacylcolistin (DAC) **3** (Figure 2) respectively, results in more potent activity with several antibiotics against *E. coli* and *Salmonella typhimurium* than PMBN (300 fold reduction in MIC compared to a 10-fold reduction effected by PMBN at 3 μg/mL).[38] However, these two compounds retain antibacterial activity, likely as a result of the six positive charges.[38] Further truncation of the polymyxin core in polymyxin B octapeptide (PBOP) **4** and polymyxin B heptapeptide (PBHP) **5** (Figure 2), results in low inherent toxicity toward *E. coli* and *S. typhimurium*. Both compounds sensitize *E. coli* and *S. typhimurium* to rifampin, erythromycin, clindamycin, and novobiocin, with the more positively charged PBOP exhibiting more potent activity.[39] PMBN, PBOP, and PBHP also act as potent synergists with erythromycin, lincomycin, linezolid, nisin, and vancomycin against *P. aeruginosa* PA14.[40]

Guanidylated-colistin (GCol) **6** and guanidylated-polymyxin B (GPMB) **7** (Figure 2) possess guanidine moieties in place of the 4-amino groups of the Dab residues of colistin and polymyxin respectively.[41] Both compounds synergize with erythromycin and rifampin against multiple strains of *P. aeruginosa, A. baumannii, E. coli, E. cloacae*, and *K. pneumoniae*, displaying superior activity than PMBN, however GCol and GPMB exhibit similar cytotoxicity in comparison to PMB toward HEK293, and HepG2 cell lines.[41]

Reducing the number of positive charges carried by the polymyxin scaffold as a means to reduce nephrotoxicity has been investigated, and several analogues with only three positive charges (compared to the five of polymyxin antibiotics and PMBN) retain potentiation activity.[42,43] NAB7061 **8** and NAB739 **9** (Figure 2), in which the Dab residue of the linear portion of the polymyxin is replaced with an aminobutyryl (Abu) or D-Ser residue respectively, both synergize potently with rifampin, clarithromycin, and vancomycin.[42] Compared to PMB, both compounds exhibit lower affinity for the brush border membrane (BBM) of the renal cortex, and exhibit no cytotoxicity against V79 Chinese hamster lung fibroblast cells at the highest concentration tested (128 μg/mL).[42]

SPR741 **10** (Figure 2), previously known as NAB741, also carries three positive charges, and has an acetyl group in place of the hydrophobic octanoyl residue.[43] SPR741 was initially reported to sensitize *E. coli, K. pneumoniae, E. cloacae*, and *A. baumannii* to rifampin, clarithromycin, azithromycin, mupirocin, fusidic acid, and vancomycin at 4 μg/mL,[43] and later reported to additionally sensitize *E. coli* and *K. pneumoniae* to erythromycin, retapamulin, and telithromycin, and *A. baumannii* to erythromycin, and retapamulin.[44] The combination of 4 μg/mL SPR741 and 1 μg/mL rifampin inhibits bacterial growth of all but one of a panel of 28 extensively drug resistant *A. baumannii* clinical isolates, and the combination is bactericidal and synergistic *in vitro*. In a murine pulmonary model of *A. baumannii* infection, 60 mg/kg SPR741 and 5 mg/kg rifampin effected a

survival rate of 90% compared to 50% for rifampin alone, and decreased the bacterial burden in lung tissue by 6 $\log_{10}$ CFU/g compared to the untreated control, an additional 2 $\log_{10}$ CFU/g compared to rifampin alone.[45]

SPR741 acts predominantly on the OM, effecting little depolarization the *E. coli* CM compared to PMB and PMBN while causing 10–15 nm undulations in the cell surface. Genetic truncation of the LPS core selectively increases sensitivity to SPR741 over PMB and PMBN, indicating heptose residues in the LPS core prevent access of SPR741 to the CM.[46] SPR741 exhibits no cytotoxicity against V79 Chinese hamster lung fibroblast cells at the highest concentration tested (128 μg/mL), while renal clearance was 400-fold higher than that of colistin.[43] SPR741 entered phase 1 clinical trials in 2016 but was discontinued in 2020 and replaced with SPR206 **11** (Figure 2), a polymyxin with standalone antibacterial activity.[47]

