OUTER MEMBRANE PERTURBATION AS AN ANTIBIOTIC APPROACH

OVERCOMING INTRINSIC AND ACQUIRED ANTIBIOTIC RESISTANCE WITH OUTER MEMBRANE PERTURBATION

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Foreword

Abstract

There is an urgent need to identify novel antibiotics for multidrug-resistant Gram-negative pathogens. These bacteria are intrinsically resistant to many antimicrobials due to a formidable outer membrane barrier. Herein we investigate the potential of perturbing the outer membrane to sensitize Gram-negative bacteria to otherwise inactive antibiotics. In chapter 2, we identify the ability of *mcr-1* mediated resistance to confer protection from the lytic but not outer membrane-perturbing activity of colistin. Exploiting this sensitivity, we show that colistin and clarithromycin in combination are efficacious against *mcr-1*-expressing *Klebsiella pneumoniae* in murine infection models. This demonstrates the viability of colistin combination therapies against Gram-negative pathogens harbouring *mcr-1*, and points to a mechanism of *mcr-1*-mediated resistance extending beyond the predicted reduction in binding affinity of polymyxins to the outer membrane. We continue to investigate the potential of using outer membrane perturbants with otherwise inactive antimicrobials in chapter 3. In this work, we identify the ability of OM disruption to change the rules of Gram-negative entry, render pre-existing resistance ineffective, reduce the development of spontaneous resistance and attenuate biofilm formation. Together, these data suggest that OM disruption overcomes many traditional hurdles encountered during antibiotic treatment and is a high priority approach for further development.

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List of abbreviations

ATP ..... adenosine triphosphate
Bam ..... β-barrel assembly machinery
CARB-X ..... combating antibiotic-resistant bacteria biopharmaceutical accelerator
CARD..... comprehensive antibiotic resistance database
CDC ..... centers for disease control and prevention
CFU ..... colony-forming units
cLogD ..... calculated logD
CLSI ..... clinical and laboratory standards institute
Core OS ..... LPS core oligosaccharide
CV ..... crystal violet
DAB ..... diaminobutyric acid
$DiSC_3(5)$ ..... 3,3′-dipropylthiadicarbocyanine iodide
EDTA ..... ethylenediaminetetraacetic acid
EPS ..... extracellular polymeric substance
FIC ..... fractional inhibitory concentration
FOR ..... frequency of resistance
GlcNAc ..... N-acetylglucosamine
Hep ..... L-glycero-D-manno-heptose
IM ..... inner membrane
Kdo ..... 3-deoxy-D-manno-oct-2-ulsonic acid
L-Ara4N 4-amino-4-deoxy-L-arabinose
LB ..... lysogeny broth
LOS..... lipooligosaccharide
LPS ..... lipopolysaccharide
Lpt ..... LPS transport system
mcr ..... mobilized colistin resistance
MHB ..... mueller hinton broth
MIC ..... minimum inhibitory concentration
MRSA ..... methicillin-resistant *Staphylococcus aureus*
MW ..... molecular weight
NPN N-phenyl-1-naphthylamine
OD ..... optical density
OM ..... outer membrane
PBS ..... phosphate buffered saline
pEtN ..... phosphoethanolamine
PMB ..... polymyxin B
PMBN ..... polymyxin B nonapeptide
RGI ..... resistance gene identifier
UDP ..... uridine diphosphate
WHO ..... world health organization

Chapter I – Introduction

Preface

Some parts of this chapter were adapted from a previously published review:

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CRM wrote the manuscript with input from CNT and EDB.

Antibiotic discovery

The discovery and clinical implementation of antibiotics mark one of the greatest achievements in human history. Modern medicine relies heavily on antibiotics for the treatment and prevention of infection during many high-risk procedures, including chemotherapy and organ transplants. Unfortunately, decades of misuse have contributed to the global dissemination of resistance and rapid erosion of our therapeutic arsenal. Overcoming antibiotic resistance is paramount to human health and will require improvements in antibiotic stewardship, reduction in agricultural use, and new antibiotic discovery. Policy-based changes to mitigate some of these issues have been implemented, and early results show agricultural and clinical use trending downward[1-3]. Slowing the spread of antibiotic resistance will help prolong the effectiveness of current antibiotics. However, the development of resistance for every antibiotic is inevitable. As such, overcoming the resistance crisis will require an ever-expanding arsenal of effective therapeutics.

After the discovery of penicillin by Alexander Fleming in 1929[4], new antibiotics were rapidly identified, with over ten additional classes discovered between 1940 and 1960[5]. This era, known as the “Golden Age” of antibiotic discovery, was ushered in on the Waksman platform, which screened Actinomycetes and other soil-dwelling bacteria for the production of secondary metabolites that inhibit bacterial growth[6]. Although initially highly successful, this approach was plagued by rediscovery, and by the 1960s, an exponentially

increasing number of producers were required to be screened for each new antimicrobial identified[7]. As such, the field moved heavily into medicinal chemistry efforts optimizing previously discovered antibiotic scaffolds for improved potency, reduced susceptibility to resistance and increased spectrum of activity. These endeavours were of critical importance to our current antibiotic arsenal, and continue to provide the next generation of antibiotics[8]. However, reliance on medicinal chemistry efforts has proven unable to keep pace with the resistance threat. Decades of optimization around the same chemical scaffolds have made even incremental improvements difficult. Additionally, many antibiotic resistance mechanisms confer cross-resistance to drugs within the same class, highlighting the importance of identifying compounds with unique chemical scaffolds.

Little research attention was directed towards developing new antibiotic scaffolds until the 1990s when advancements in computation, robotics, and recombinant DNA techniques allowed for the advent of high-throughput screening in antibiotic discovery. This enabled the examination of hundreds of thousands of synthetic molecules for activity against purified proteins. While this approach was expected to usher in the next “Golden Era” of antibiotic discovery, identifying potent inhibitors of *in vitro* enzyme activity proved more difficult than expected. Putative inhibitors often lacked the necessary physical, and chemical properties for further development[9,10], and promising leads were unsuccessful in transitioning from potent *in vitro* inhibitors to whole-cell actives[11]. This was a particular challenge in Gram-negative bacteria due to a formidable outer

membrane (OM) barrier and efflux machinery. The technologies that brought so much promise to the fields of cancer and HIV[12] failed to bring even a single novel antibiotic into the clinic.

With an increasing number of pharmaceutical companies exiting the antibiotic space, the onus has fallen on academic labs and small R&D companies to advance discovery. Policies makers have begun to take notice and pull incentives are being put in place to help alleviate some of the financial hurdles in bringing new antibiotics into the clinic[13]. Additionally, private-public partnerships such as Combating Antibiotic-Resistant Bacteria Biopharmaceutical Accelerator (CARB-X), have begun to provide funding and resources to spearhead antibiotic development. In contrast to large pharmaceutical ventures, antibiotic discovery from academic labs explores more innovative, high-risk, high-reward approaches including anti-virulence, immunotherapy and drug-resistance modulation. Indeed, the next generation of antimicrobials may employ a completely different approach to treating bacterial infection than our current antibiotic arsenal.

The Gram-negative resistance problem

Antibiotics can be categorized by their spectrum of activity into broad-spectrum (active against both Gram-positive and Gram-negative bacteria) or narrow-spectrum (active against one or the other) agents. With the exception of polymyxins, narrow-spectrum antibiotics are predominantly active against Gram-positive bacteria. In this category, development of novel antibiotics has shown slow, but notable progress in the last 20 years, with the introduction of three

novel Gram-positive active antibiotic classes: the cyclic lipopeptide daptomycin[14], the oxazolidinone linezolid[15] and the pleuromutilin retapamulin[16]. Further, two additional classes of Gram-positive active antibiotics are currently in development, the FabI inhibitor Afabicin and FtsZ inhibitor TXA709[8]. In contrast, no Gram-negative active antibiotic with a novel target has been introduced into the clinic since the quinolones in 1967[17], and none are currently in clinical development[8].

In 2017, the World Health Organization (WHO) published a list of twelve high-priority pathogens for which new antibiotics are urgently needed[18]; nine of the twelve were Gram-negative. In 2019, the Centers for Disease Control and Prevention (CDC) echoed this sentiment by releasing its own Antimicrobial Resistance Threats Report characterizing antibiotic-resistant bacteria and fungi based on their threat level to human health[19]; three of the five most urgent threats on this list were Gram-negative pathogens. These reports underscore the rising concern amongst the medical and scientific community on the global threat of multidrug-resistant Gram-negative bacterial infections.

Gram-negative resistance elements can be divided into three major categories: intrinsic, spontaneous and horizontally acquired[20]. Intrinsic resistance elements are defined as those conserved across the genomes of individual bacterial species, independent of antibiotic-mediated selective pressure. Intrinsic resistance in Gram-negative bacteria is derived from both the inherently impermeable OM and efflux machinery, which together reduce the efficacy of

many classes of clinically effective Gram-positive antibiotics. Spontaneous resistance occurs by mutations in strains of previously susceptible bacteria upon antibiotic selection. This type of resistance is limited to vertical transmission through replication of the resistant strain. Horizontal gene transfer occurs between bacterial strains or species, when resistance elements, located on mobile DNA elements (plasmids, transposons) are acquired[21]. These mobile elements often rapidly spread antibiotic resistance genes across populations and have garnered much attention for their contribution to the current resistance crisis.