Several other peptide membrane disrupters have been reported. The cathelicidin peptides L-11 **12** (RIVQRIKKWLR-NH_2) and its D-amino acid counterpart D-11 **13** sensitize *E. coli*, *P. aeruginosa*, *K. pneumoniae*, and *A. baumannii* to vancomycin,[48] while D-11 also sensitizes *K. pneumoniae* to rifamycins, aminocoumarins, and macrolides.[49] Activity is lost upon addition of exogenous LPS or Mg^{2+}, indicating that the peptide binds LPS.[48] Linear lipopeptide paenipeptin analogues **14** and **15** (Figure 3) potentiate rifampin and clarithromycin against *A. baumannii* and *K. pneumoniae* to nanomolar concentrations, and additionally potentiate erythromycin and vancomycin against *A. baumannii*. Once again, addition of exogenous LPS neutralizes activity indicating LPS binding is driving the increase in permeability.[50] Short linear antibacterial peptide (SLAP)-S25 **16** (Figure 3), increases the efficacy of rifampin and vancomycin against *E. coli*. Exogenous LPS or addition of divalent cations reduces activity and SLAP-S25 binds LPS, even when phosphoethanolamine (PetN) modified, with high affinity *in vitro*. Exogenous CM phospholipids also reduce activity, and phosphatidylglycerol (PG) abolishes activity, with SLAP-S25 binding PG with higher affinity than LPS, suggesting the adjuvant activity of SLAP-S25 is a result of triggering membrane damage through binding both the OM and CM.[51]

The anti-protozoal drug pentamidine **17** (Figure 3) is an OM permeabilizer that synergizes potently with rifampin, novobiocin, and erythromycin, but not vancomycin, against several Gram-negative species and, unlike for PMBN, activity is retained against polymyxin resistant isolates (harboring *mcr-1*).[52] In a mouse model of colistin-susceptible *A. baumannii* infection, 10 mg/kg pentamidine and 5 mg/kg novobiocin effected 100% survival, while in a colistin-resistant *A. baumannii* model, 10 mg/kg pentamidine and 50 mg/kg novobiocin rescued 90% of the mice.[52] Pentamidine causes 40 nm undulations on the surface of treated *E. coli*, cells, binds purified lipid A *in vitro* with high affinity (K_d ~120 nM), results in enhanced LPS release similar to polymyxins, and its activity is impeded by both exogenous LPS and Mg^{2+}, all of which indicate direct association with LPS.[52] However, pentamidine is beset by side effects including: nephrotoxicity, hypotension, hypoglycemia, and QT prolongation, limiting its clinical potential.[53]

Preliminary structure functions studies on pentamidine revealed that each of the amidine groups is necessary for activity, and increased hydrophobicity, inter-amidine distance, and rigidity leads to increased rifampin potentiation, with compounds **18** and **19** exhibiting increased potency compared to pentamidine.[52] From a series of bis-amidines with reduced linker flexibility, **20**, **21** and **22** exhibited the greatest activity, synergizing to a greater degree than pentamidine with erythromycin, rifampin, and novobiocin against *E. coli*, *K. pneumoniae*, *A. baumannii*, and *P. aeruginosa*, although exhibiting lower activity than PMBN in most cases.[54] P35 **23**, in which an *N*-phenyl group replaces the central carbon of pentamidine, synergizes with novobiocin to a much greater degree than pentamidine, and also potentiates rifampin, rifaximin, fusdic acid, roxithromycin, erythromycin, clarithromycin thiostrepton, trimethoprim, and linezolid at 50 μg/mL against *A. baumannii*, the latter three of which pentamidine does not potentiate.[53] P35 has an improved toxicity profile compared to pentamidine, with lower cytotoxicity against HEK293 cells, a reduced impact on HepG2 cell cycle arrest, and lower hERG trafficking inhibition.[53]

The diamidine MD-124 **24** was developed following an SAR study on the MD-100 (**25**) scaffold, which was identified from a screen of cationic compounds for rifampin potentiation in *E. coli*. MD-124, sensitizes *E. coli*, *A. baumannii,* and *K. pneumoniae* to rifampin and clarithromycin, with strain specific activity. Activity of MD-124 is reduced upon addition of divalent cations or exogenous LPS, and in an *E. coli* strain with a compromised OM and. A dansyl-PMBN fluorescent indicator suggested MD-124 binds to LPS, and molecular dynamics simulations more specifically indicate binding at the hydrophilic portion of LPS.[55] The addition of phosphatidylcholine has a much lower effect on activity than addition of exogenous LPS, indicating selectivity toward LPS over eukaryotic phospholipids, and MD-124 exhibits low cytotoxicity toward HEK293 and NIH3T3 cells.[55]

The carbazoles MAC-0493157 **26** and MAC-0483351 **27** (Figure 3) sensitize *E. coli* to linezolid, while MAC-0493157 also potentiates novobiocin. Both compounds physically disrupt OM integrity to a similar extent as SPR741, in addition to disrupting the CM; however, both are cytotoxic toward HEK293 cells.[56] Liproxstatin-1 **28**, a ferroptosis inhibitor in human cells, and MAC-0568743 **29**, potentiate large hydrophobic antibiotics (rifampin, novobiocin, erythromycin) in addition to the small hydrophilic linezolid in *E. coli,* but do not potentiate large, hydrophilic antibiotics. Both compounds retain activity with rifampin in *K. pneumoniae* and *A. baumannii*, while MAC-0568743 also exhibits activity in *P. aeruginosa.* Both compounds disrupt the OM and increase cell membrane roughness but have limited impact on CM integrity. Again, addition of Mg^{2+} or exogenous LPS suppresses activity, indicating LPS binding, which was confirmed with a BODIPY-cadaverine assay. However, against an *E. coli waaC* deletion mutant, in which LPS is truncated, both compounds exhibited more pronounced synergy with rifampin than in wild type *E. coli*, suggesting that LPS binding is independent of the LPS core. The two compounds may act through distinct or multiple mechanisms, as MAC-0568743 has no interaction with PetN modified LPS, while liproxstatin-1 has comparatively lower affinity for LPS.[57]

The bis-2-aminoimidazole **30** potentiates azithromycin against *P. aeruginosa*, reducing the MIC against PA01 from 256 μg/mL to 0.25 μg/mL at 60 μM, and to 1 μg/mL at 40 μM. Activity is retained against several cystic fibrosis clinical isolates including two mucoid

strains, and **30** displays activity in a *Galleria mellonella* infection model. Activity is correlated with an increase in cell membrane permeability though direct binding of LPS has not been established.[58]

Interference with LPS synthesis or OM assembly.

Although the use of membrane permeabilizing adjuvants may prove to be a viable approach for the treatment of infections caused by some bacteria, toxicity issues and a potential lack of efficacy against increasing numbers of polymyxin resistant strains necessitate alternative approaches.

LPS/LOS is essential in most Gram-negative bacteria, however some species, including *A. baumannii*, remain viable upon complete loss of LPS/LOS.[59] In *A. baumannii* this phenotype imparts attenuated fitness and virulence, and reduced susceptibility to polymyxin antibiotics,[60] but also results in increased susceptibility to other antibiotic classes including macrolides and glycopeptides.[61] Inducing the LOS deficient phenotype therefore represents a novel approach to sensitizing *A. baumannii* to these antibiotics.

Several aryl 2-aminoimidazole adjuvants enhance the sensitivity of *A. baumannii* to macrolide and glycopeptide antibiotics. Compounds **31** and **32** (Figure 4), reduce the clarithromycin MIC against *A. baumannii* 5075 (AB5075) from 32 μg/mL to 0.25 μg/mL at 30 μM. Adjuvant activity (defined here as lowering the clarithromycin MIC to 2 μg/mL or lower) is retained down to 10 μM, and compound **31** is active in a *G. mellonella* model of infection.[62] Both compounds also sensitize *A. baumannii* to vancomycin, reducing the MIC of AB5075 from 256 μg/mL to 1 μg/mL at 30 μM. Activity is conserved with both classes of antibiotics, against a panel of primary clinical *A. baumannii* isolates that encompasses nearly all clinically relevant *A. baumannii* clades.[63, 64] Compound **31** is not hemolytic and has low cytotoxicity against HaCaT keratinocyte cells.[62]