Notably, difficulties in the discovery of Gram-negative active antibiotics can largely be attributed to intrinsic resistance mechanisms preventing molecules from accumulating within bacteria. All Gram-negative bacteria are protected by a formidable OM barrier, which acts to slow the influx of noxious chemicals[22,23]. In combination with robust efflux machinery, the OM severely impedes the sufficient accumulation of small molecules for growth-inhibition. This problem has been traditionally overcome with antibiotics that pass through porins. These β-barrel proteins, permit the diffusion of small hydrophilic molecules through the OM. However, this entry route significantly restricts the range of physicochemical properties compatible with Gram-negative activity. Indeed, the vast majority of Gram-negative active compounds are hydrophilic with a molecular weight less than 600 Da[24,25]. Unfortunately, biochemical screens typically yield potent inhibitors that do not adhere to these requirements, as *in vitro* actives are often

hydrophobic and non-potent against whole cells[11]. The limited range of hydrophobicity compatible with Gram-negative activity has proven particularly difficult to overcome, as efforts to increase permeability often concur with a decrease of *in vitro* potency. As such, traditional Gram-negative drug discovery efforts are forced to find a delicate balance between permeability and *in vitro* activity.

Structure and synthesis of LPS

The OM is an asymmetric bilayer composed of predominantly lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet. LPS is stabilized by divalent cation bridging to form a robust permeability barrier. Additionally, polarity in the carbohydrate chains of LPS excludes hydrophobic compounds, making it unique from other biological membranes. LPS can be divided into three major components: lipid A, core oligosaccharide and O-antigen polysaccharide (Figure 1a,b). LPS biosynthesis has been thoroughly characterized in *Escherichia coli* and is primarily conserved across Gram-negative species (Figure 1c). In short, lipid A is assembled from uridine diphosphate N-acetylglucosamine (UDP-GlcNAc), which is first modified with the addition of two fatty acid chains to form UDP-diacyl-GlcN through the successive action of LpxA[26], LpxC[27], and LpxD[28]. LpxH then hydrolyzes UDP-diacyl-GlcN to form lipid X[29], which is condensed with its precursor by LpxB to form lipid A disaccharide[30]. After 4'-phosphorylation by LpxK[31], two 2-keto-3-deoxymanno-octulosonic acid (Kdo) residues are incorporated by WaaA to form Kdo_2-lipid

IV_A[32]. The acyl chains of Kdo_2-lipid IV_A undergo further modifications by LpxL[33] and LpxM[34] to form Kdo_2-lipid A, which is the minimum composition of LPS required for viable *E. coli* growth.

Core oligosaccharides are sequentially assembled onto Kdo_2-lipid A using nucleotide sugars as donors for a number of glycosyltransferases. Core oligosaccharide structure is divided into two distinct regions, inner and outer core. The inner core is primarily conserved across species and typically contains Kdo and L-glycero-D-mannoheptose. Heptose is biosynthesized by GmhA, GmhB, and GmhD and transferred onto Kdo_2-lipid A by the heptosyltransferases WaaC and WaaF[35]. The biosynthesis of both outer core and O-antigen is highly variable across bacterial species.

After biosynthesis along the cytoplasmic face of the inner membrane (IM), LPS is flipped by MsbA to the outer leaflet of the IM, where a terminal O-sugar chain can be incorporated[36]. Before integration into the OM, LPS must travel across the periplasmic space. Due to its amphipathic nature, LPS cannot passively travel through the periplasm to the OM. To overcome this hurdle, Gram-negative bacteria use a transmembrane LPS transport system (Lpt) to form a bridge between the IM and OM (Figure 1a). The structure of this bridge was recently elucidated in work by Li *et al.*[37] and Owens *et al.*[38], in which the complete structure of the Lpt complex was solved, and a new model was proposed for LPS transport: LPS is first detached from the IM by the ATP-binding cassette transporter $LptB_2FG$, which acts as a pump to power LPS transit. LptC receives

LPS from $LptB_2FG$, and by constriction, LPS is moved along LptC and LptA. LPS is then passed to LptD, which is connected to the OM translocon (LptD-LptE). Once through the periplasmic space, LPS is then integrated into the OM.

The outer membrane as an antibiotic target

The OM renders Gram-negative pathogens intrinsically resistant to most potent *in vitro* enzyme inhibitors, resulting in the failure of countless antibiotic discovery efforts. However, the importance of a robust OM in bacterial fitness and virulence should not be overlooked. Indeed, several research efforts have demonstrated the encouraging potential of targeting the OM through perturbation of LPS biosynthesis, LPS transport and OM structure.

The conservation of many enzymatic steps in Kdo_2-lipid A biosynthesis, required for Gram-negative viability, has made these aspects of the pathway desirable targets. Further, the inhibition of many LPS biosynthetic enzymes disrupts OM integrity, increasing vulnerability to otherwise inactive antibiotics[39], as well as complement-mediated serum activity, and phagocytosis[40]. As such, a number of research groups have identified novel inhibitors of the enzymes involved in LPS biosynthesis.

One such example, LpxC, has an extensive history as an antimicrobial target, with the first inhibitor L-573,655 identified by Merck & Co. in the mid 1980s[41]. At the time, the perceived market for a narrow-spectrum Gram-negative active antibiotic was limited, and the inability to expand activity from

Enterobacteriaceae to *Pseudomonas aeruginosa* or *Serratia* spp. resulted in the termination of the project[42]. Over the years, dozens of patents for novel LpxC inhibitors have since been filed by research groups, including Merck & Co, Pfizer, AstraZeneca and Achaogen[43]. Notably, LpxC is a rare example of *in vitro* inhibition translating to whole-cell potency in Gram-negative bacteria. Sub-lethal levels of LpxC inhibition disrupts OM integrity, which may allow these inhibitors to facilitate their uptake into the cell. Despite the overwhelming number of identified leads, no inhibitor has advanced past Phase I trials[44,45].

Once synthesized and transported through the periplasm, LPS integrates into the OM and is stabilized by divalent cation bridging[23]. The distinctive properties of LPS and, therefore, the OM, distinguish it from eukaryotic membranes making it an exploitable antimicrobial target. Indeed, a range of antimicrobial compounds selectively target and disrupt the OM by displacing cations from the phosphates of lipid A. This approach is exploited by host immune factors such as cationic antimicrobial peptides as well as by natural product metabolites like polymyxins.

The most successful family of LPS-targeting antimicrobials are the polymyxins, particularly polymyxin B (PMB) and polymyxin E (colistin). Discovered in the 1940s[46,47], these cyclic lipodecapeptide antibiotics are highly effective against Gram-negative pathogens and remain a critical last-line therapeutic[48]. Polymyxins contain a cyclic heptapeptide core linked to a tripeptide “panhandle” and an N-terminal fatty acyl tail (Figure 2). Polymyxins rely on the

positively charged amino groups of diaminobutyric acid (DAB) to interact with the phosphates of lipid A. Polymyxin destabilizes the OM by displacing the magnesium bridging between LPS molecules, leading to increased cell envelope permeability, disruption of the IM, and lytic cell death[49,50] While polymyxins demonstrate potent antimicrobial activity, their considerable nephrotoxicity complicates therapeutic use. A number of groups are developing polymyxin derivatives with a focus on reducing nephrotoxicity[51]. Because the activity of polymyxin can be broadly characterized into two components (OM disruption and lysis)[52], two divergent strategies are under investigation for the optimization of polymyxins: OM disruption for use as part of combination therapy or a lytic monotherapy approach.

Attempts to optimize the bactericidal activity of polymyxin have employed structural modifications to the hydrophobic tail and reductions in the number of positive charges[53]. It remains to be seen how the reported reduction in toxicity for these molecules will translate into humans. The previous polymyxin derivative CB-182 804 (Cubist)[54] was also predicted to have reduced toxicity profiles based on *in vitro* and *in vivo* assays, yet ultimately abandoned due to nephrotoxicity once advanced into human trials. However, many groups continue to use polymyxin as a template for optimization, irrespective of the inevitable association between IM disruption and toxicity. Indeed, a high degree of conservation between the bacterial IM and eukaryotic cell membrane has led to the typical abandonment of molecules with IM activity in drug discovery efforts due to toxicity

concerns[9]. Optimistically, it is possible that a redirection of focus towards optimizing the OM- and not IM-disrupting activity of polymyxins may offer more flexibility in ameliorating nephrotoxicity.

mcr-1 mediated polymyxin resistance

Resistance to colistin and other polymyxin antibiotics is predicted to occur through a reduction in the electrostatic attraction between the cationic antibiotic and anionic LPS. Resistance is conferred by the addition of cationic moieties, phosphoethanolamine (pEtN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) onto the phosphates on lipid A[55]. Until recently, polymyxin resistance was believed to result solely from mutations that constitutively activate two-component regulatory systems PhoP-PhoQ and PmrA-PmrB[56,57]. As this spontaneous resistance is unable to rapidly spread through bacterial populations, the clinical impact of resistance was limited[58]. However, this assumption was overturned in 2015, with the identification of the first mobile colistin resistance gene (*mcr-1*) in *E. coli*[59]. The plasmid borne *mcr-1* is capable of rapid dissemination through horizontal gene transfer, and since its initial discovery, nine variants have been identified within isolates around the globe. Losing colistin from the antibiotic arsenal would leave little or no therapeutic options for many multi-drug resistant Gram-negative infections.