A structure activity relationship study on the aryl 2-aminoamidazole scaffold identified several compounds with increased macrolide potentiation activity, with **33** and **34** (Figure 4) reducing the clarithromycin MIC against AB5075 from 32 μg/mL to 1 μg/mL and 0.25 μg/mL respectively at 7.5 μM, and activity is retained across the same *A. baumannii* panel mentioned above.[65] Although this series does cause membrane perturbation as determined by a BacLight assay, this appears to be decoupled from resistance suppression activity when compared to pentamidine.[62] Instead compound **31** effects a shift toward palimitoylated lipid A species, an overall reduction of lipid A hydroxylation, and the absence of one lipid A species. This, along with the fact that the compound does not further sensitize a LOS-deficient strain to macrolides or vancomycin, indicates that this class of adjuvants is likely acting through modulation of LPS synthesis or assembly.[62]

Inhibitors of LpxC, which catalyzes the first committed step in lipid A biosynthesis, have potent antibacterial activity against several Gram-negative species, however inhibitors that weakly bind LpxC have potential as adjuvants. One weak LpxC inhibitor MAC-0485042 **35** (Figure 4) potentiates rifampin and vancomycin without physical disruption of the OM. MAC-0485042 exhibits increased potency in an *E. coli* strain containing a H19Y mutation

in LpxC (*lpxC101)*, known to sensitize cells to LpxC inhibitors, which along with *in vitro* binding indicates that LpxC is a target of this compound.[56] LpxC inhibitors exhibit poor antimicrobial activity against *A. baumannii*, likely because of non-essential nature of LOS in the species, but can increase susceptibility to other antibiotic classes as in the case of PF-5081090 **36** with rifampin, azithromycin, and vancomycin. PF-5081090 **36** significantly reduces lipid A, with a concomitant increase in OM permeability.[66]

The azaindole BWC-Aza2 **37** potentiates rifampin, erythromycin, solithromycin, and novobiocin in *P. aeruginosa, A. baumannii, Enterobacter cloacae,* and *Citrobacter freundii* on par with PMBN. Activity is significantly impaired in a ΔLPS *E. coli* strain, and no change in activity is observed in the presence of exogenous LPS, indicating inhibition of LPS biosynthesis or assembly rather direct LPS binding, however the exact target is unknown as the ΔLPS strain harbors deletion of seven LPS synthesis genes.[67] MRL-494 **38** (Figure 4) inhibits assembly of proteins into the OM by binding directly or proximally to the essential exposed protein BamA of the β-barrel assembly machine, inhibiting the assembly of proteins into the OM to and sensitizing *E. coli* to rifampin.[68] MAC13243 **39** (Figure 4) is an inhibitor of LolA, a periplasmic chaperone that transports lipoproteins from the CM to the OM and partial depletion of which is enough to permeabilize the OM. MAC13243 increases membrane permeability and exhibits synergy against *E. coli* with erythromycin and novobiocin but not vancomycin or rifampin.[69]

The proline-hinged peptide KL-L9P (Ac-KLLKLLKKPLKLLK-NH_2) **40** sensitizes MDR *E. coli* to linezolid, rifampin, and clarithromycin, MDR and non-MDR strains of *K. pneumoniae* and *P. aeruginosa* to erythromycin and rifampin and is active in a murine skin *A. baumannii* infection model. KL-L9P does not compromise membrane integrity but induces a morphological change that results in a large increase in fine surface roughness and fluidity. The authors hypothesize that KL-L9P binds to lipid A in a hinged helical formation, and creates a hard gel-like LPS domain, affecting arrangement of the OM.[70]

The peptidomimetic CEP-136 **41** (Figure 4) sensitizes *E. coli, K. pneumoniae, A. baumannii,* and *P. aeruginosa* to rifampin, clarithromycin, and azithromycin in both lab strains and clinical isolates. While adjuvant activity is reduced in LPS-deficient *E. coli,* the MIC of CEP-136 is not affected indicating that the compound does not bind LPS. Synergy is particularly strong in a Δ*rfaG* mutant that has no outer core sugar, suggesting that CEP-136 facilitates antibiotic uptake via destabilization of the LPS inner core.[71]

Inhibition of efflux.

In addition to limited permeation of many antibiotics into Gram-negative cells, efflux pump systems contribute to intrinsic resistance in Gram-negative bacteria by keeping intracellular antibiotic concentrations low. Efflux pump inhibitors have long been investigated,[72] and although none has yet made it to the clinic, this represents another adjuvant approach that has the potential to overcome intrinsic resistance in Gram-negative bacteria.