MCR-1 and its variants are phosphoethanolamine (pEtN) transferase enzymes that catalyze the reaction of pEtN-4'-lipid A from lipid A plus phosphatidylethanolamine (Figure 3)[59,60]. Unmodified lipid A contains two

negatively charged phosphates at the 1' and 4' position (Figure 1b), which act as a binding site for polymyxin antibiotics and other positively charged molecules[23]. The addition of pEtN reduces the negative charge on lipid A from -1.5 to -1. This reduction in charge is predicted to reduce polymyxin binding at the OM, impeding self-promoted update and lysis[23]. In chapter 2, I identify that while expression of *mcr-1* does reduce the lytic activity of colistin, it does little to prevent perturbation of the OM, which lends itself to exploitation with a combination antibiotic approach.

Overcoming Gram-negative permeability

The target of many Gram-positive active antibiotics is present in Gram-negative bacteria; arguably, the only barrier to their activity against Gram-negative bacteria is their inability to permeate the OM. Indeed, strains of *E. coli* with compromised OM or efflux capability are highly sensitized to many of these traditionally Gram-positive active antibiotics[11,39,61]. Antibiotic discovery efforts have thus attempted to increase the intracellular concentration of Gram-positive active antibiotics in Gram-negative pathogens through a variety of approaches, including inhibition of efflux machinery, medicinal chemistry efforts and chemical perturbation of the OM.

Gram-negative bacteria harbour robust efflux machinery that expels a multitude of structurally diverse compounds from the bacterial cell. The identification of an efflux pump inhibitor (EPI) has the potential to sensitize bacteria to a range of antibiotics. Two of the most well-studied efflux systems are

the *E. coli* AcrAB-TolC and *P. aeruginosa* MexAB-OprM systems[62]. One of the first EPIs in Gram-negative bacteria was PAβN, which impedes both *P. aeruginosa* MexAB and *E. coli* AcrAB[63]. PAβN sensitizes these Gram-negative pathogens to a number of Gram-positive active antibiotics, including oxazolidines, macrolides and rifamycins[64]. Unfortunately, serum-binding and toxicity concerns have hampered further development of PAβN and analogues[65]. Several efflux inhibitors have been identified over the last 20 years[66]. However, optimization and advancement into preclinical development has proven difficult due to broad substrate specificities and redundancy in efflux systems[67,68]. Alternative medicinal chemistry approaches may prove more successful in modifying compounds to avoid efflux[69], although an incomplete understanding of substrate specificity continues to burden this approach.

Efforts to increase compound permeability through the OM have also suffered from an incomplete understanding of Gram-negative entry. The traditional “rules” of permeability state that to be active against Gram-negative bacteria, molecules must be less than 600 Da and hydrophilic. Recent work by Richter *et al.* has expanded these rules, identifying the benefits of flatness, rigidity, and positive charge in OM-permeating molecules[70]. These properties are predicted to better facilitate passage through the porins, increasing compound influx. Applying these observations to medicinal chemistry efforts has allowed the modification of a select number of Gram-positive active antibiotics into gaining Gram-negative activity[70,71]. This approach is, however, limited to scaffolds for

which modifications do not alter affinity for the intracellular target. Advancing our understanding of Gram-negative permeability is sure to further improve this promising approach.

An alternative to modifying Gram-positive active antibiotics for improved entry is directly perturbing the OM barrier. Disruption of the OM sensitizes bacteria to many Gram-positive antibiotics[72], an approach that has been rigorously studied with the OM perturbant, polymyxin B nonapeptide (PMBN). Reported by Vaara et al. in 1983, PMBN is a polymyxin B analog lacking a hydrophobic tail, a modification that largely eliminates the antimicrobial (lytic) activity but retains OM disruption[73]. This compound displaces ion bridging between LPS to perturb the OM and allow the entry of antibiotics traditionally active against only Gram-positive bacteria into Gram-negatives. PMBN displays reduced cytotoxicity to polymyxin B but nearly identical nephrotoxicity in rats[72]. Recent attempts to decrease renal toxicity resulted in the development of a PMBN derivative with only three positive charges, SPR741(Figure 2)[74].

In-licensed by Spero Therapeutics, SPR741 potentiates a range of antimicrobials, including rifampicin and clarithromycin, against *E. coli*, *K. pneumoniae* and *A. baumannii*[75]. When partnered with rifampicin, SPR741 reduces bacterial burden in a murine *A. baumannii* lung infection model[76]. Toxicology is also encouraging with reduced nephrotoxicity when compared to polymyxin B[77]. SPR741 has completed Phase Ia and Ib trials, demonstrating a promising pharmacokinetic profile and tolerability[78]. Unfortunately, as with other

polymyxin derivatives containing a reduced number of positive charges, SPR741 lacks *P. aeruginosa* activity and is ineffective against polymyxin resistant strains[39]. It is currently unknown if SPR741 will advance into Phase II trials.

Our lab previously identified the antiprotozoal drug pentamidine as another OM perturbant. Screening for potentiator molecules typically looks for growth inhibition at sub-MIC concentrations of an otherwise inactive compound. However, these conventional antibiotic sensitization screens capture a large number of hit compounds with extraneous activity. Stokes *et al.* developed a unique whole-cell screening platform for molecules that perturb the OM[39]. At 15°C, *E. coli* becomes susceptible to vancomycin[79]. Paradoxically, this phenotype is reversed through the inactivation of genes involved in OM biosynthesis and by non-lethal molecules that interact with the OM[39]. This screening approach was exploited to identify the cryptic capacity of the antiprotozoal drug pentamidine to disrupt the OM and potentiate a range of Gram-positive active antibiotics against Gram-negative pathogens. The combination of novobiocin and pentamidine demonstrated encouraging efficacy in a systemic *A. baumannii* murine infection model[39]. Pentamidine maintains potentiation activity against polymyxin resistant bacteria but lacks *P. aeruginosa* activity, and concerns surrounding nephrotoxicity have limited further development.

In all, the looming threat of Gram-negative resistance has resulted in an increase in investigations of OM perturbants. Recent studies have identified a number of small molecules[80,81], peptides[82,83], polymers[84] and host immune

factors[85,86] that sufficiently disrupt the OM barrier to sensitize bacteria to Gram-positive active antibiotics. Indeed, the discovery of a potent, broad-spectrum OM disrupter has incredible potential as a combination therapy. Clinical implementation would allow for the immediate use of dozens of Gram-positive active antibiotics against Gram-negative pathogens, dramatically increasing the antibiotic arsenal.

Purpose and goals of this thesis

My thesis work has focused on the potential to use OM-perturbing chemicals alongside traditionally Gram-positive active antibiotics against Gram-negative bacteria. In chapter 2, I describe our identification of the ability for *mcr-1* to protect bacteria from the lytic, but not OM-disrupting, activity of colistin. Exploiting this sensitivity using antibiotic combinations may provide a therapeutic option for an otherwise difficult to treat resistance mechanism. I next sought to address the possible limitations or unexpected benefits of the use of OM perturbants in combination with Gram-positive active antibiotics, given the lack of attention towards this research area. Chapter 3 comprises our work on the interaction between OM disruption and Gram-negative resistance, in which I uncover the ability for OM perturbation to overcome intrinsic, spontaneous and horizontally acquired antibiotic resistance. In chapter 4, I integrate the results of chapter 2 with recent advances in our understanding of *mcr-1* and review the potential for OM perturbation in clinical applications. Finally, I provide suggestions on how to further expand our understanding of this intriguing antibiotic approach.

Figures and Legends

Figure 1

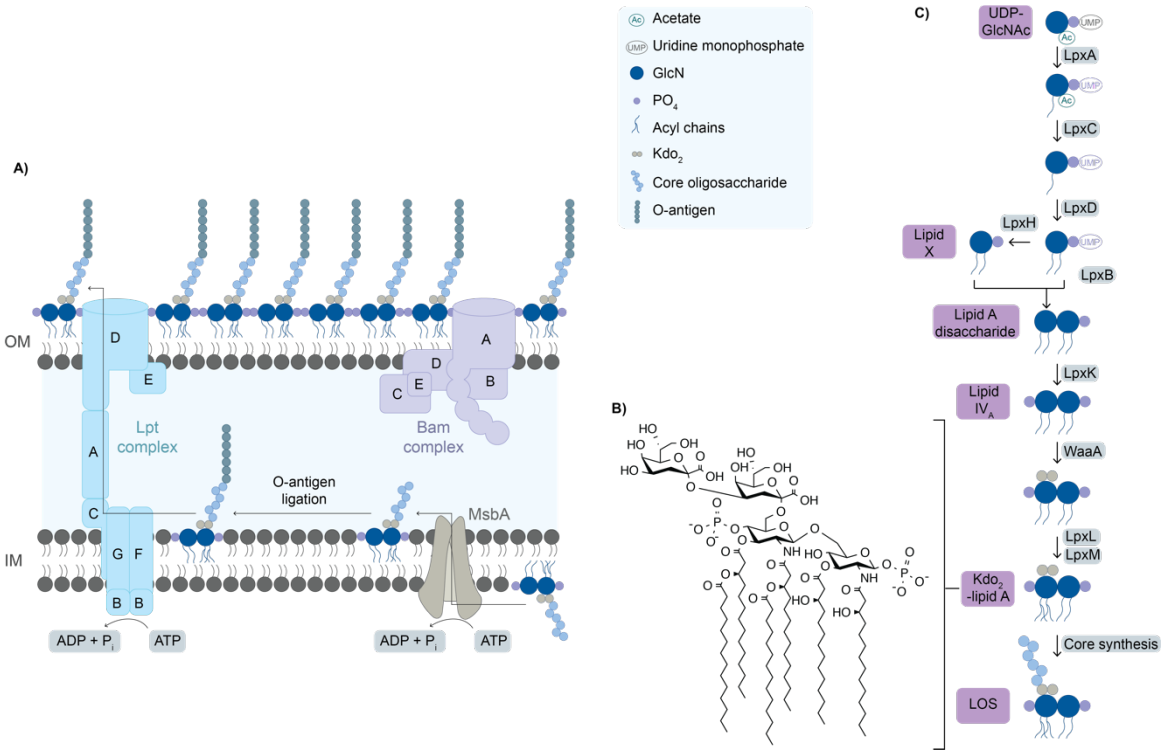

Figure 1. Lipopolysaccharide biosynthesis and transport. a) After synthesis, lipooligosaccharide (LOS) is flipped to the periplasmic face of the inner membrane by MsbA and if present, O-antigen is attached to form lipopolysaccharide (LPS). Transport through the periplasm and integration into the OM is then facilitated by the LPS transport (Lpt) complex. b) *E. coli* K-12 Kdo_2-lipid A structure. c) Schematic of lipopolysaccharide (LPS) biosynthesis in *E. coli*. UDP-GlcNAc is converted to LOS at the inner-leaflet of the inner membrane. Abbreviations; Kdo (3-deoxy-D-manno-octulosonic acid), Bam (β-barrel assembly machinery) GlcN (glucosamine), GlcNAc (*N*-acetylglucosamine).

Figure 2

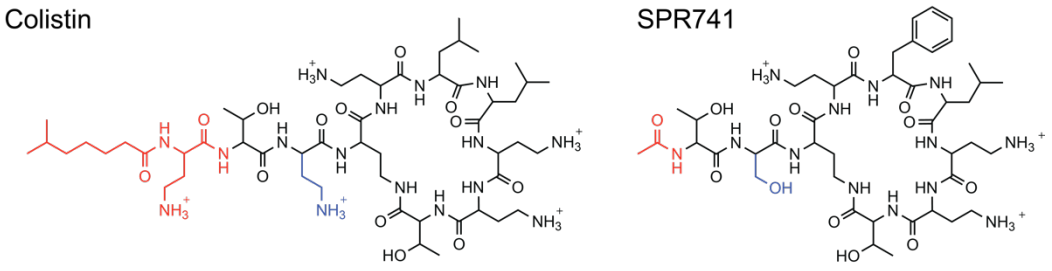

Figure 2. Chemical structure of colistin and SPR741. Modification to the hydrophobic N-terminal fatty acyl chain (red) and diaminobutyric acid (DAB) residue (blue) are highlighted.

Figure 3

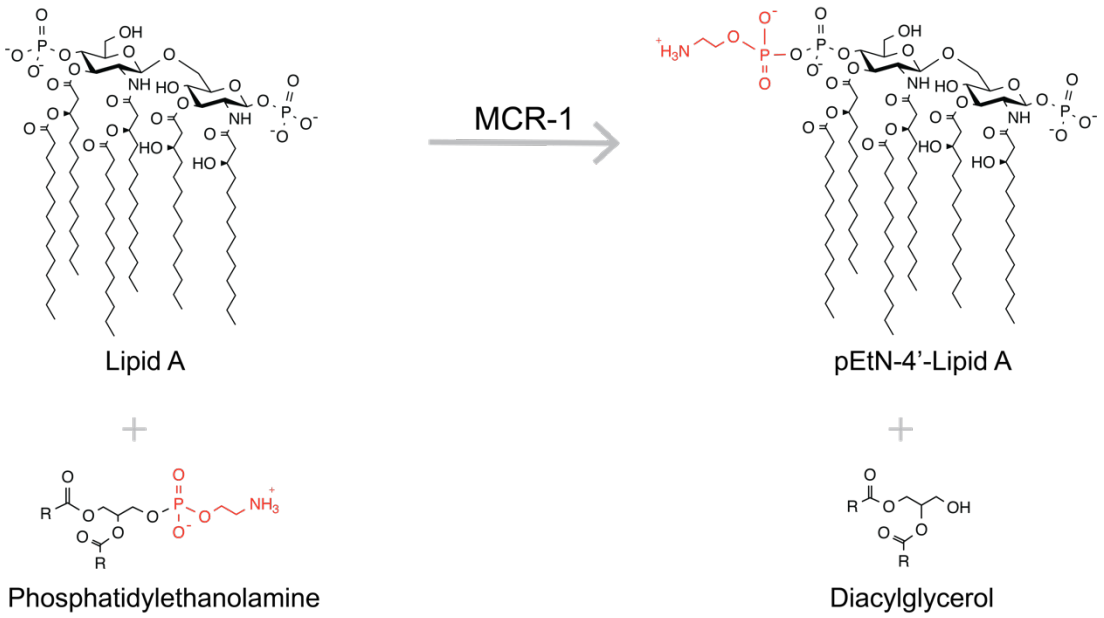

Figure 3. MCR-1 mediated colistin resistance. The chemical reaction for the modification of lipid A by MCR-1 in *E. coli.* MCR-1 catalyzes the reaction of phosphatidylethanolamine and lipid A to form phosphoethanolamine (pEtN)-4'-lipid A and diacylglycerol.

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Chapter II – Overcoming *mcr-1* mediated colistin resistance with colistin in combination with other antibiotics

Preface

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Abstract

Plasmid-borne colistin resistance mediated by *mcr-1* may contribute to the dissemination of pan-resistant Gram-negative bacteria. Here, we show that *mcr-1* confers resistance to colistin-induced lysis and bacterial cell death, but provides minimal protection from the ability of colistin to disrupt the Gram-negative outer membrane. Indeed, for colistin-resistant strains of Enterobacteriaceae expressing plasmid-borne *mcr-1*, clinically relevant concentrations of colistin potentiate the action of antibiotics that, by themselves, are not active against Gram-negative bacteria. The result is that several antibiotics, in combination with colistin, display growth-inhibition at levels below their corresponding clinical breakpoints. Furthermore, colistin and clarithromycin combination therapy displays efficacy against *mcr-1*-positive *Klebsiella pneumoniae* in murine thigh and bacteremia infection models at clinically relevant doses. Altogether, these data suggest that the use of colistin in combination with antibiotics that are typically active against Gram-positive bacteria poses a viable therapeutic alternative for highly drug-resistant Gram-negative pathogens expressing *mcr-1*.

Introduction

The widespread emergence of carbapenem-resistant Enterobacteriaceae has significantly increased dependence on the cationic peptide colistin, widely regarded as an antibiotic of last resort. Colistin acts by associating with the anionic lipopolysaccharide (LPS) component of the Gram-negative outer membrane, causing membrane destabilization that leads to cell envelope permeability, leakage of cellular contents, and ultimately lytic cell death[1,2]. The recent increase in the use of colistin in clinical practice, accompanied by its unbridled use in agriculture, have contributed to the rapid dissemination of resistance.

Colistin resistance is predominantly achieved through a reduction of the electrostatic attraction between colistin and the Gram-negative outer membrane. This is typically facilitated by the addition of cationic phosphoethanolamine (pEtN) and/or 4-amino-4-deoxy-L-arabinose (L-Ara4N) moieties to phosphate groups on the lipid A component of LPS, which reduces the net anionic charge of the cell surface[3]. Formerly, these LPS modifications were thought to be solely the result of chromosomal mutations that constitutively activate the two-component regulatory systems PhoP-PhoQ and PmrA-PmrB[4,5]. The inability of this form of chromosomal colistin resistance to rapidly spread through bacterial populations via horizontal gene transfer has limited its clinical impact to localized and controllable outbreaks[6]. However, the recent discovery of *Escherichia*

coli harbouring plasmid-borne colistin resistance via the *mcr-1* gene provides a mechanism for rapid dissemination[7].