Anthracycline antibiotic $301A_1$ **42** (Figure 5) is highly synergistic with rifampin and moderately synergistic with linezolid in *E. coli* and *A. baumannii*. Antibiotic $301A_1$

competes with Nile Red for efflux, and synergy with linezolid is lost in an *E. coli* strain overexpressing AdeIJK, pointing toward $301A_1$ competing with linezolid for efflux. Activity with rifampin is lost in *E. coli rpoB* mutants, indicating that $301A_1$ is directly involved in rifampin reaching RNA polymerase, and as there was no indication of membrane perturbation, the authors hypothesize $301A_1$ also competes with rifampin for efflux.[73] A screen for inhibitors of AcrA of the AcrAB-TolC efflux pump system with sub-inhibitory concentrations of novobiocin in an OM permeable strain of *E. coli* identified NSC60339 **43** (Figure 5), which potentiates novobiocin and erythromycin and dramatically inhibits the rate of efflux of the AcrAB-TolC substrate H33342. Proteolysis suggests NSC60339 binds AcrA and causes structural changes to the protein. Analogues **44** and **45** exhibit increased activity over NSC60339.[74] The marine metabolite 3,4-dibromopyrrole-2,5-dione **46** (Figure 5) is synergistic with erythromycin in *E. coli*, inhibits RND pumps as shown by accumulation of H33342, and is inactive in a strain lacking the target RND pump (*E. coli* AG100A).[75]

Finally, some adjuvants likely act through more than one mode of action, typically affecting both the OM and efflux, either in concert to potentiate one antibiotic, or with different actions depending on the antibiotic. The triamine derivative of the known efflux inhibitor PAβN, compound **47** (Figure 5), potentiates clarithromycin against *E. coli*, reducing the MIC 32-fold to 2 μg/ml. The compound increases OM permeability and decreases efflux pump activity.[76] Other PAβN analogues: **48** and **49**, potentiate clarithromycin, azithromycin, and novobiocin in *E. coli*, with **48** having 128-fold greater potency than PAβN with clarithromycin. Both compounds affect efflux and also disrupt both the OM and CM.[77]

The dilipid ultrashort tetrabasic peptidomimetic dUSTBP8 **50** (Figure 5) potentiates novobiocin and rifampin against MDR clinical isolates of *P. aeruginosa, A. baumannii, K. pneumoniae, E. cloacae,* and *E. coli,* with activity comparable to PMBN. Preliminary studies suggest OM permeabilization and/or disruption of efflux, potentially by sequestering lipids around transmembrane proteins of efflux pumps, or by altering the proton motive force required to power active efflux.[78] Norspermidine derivatives with hydrophobic substituents show nonspecific, but weak membrane permeabilization and depolarization, and efflux inhibition in *A. baumannii* and *P. aeruginosa*. NAda **51** and NDiphe **52** (Figure 5) potentiate rifampin, erythromycin, and vancomycin in MDR *K. pneumoniae*, *P. aeruginosa,* and *E. coli*. Both compounds increase membrane permeability and effect small reductions in efflux, and the authors hypothesize that membrane depolarization may lead to an ion imbalance that indirectly slows efflux.[79]

In conclusion.

Infections caused by Gram-negative bacteria are becoming increasingly difficult, and in some cases impossible, to treat as a result of rising levels of resistance and a lack of novel Gram-negative acting antibiotics. Small molecule adjuvants that overcome intrinsic resistance in Gram-negative bacteria and sensitize the bacteria to approved antibiotics that are otherwise limited to treating Gram-positive infections have the potential to significantly expand therapeutic options for treating Gram-negative infections. Adjuvants that enhance antibiotic uptake through directly binding components of the OM are one approach, though many are beset with toxicity issues. Inhibitors of enzymes, chaperones, and assembly

proteins involved in the synthesis, transport and assembly of the OM also show promise in overcoming intrinsic resistance, as do inhibitors of efflux.