Encoding a pEtN transferase, *mcr-1* confers colistin resistance through the addition of pEtN to the lipid A component of LPS. Since its discovery in late 2015, bacteria harbouring *mcr-1* have been detected in environmental and hospital isolates worldwide[7,8]. Particularly concerning are those multidrug-resistant strains containing *mcr-1* alongside extended-spectrum β-lactamase and carbapenemase resistance genes[9]. Indeed, the spread of *mcr-1* threatens to decrease the therapeutic utility of colistin from an already shrinking antibiotic arsenal.

With no reprieve from our therapeutic reliance on colistin in the current antibiotic pipeline, bridging the gap between widespread colistin resistance and the development of new antibiotics will require the creative use of available treatment options. To this end, the documented ability of colistin to potentiate a variety of antibiotics against Gram-negative pathogens provides an attractive therapeutic opportunity. Synergy with colistin has been explored for a range of antimicrobial agents, most commonly rifampicin[10,11] and carbapenems[12], but also macrolides[13], minocycline[14], tigecycline[15], and glycopeptides[16] Remarkably, colistin can impact the surface integrity of intrinsically colistin-resistant bacteria[17,18] and antibiotic potentiation is maintained within many pathogens expressing chromosomally mediated resistance to colistin monotherapy[19-21].

Unlike the monogenetic nature of *mcr-1*, chromosomal colistin resistance is mediated through mutations in two-component regulatory systems PhoPQ and

PmrAB that activate numerous genes involved in LPS modifications (i.e., *pmrHFIJKLM*) and a plethora of other cellular processes. For example, the PhoPQ system controls the expression of ~ 3% of the *Salmonella* genome[22]. Indeed, in PhoPQ-mediated colistin-resistant *Klebsiella pneumoniae*, additional components of the PhoPQ regulon such as the outer membrane lipoprotein *slyB* and the magnesium-importing ATPase *mgtA* are upregulated, potentially further contributing to resistance beyond pEtN/L-Ara4N modification of lipid A[23]. Comparatively, *mcr-1* reduces colistin efficacy through the expression of a single pEtN transferase and does not confer co-resistance to other cationic antimicrobial peptides, as observed in chromosomally regulated colistin resistance[24]. Additionally, *mcr-1*-expressing strains are unaffected by the outer membrane disruption and potentiation of antibiotics by polymyxin B nonapeptide (PMBN)[25]. Encouragingly, the combination of amikacin and colistin has shown promising combinatorial synergy in carbapenem-resistant *E. coli* carrying *mcr-1*[26]. While significant attention has been directed towards exploring combination treatment options to combat chromosomally mediated colistin resistance, the unique monogenetic nature of *mcr-1* largely differentiates this form of colistin resistance. As such, data generated from chromosomal colistin resistance studies may not accurately predict *mcr-1* susceptibility to antibiotic potentiation with colistin. Therefore, it is essential to thoroughly investigate colistin-based combination therapies against pathogens expressing *mcr-1*.

Here, we screened a collection of Enterobacteriaceae expressing the *mcr-1* gene against a range of antibiotics, representing all major drug classes, for a reduction in minimum inhibitory concentration (MIC) in the presence of colistin. Large, hydrophobic antibiotics conventionally active against Gram-positive bacteria such as rifamycins and macrolides demonstrated the greatest decrease in MIC in combination with colistin. Investigating the mechanism underlying this antibiotic potentiation, we observed that *mcr-1* provides a high degree of resistance to the bactericidal and lytic activity of colistin, but confers minimal protection to its outer membrane perturbation. Exploiting this susceptibility through colistin combination treatment demonstrated encouraging efficacy in two mouse models of *mcr-1*-positive *Klebsiella pneumoniae* infection. Additionally, unlike traditional monotherapy antibiotic treatments, resistance to colistin combination therapy can be readily overcome through the exchange of the antibiotic partner. With the anticipated spread of plasmid-mediated colistin resistance, we propose further investigation into colistin combination therapy as a potential last resort therapeutic option.

Results

Colistin potentiates antibiotics in *mcr-1*-positive bacteria

To date, all clinical and environmental isolates expressing *mcr-1* have been members of the Enterobacteriaceae and, as such, many of these pathogens, in addition to *Pseudomonas aeruginosa*, have been shown to capably express *mcr-1* in a laboratory setting[7]. *E. coli, Salmonella* Typhimurium,

Enterobacter aerogenes, Enterobacter cloacae, and *K. pneumoniae* are all major pathogens of clinical importance known to harbour *mcr-1*. Therefore, these species were engineered to express *mcr-1* from the pGDP2 plasmid and investigated for susceptibility to colistin combination treatment. We screened > 40 antibiotics covering all major drug classes for changes in MIC in the presence of colistin. Fold reduction in MIC was quantified by dividing the MIC of an antibiotic alone by its MIC in the presence of a therapeutic concentration of colistin (2 μg/mL).

As expected, *mcr-1* confers no change in susceptibility (<4-fold change) to antibiotics outside of the polymyxin class (Supplemental Table 1). However, in the presence of colistin, several antibiotics are highly potentiated, as characterized by a greater than an eightfold reduction in MIC (Figure 1, Supplemental Table 1). Importantly, the 2 μg/mL colistin concentration used when determining fold reduction in MIC represents a concentration obtainable during standard therapeutic colistin dosing[27-29]. In combination with colistin, the antibiotics rifampicin, rifabutin, clarithromycin, minocycline, and novobiocin achieve the highest therapeutic potential as combinatorial partners, with an observed reduction in MIC below the corresponding Gram-positive clinical breakpoint for all Enterobacteriaceae tested. Of note, with the removal of novobiocin from market in 2011, there is no currently listed CLSI or EUCAST breakpoint. However, we classify novobiocin as having high clinical potential as a combination agent with colistin, as it is potentiated to active concentrations below

steady-state serum levels (5 µg/mL)[30]. Indeed, the unique mechanism of action and potential of novobiocin as a combination partner with colistin and other Gram-negative outer membrane disrupting compounds[25] may justify a re-evaluation of clinical use. Additionally, the topical antibiotic mupirocin is potentiated to clinically pertinent levels of susceptibility, representing a potential treatment strategy for the increasing threat of multidrug-resistant skin and soft-tissue infections[31].

To investigate whether colistin potentiation is conserved beyond laboratory-generated *mcr-1* strains, we tested nine clinical and retail food-derived *mcr-1*-positive *E. coli* strains with clarithromycin, novobiocin, and rifampicin. Encouragingly, all nine strains were susceptible to potentiation, with an observed fold reduction in MIC greater than eight for all three antibiotics (Supplemental Figure 1). Susceptibility below clinical breakpoint is achieved in the presence of colistin for clarithromycin or rifampicin in six of nine and, for novobiocin, in seven of nine strains. Overall, eight of nine strains demonstrate therapeutic levels of sensitivity to at least one of the three antibiotic-colistin combinations. In addition to the broad conservation of susceptibility to colistin combination treatment observed across the *mcr-1*-positive Enterobacteriaceae species tested, potentiation was highly dependent upon the antibiotic class. Notably, broad-spectrum antibiotics, such as those within the fluoroquinolone and beta-lactam classes, displayed a limited reduction in MIC and no impact on clinical breakpoints. Antibiotics in the rifamycin and macrolide class, as well as

minocycline and novobiocin, displayed the highest levels of synergy with colistin in *mcr-1*-positive Enterobacteriaceae.

To determine if the expression of *mcr-1* increases resistance to the outer-membrane disruption activity of colistin proportionally to the increased resistance to growth-inhibition, we investigated the concentration range of colistin capable of potentiating rifampicin (1 μg/mL) into wild-type and *mcr-1*-expressing *E. coli*. Due to the physicochemical properties of rifampicin, outer-membrane disruption is essential to facilitate entry and growth-inhibition. In a wild-type background, growth-inhibition occurs at a colistin concentration of 0.15 μg/mL and decreases twofold to 0.075 μg/mL in the presence of rifampicin at 1 μg/mL (Figure 2a). Therefore, while a concentration of 0.075 μg/mL colistin is insufficient to inhibit bacterial growth, it provides sufficient outer-membrane disruption to allow rifampicin uptake and rifampicin-mediated growth-inhibition. Expression of *mcr-1* increases the concentration of colistin required for growth-inhibition to 5 μg/mL (Figure 2b), which is 32-fold greater than wild-type. However, in the presence of rifampicin, the concentration of colistin required to inhibit growth is 0.15 μg/mL (Figure 2b) or only twofold greater than the concentration required to potentiate rifampicin in wild-type cells. Thus, expression of *mcr-1* appears to provide significant protection against the monotherapy activity of colistin, but not its capacity to perturb the outer membrane.

To further characterize the impact of *mcr*-1 on the outer-membrane perturbation activity of colistin, we measured uptake of the hydrophobic

fluorophore *N*-phenyl-1-naphthylamine (NPN). An intact outer membrane prevents entry of NPN to the phospholipid layer and the subsequent fluorescence. Therefore, NPN uptake represents a quantitative read-out for colistin-mediated outer-membrane disruption. We observed only a 2- to 4-fold increase in colistin concentrations required to reach comparable levels of NPN uptake in *mcr-1*-expressing *E. coli* compared to wild-type (Figure 2c). For example, initial uptake (>5%) occurs at 0.39 μg/mL of colistin in wild-type *E. coli* and 0.78 μg/mL in an *mcr-1* background (Figure 2c). Saturation of NPN uptake (>95%) occurs at 6.25 μg/mL in wild-type and 12.5 μg/mL when expressing *mcr-1* (Figure 2c). All additional levels of NPN uptake observed in a wild-type background between the initial uptake and saturation can be achieved by increasing the concentration of colistin 2- to 4-fold in an *mcr-1*-expressing strain (Figure 2c). Despite a 32-fold change in MIC observed with expression of *mcr-1*, the concentration of colistin required for significant outer-membrane perturbation is only increased 2- to 4-fold.

With the observed discrepancy between susceptibility to outer-membrane perturbation and resistance to growth-inhibition conferred by *mcr-1*, we hypothesized *mcr-1* may provide resistance through a mechanism beyond reducing the initial electrostatic interaction with the outer membrane. To determine if *mcr-1* expression alters the rate at which colistin-mediated lysis occurs, we monitored the reduction of optical density (OD) over 18 h in wild-type and *mcr-1*-expressing *E. coli*. To isolate the impact of *mcr-1* on lytic rate and

normalize for differences in strain susceptibility, we compared the lowest concentration of colistin capable of causing growth-inhibition after 18 h when the initial inoculum is ~ 8×10^8 colony-forming units (CFU) mL^{-1}. Typically, exposure to inhibitory concentrations of colistin results in rapid bacterial lysis and, therefore, a reduction in culture turbidity. Indeed, wild-type *E. coli* displayed an exponential decrease from the starting ~ 0.5 OD (600 nm) over 6 h with clearance plateauing at ~ 0.1 OD (600 nm) (Figure 2d). However, expression of *mcr-1* drastically reduced the rate of bacterial lysis, with OD (600 nm) increasing over the first 3 h followed by a slow decline to ~ 0.4 OD (600 nm) after 18 h (Figure 2d). Notably, we were unable to return the rate of lysis to wild-type levels by increasing the concentration of colistin (Supplemental Figure 2 a,b). The observed change in lytic susceptibility appears to be polymyxin specific, as lysis by ampicillin occurs as previously described[32] for both strains (Supplemental Figure 2c).

Overcoming resistance to colistin combination therapy

In cases where colistin combination treatment effectively inhibits the growth of an *mcr-1*-positive strain, antimicrobial activity is driven solely by the antibiotic partner. Consequently, reduced efficacy in this form of treatment would likely be facilitated by resistance to the antibiotic partner, rather than to the potentiation ability of colistin[33]. As such, colistin combination therapy offers the unique advantage that, despite the potential for the evolution of resistance, substitution of the antibiotic partner should renew treatment efficacy. Indeed,

spontaneous *E. coli* mutants expressing *mcr-1* generated in the presence of rifampicin and colistin were no longer susceptible to colistin/rifampicin combination treatment but remained susceptible if rifampicin was exchanged for either novobiocin or clarithromycin at therapeutically relevant levels (Figure 3a,b and Supplemental Table 2).

In vivo efficacy

Given the immediate therapeutic potential of combination therapy, we investigated clarithromycin in combination with colistin using two murine models of *mcr*-1-positive *K. pneumoniae* infection. Recognizing the potential for dose-sparing during drug combination therapies and noted concerns of colistin toxicity, we tested the efficacy of colistin at approximately one-fifth of the human equivalent dose[34]. Clarithromycin was selected for its high propensity for *in vitro* potentiation among all Enterobacteriaceae tested (Figure 1) and dosed at approximately the standard human equivalent dose[35]. During a thigh infection, neither colistin (7.5 mg kg^{-1}) nor clarithromycin (200 mg kg^{-1}) showed a significant reduction in bacterial load when treatment was initiated 1 h post-infection (Figure 4a). However, the combination of colistin and clarithromycin proved efficacious, resulting in a 2.9-log_{10} reduction ($p < 0.0001$, Mann–Whitney *U*-test) in CFU when compared to the untreated control 8 h after infection (Figure 4a). *K. pneumoniae* bloodstream infections cause a high level of patient mortality and, therefore, represent a prime candidate for novel last resort therapeutics. To produce a murine bacteremia infection, mice were inoculated with a dose of *mcr-*

1-expressing *K. pneumoniae* that led to 100% lethality within 12 h. Monotherapy treatments administered 1 h post-infection did not demonstrate significant survival beyond that of the untreated group (Figure 4b). Importantly, within 1 h post-infection, *mcr-1*-expressing *K. pneumoniae* was detected at high burdens throughout the animal (Supplemental Figure 3). Animals receiving clarithromycin and colistin combination therapy daily for 5 days rescued 60% of those treated (Figure 4b), highlighting the *in vivo* efficacy of colistin combination therapy against *mcr-1*-positive infections.

Discussion

A diminishing antibiotic pipeline and increasing clinical reliance on colistin have magnified the threat of horizontally transferrable colistin resistance via the *mcr-1* gene. Similar to many other cationic peptides, the impact of colistin on bacterial cells can be broadly classified into three major events, outer-membrane disruption through interaction with surface LPS, self-promoted uptake through the outer membrane, and the formation of regions of instability in the cytoplasmic membrane leading to lysis[1,2,36]. Previous studies have leveraged the outer-membrane disruptive activity of colistin by combining it with antibiotics that otherwise are unable to traverse an intact outer membrane[10-16]. Notably, this susceptibility to combination therapy is maintained in strains with a number of conventional chromosomal colistin resistance mechanisms[19-21].

As *mcr-1* represents the predicted driver of pervasive colistin resistance, we investigated the potential of combination therapy for the treatment of

pathogens expressing *mcr-1*. Our data shows that *mcr-1*-expressing Enterobacteriaceae can be sensitized to a range of antibiotics in the presence of colistin. Importantly, sensitization occurs at concentrations below clinical breakpoint for colistin as well as the partnered antibiotic and is efficacious in treating murine models of *mcr-1*-expressing *K. pneumoniae*. The amenability of this form of combination therapy to the unconventional use of narrow-spectrum, Gram-positive active antibiotics poses an advantage over traditional broad-spectrum approaches. Specifically, Gram-negative pathogens are unlikely to be harbouring the appropriate intrinsic resistance mechanisms that would render combination treatment with such Gram-positive active antibiotics ineffective, due to a lack of selective pressure. However, should resistance develop to combination treatment, our results suggest that resensitization may be achieved with an exchange of the antibiotic partnered with colistin.

The traditional focus of the proposed mechanisms for *mcr-1* and other polymyxin resistance is that the addition of cationic groups to the phosphates of lipid A reduces the electrostatic interaction between colistin and lipid A, preventing localized disruption of the outer membrane and, therefore, self-promoted uptake and lysis[3,7,37,38]. However, this proposed mechanism would not appear to predict the observed susceptibility of *mcr-1*-expressing bacteria to outer-membrane disruption. It is interesting to note the pEtN modification of *mcr-1* provides only limited reduction to the formal charge of the phosphate ester of lipid A from −1.5 to −1[39]. Despite the significant increase in the concentration of

colistin required for growth-inhibition in an *mcr-1*-expressing strain, both our rifampicin susceptibility and NPN uptake data show that colistin is able to interact with and disrupt the outer membrane sufficiently to allow the uptake of large hydrophobic compounds largely irrespective of *mcr-1* expression. Additionally, we demonstrate that *mcr-1* not only increases the required colistin concentration to inhibit growth but also reduces the rate at which lysis occurs. With continued susceptibility to outer-membrane perturbation and reduction in the rate of colistin-mediated bacterial lysis, we hypothesize that despite the monogenetic nature of *mcr*-1, colistin resistance is conferred through a mechanism that extends beyond decreasing the strength of the electrostatic interaction between colistin and lipid A in the outer membrane.