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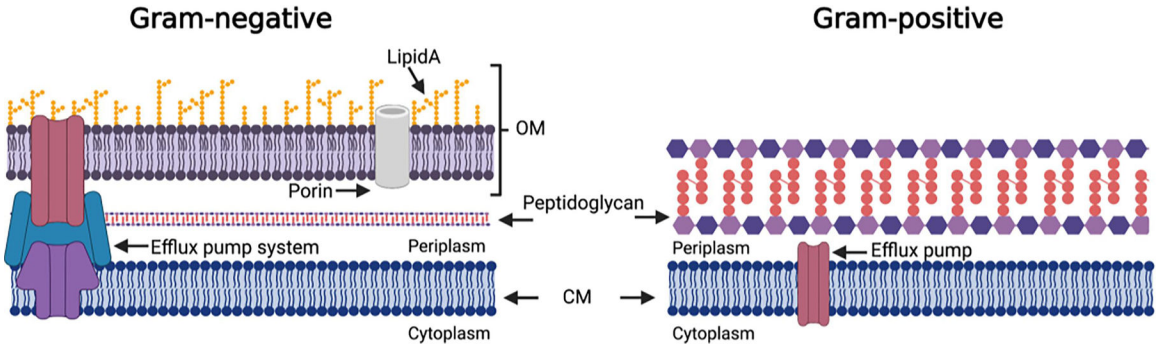

Figure 1.

Schematic representation of major structural differences between the Gram-negative and Gram-positive cell envelopes.

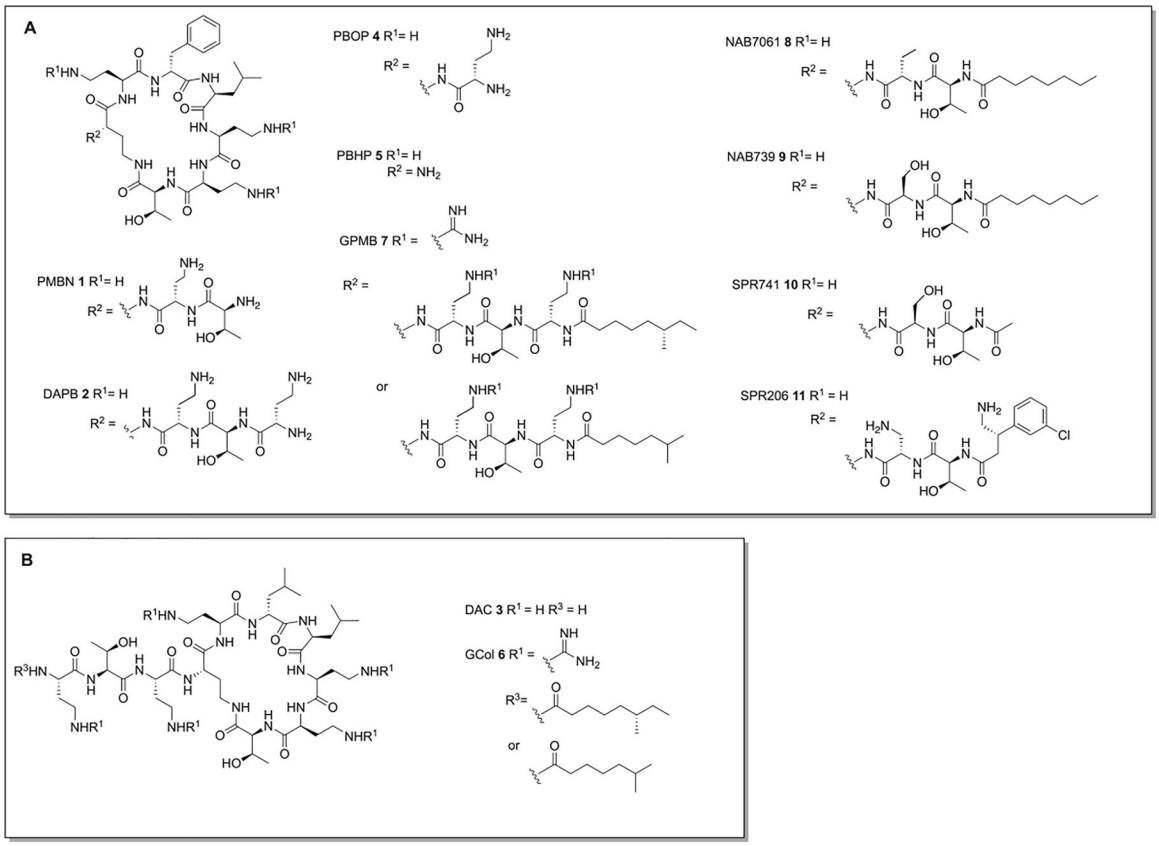

Figure 2.