The relationship between bactericidal activity and the ability of polymyxins to interact with and disrupt the outer membrane is poorly characterized. Indeed, a growing range of evidence, including the data presented in this study, suggests these two processes may not be highly linked[40,41]. For example, in wild-type backgrounds, outer-membrane disruption can be achieved with PMBN[42], which lacks the fatty-acyl moiety found on polymyxin B and colistin, and is incapable of inducing lysis. The lytic antimicrobial activity of colistin is thought to require insertion of the fatty-acyl chain into the outer membrane[42], which weakens the packing of adjacent lipid A molecules and facilitates transit to the inner membrane via self-promoted uptake. While the mechanism behind self-promoted colistin uptake is not yet fully elucidated, this step is crucial in advancing from

outer-membrane disruption to lysis[43]. One potentially underappreciated aspect of *mcr-1* that may contribute to its ability to resist the growth-inhibitory activity of colistin is that the addition of cationic groups like pEtN can alter outer-membrane architecture through a reduction in repulsion between neighbouring LPS molecules that strengthens membrane packing[39,44]. It has been speculated the high intrinsic polymyxin resistance observed in *N. meningitides* can be attributed to this phenomenon[39]. Notably, the main component of polymyxin resistance in *N. meningitides* is the phosphoethanolamine transferase LptA[45], which is closely related to the structure of MCR-1[7]. Therefore, we hypothesize that strengthened LPS packing provided by *mcr-1* could play an important role in reducing the uptake and lytic activities of colistin.

The ability of colistin to potentiate antibiotics against colistin-resistant bacteria is not well understood. Interestingly, expression of *mcr-1* reduces the potentiation ability of PMBN[25] but does not alter appreciably the susceptibility to the outer-membrane disruption of colistin. Thus, it may be that the role of the fatty-acyl tail in self-promoted uptake and lysis is impaired by *mcr-1*, but its importance in outer-membrane disruption is unaffected. Interestingly, many of the toxicity concerns associated with polymyxins can be attributed to this fatty-acyl tail[42] and changes or elimination of this hydrophobic moiety may represent a challenging avenue to combination therapy strategies in the face of *mcr-1*-mediated resistance. Overall, exploiting the susceptibility of *mcr-1*-expressing pathogens to colistin combination therapy may represent an Achilles heel for an

otherwise difficult resistance mechanism, and we recommend a re-evaluation of its clinical utility.

Methods

Colistin combination susceptibility testing

Fourteen Enterobacteriaceae strains harbouring *mcr-1* were investigated for susceptibility to colistin combination therapy. Specifically, *E. coli* K-12 BW25113[46], *K. pneumoniae* ATCC 43816, *S.* Typhimurium ATCC 14028, *E. cloacae* GN687 (Public Health Ontario), and *E. aerogenes* C0064 (Public Health Ontario) were transformed with the pGDP2:*mcr-1* plasmid. Additionally, the National Microbiology Laboratory of Canada provided *mcr-1*-positive clinical and retail food-derived *E. coli* isolates; N12-00130 (ST624), N15-02865 (ST648), N15-02866 (ST398), N16-00121 (ST3944), N16-00319 (ST156), N16-00487 (ST648), N16-01175 (ST515), N16-03711 (ST10), and N17-00323 (ST101). Lab generated strains were validated for colistin resistance above EUCAST breakpoint using Clinical and Laboratory Standard Institute (CLSI) guidelines for MIC[47]. Fold reduction of MIC was determined by dividing the MIC of the antibiotic alone by its MIC in the presence of 2 μg/mL colistin. Compounds demonstrating ≥ 8-fold reduction were prioritized based on sensitization below their corresponding Enterobacteriaceae or *Staphylococcus aureus* clinical breakpoint. Checkerboard analyses were conducted with each drug serially diluted at eight concentrations to create an 8 × 8 matrix. At least two biological replicates were done for each combination, and the means were used for FIC

calculation. The FIC for each drug was calculated by dividing the concentration of drug in the presence of codrug in a combination for a well showing < 10% growth by the MIC for that drug alone[48]. The FIC index is the sum of the two FICs, with an FIC index ≤ 0.5 deemed synergistic.

Membrane integrity assays

All assays were performed with wild-type and pGDP2:*mcr-1*-expressing *E. coli* BW25113. MICs were conducted as outlined above in the presence and absence of 1 μg/mL rifampicin. NPN assays were conducted as previously established[49]. Cells from an overnight culture were diluted 1/50 and incubated until mid-log (~ 0.5 OD 600 nm), centrifuged, washed in 5 mM HEPES buffer containing 20 mM glucose, spun down and resuspended in the same buffer to an OD (600 nm) of 1. A volume of 100 μL of cells was added to 100 μL of buffer containing NPN and varying concentrations of colistin in black clear-bottom 96-well plates. After a 1 h incubation at room temperature, fluorescence was read in a Tecan® infinite M1000 Pro, excitation 355 ± 5 nm and emission 420 ± 5 nm. Percent NPN uptake is calculated for each strain according to ref.[50]

$$\text{NPN uptake (\%)} = (F_{obs} - F_0)/(F_{100} - F_0) \times 100\%$$

where F_{obs} is the observed fluorescence at a given colistin concentration, F_0 is the initial fluorescence of NPN with *E. coli* cells in the absence of colistin, and F_{100} is the fluorescence of NPN with *E. coli* cells upon addition of

100 µg/mL of colistin, which is beyond the observed plateau in fluorescence for both strains.

To determine the rate of colistin-induced lysis, strains were grown to mid-log (2–3 h), centrifuged, suspended in fresh Mueller Hinton broth to an OD (600 nm) of 1 and added to varying concentrations of colistin to a total volume of 250 µL in a standard 96-well plate, giving a starting OD (600 nm) of ~ 0.5 in the plate reader. OD (600 nm) was measured every 30 min for 18 h during incubation at 37°C with shaking using a Tecan® Sunrise.

Generation of combination-resistant mutants

E. coli BW25113 transformed with pGDP2:*mcr-1* was used to generate mutants resistant to rifampicin and colistin combination therapy through the plating of bacteria onto 1 µg/mL colistin and 2 µg/mL rifampicin and incubated until colony growth. Single colonies were passaged overnight in rifampicin and colistin to reconfirm resistance prior to investigation with MIC and fold change assays, which were performed as outlined above.

Animal studies

All animal studies were conducted according to guidelines set by the Canadian Council on Animal Care using protocols approved by the Animal Review Ethics Board at McMaster University under Animal Use Protocol # 17-03-10. All animal studies were performed with 6–8-week-old female CD-1 mice from Charles River. Female mice were used in accordance with previously established

models[51] as well as ease of housing and randomization. The sample size was selected based on the results of a preliminary infection trial. Before infection, mice were relocated at random from a housing cage to treatment or control cages. No animals were excluded from analyses, and blinding was considered unnecessary.

Mouse thigh infection model

The combination of colistin and clarithromycin was tested against *K. pneumoniae* ATCC 43816 transformed with pGDP2:*mcr-1* in a neutropenic mouse thigh infection model. Female CD-1 mice were rendered neutropenic by cyclophosphamide, dosed at 150, and 100 mg kg^{-1} delivered on days -4 and -1 prior to infection. Bacteria were suspended in sterile saline and adjusted to a concentration of ~ 1×10^6 CFU per infection site and injected into the right and left thighs of five mice per treatment group. At 1 h post-infection, mice received either colistin (7.5 mg kg^{-1}, i.p. $n = 10$), clarithromycin (200 mg kg^{-1}, p.o. $n = 10$), untreated ($n = 8$), or the combination ($n = 10$). Mice were euthanized 8 h after infection; thigh tissue was aseptically collected, weighed, homogenized, serially diluted in PBS and plated onto solid LB supplemented with kanamycin (50 µg/mL). Plates were incubated overnight at 37°C and colonies were quantified to determine bacterial load.

Mouse bacteremia model

The combination of colistin and clarithromycin was tested against *K. pneumoniae* ATCC 43816 transformed with pGDP2:*mcr-1* in an immunocompetent bacteremia infection model. Female CD-1 mice were infected intraperitoneally with ~ 1×10^6 CFU of bacteria with 5% porcine mucin (Sigma-Aldrich). Infections were allowed to establish for 1 h prior to treatment with colistin, clarithromycin, or the combination. With the encouraging reduction in CFU observed in the thigh infection model, dosing was administered as described above. Clinical endpoint was determined using a five-point body condition score, analyzing weight loss, body temperature, respiratory distress, hampered mobility, and hunched posture. Experimental endpoint was defined as 10 days post-infection for mice not reaching the clinical endpoint.

Data availability

The data that support the findings of this study are available in this article and its Supplemental Information files, or from the corresponding author upon request.

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Author contributions

C.R.M., J.M.S., L.A.C., M.R.M., and E.D.B. designed the experiments. C.R.M. and J.M.S. designed and conducted the *in vitro* potentiation assays. M.R.M. provided access to clinical and food-derived *mcr-1*-expressing *E. coli* isolates and assisted in colistin potentiation screening. C.R.M. and L.A.C. designed and performed *in vivo* infection model experiments. B.K.C. and A.A.F.-C. supported murine model development. C.R.M. and E.D.B. wrote the manuscript with input from all authors.