Polymyxin derived adjuvants. **A**: Adjuvants derived from the polymyxin B scaffold. **B** Adjuvants derived from the colistin scaffold.

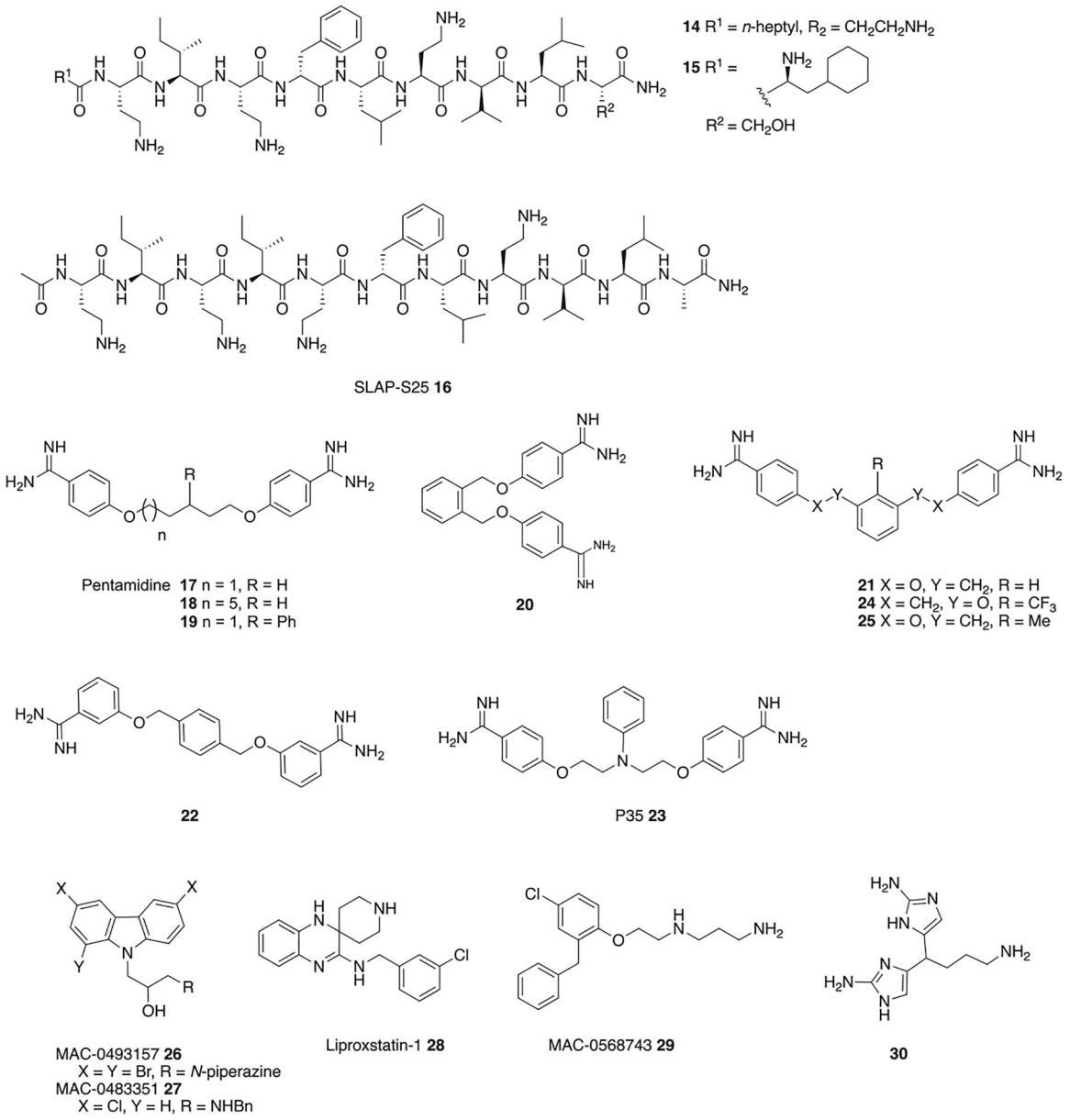

Figure 3.
Adjuvants that act via OM disruption.

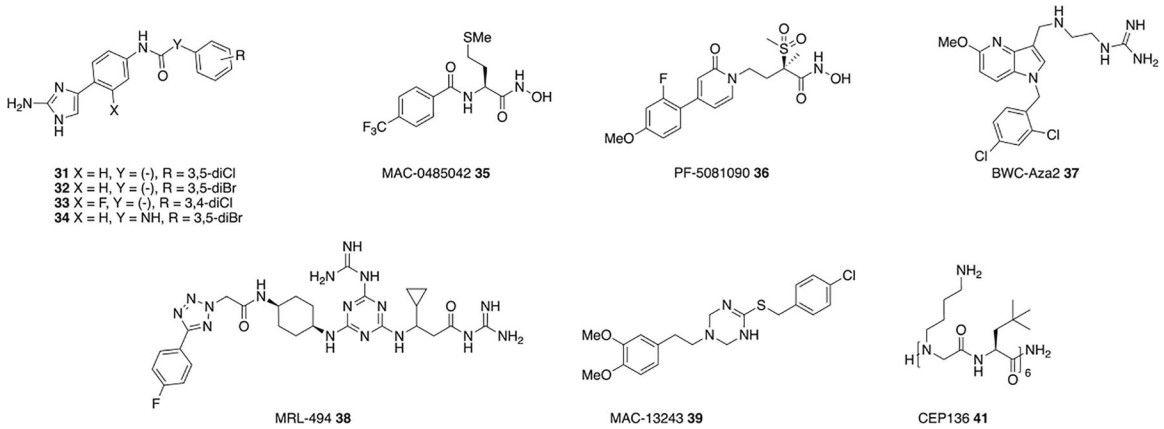

Figure 4.
Adjuvants that interfere with OM component synthesis or assembly.

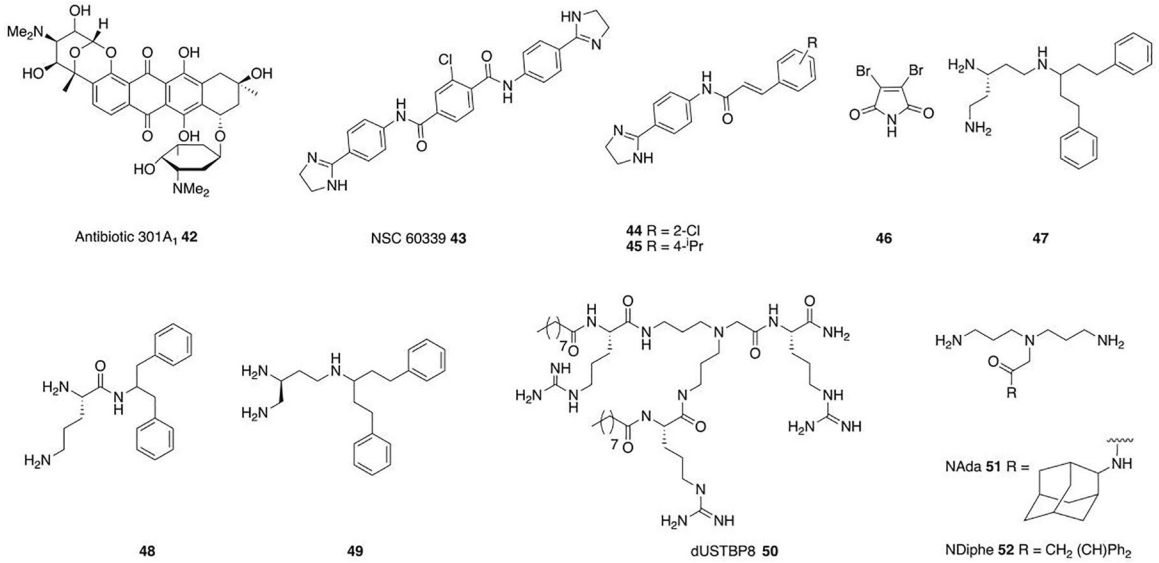

Figure 5.
Adjuvants that inhibit efflux or act through multiple pathways.