Figures and Legends

Figure 1

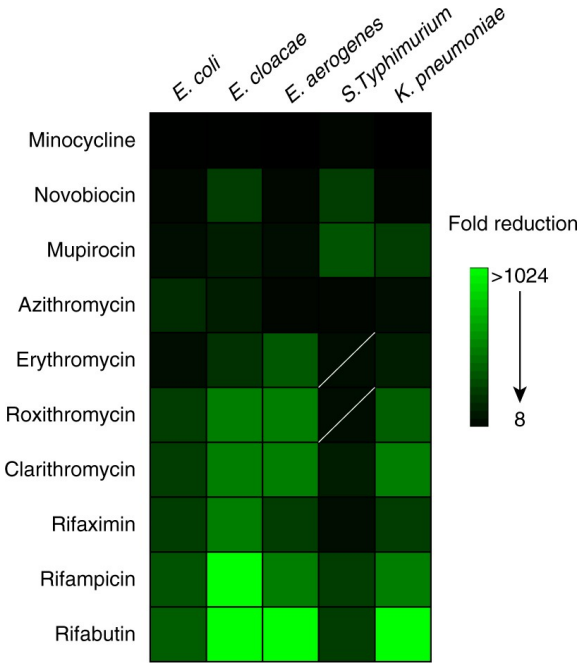


Figure 1. Colistin potentiates antibiotics conventionally used against Gram-positive bacteria in Enterobacteriaceae expressing *mcr-1*. Heat map showing the mean fold reduction of MIC in the presence of 2 μg/mL colistin for strains transformed with pGDP2:*mcr-1*. Antibiotics listed were potentiated ≥ 8-fold across all lab generated Enterobacteriaceae strains. A lack of potentiation below clinical breakpoint is indicated by a diagonal white line. Data are representative of two biological replicates.

Figure 2

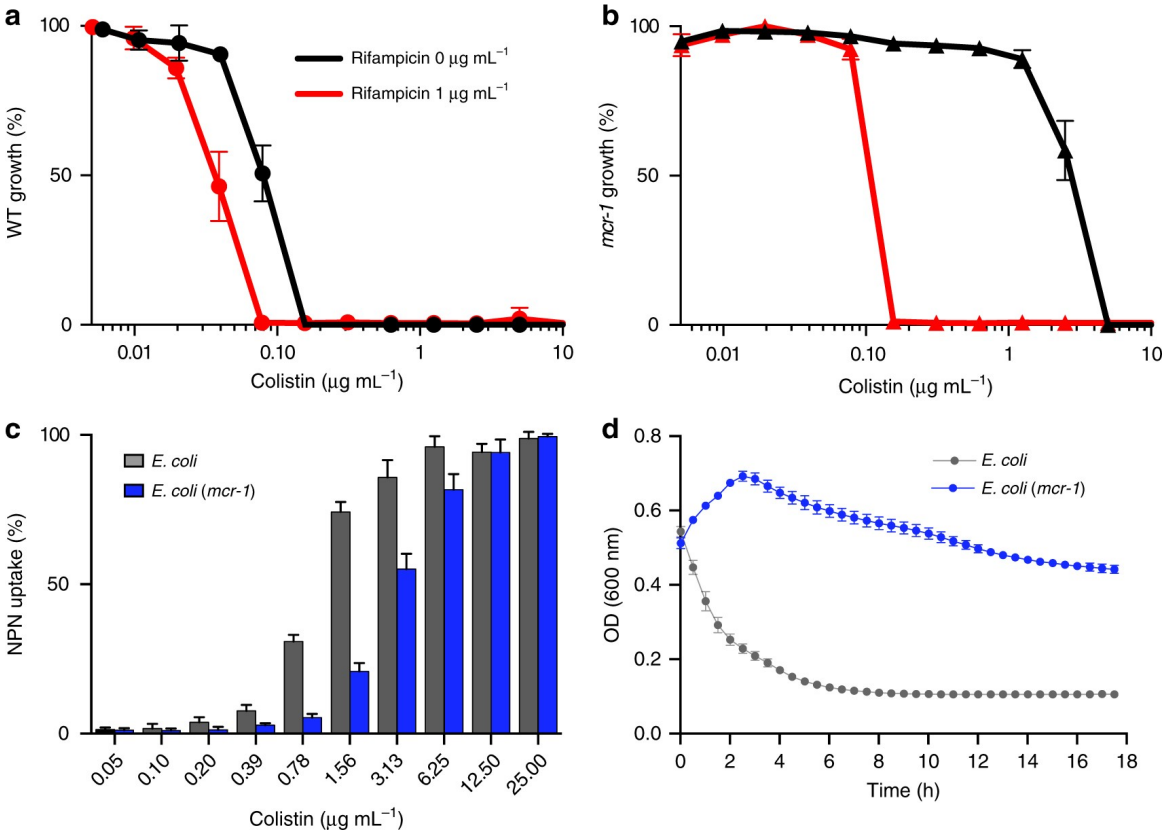


Figure 2. Expression of *mcr-1* provides limited protection to colistin-mediated outer-membrane disruption. a, **b** Potency analysis of wild-type (**a**) and *mcr-1*-expressing (**b**) *E. coli* in the presence (red) and absence (black) of rifampicin (1 μg/mL). **c** *N*-Phenyl-1-naphthylamine (NPN) uptake of wild-type (gray) and *mcr-1*-expressing *E. coli* (blue) induced by colistin. NPN uptake (%) represents the background-subtracted fluorescence divided by the fluorescence observed at 100 μg/mL of colistin. **d** Kinetic analysis of colistin-mediated lysis in wild-type (gray) and *mcr-1*-expressing (blue) *E. coli*. Optical density (OD) at 600 nm was monitored every 30 min for 18 h in the presence of colistin at 50 μg/mL and 6.25 μg/mL in *mcr-1* and wild-type, respectively. Concentrations selected are the lowest values capable of inhibiting growth with a starting cell density of OD (600 nm) 0.5. Data in **a**, **b** ,**c**, and **d** represent means with standard deviation from two biological replicates.

Figure 3

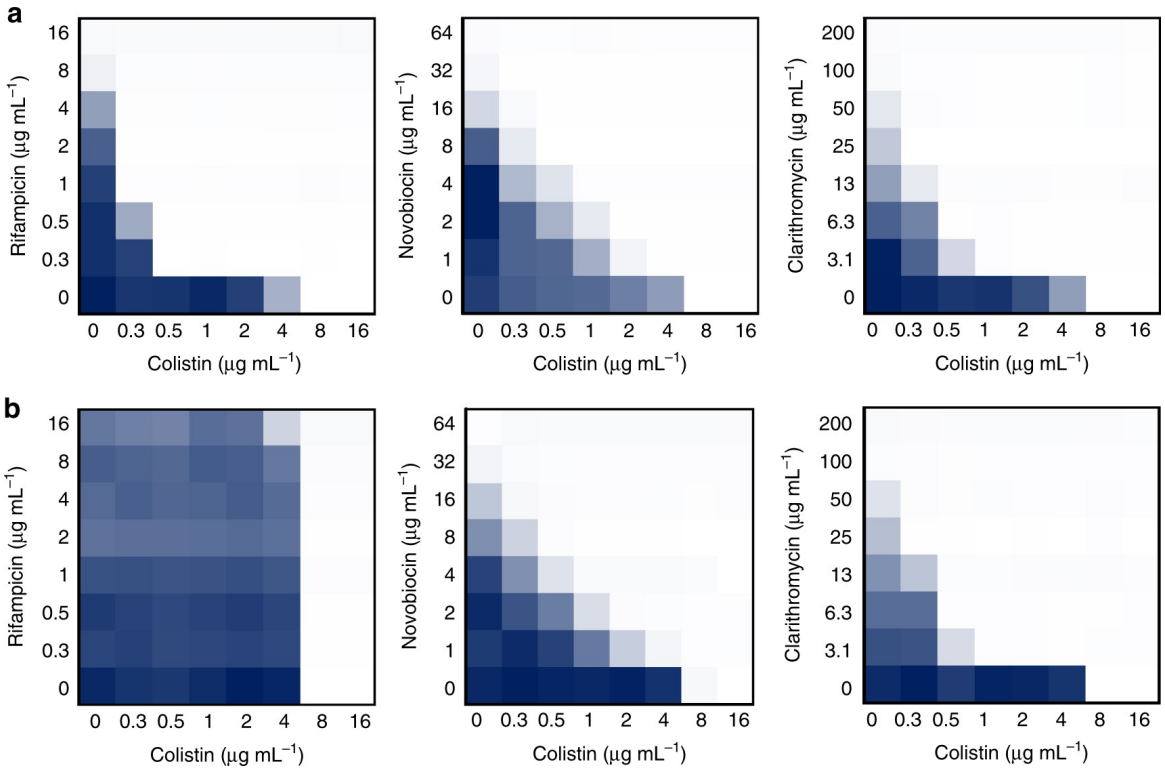


Figure 3. Resistance to colistin combination therapy can be overcome by exchange of the partnered antibiotic. **a**,**b** Checkerboard broth microdilution assays showing dose-dependent potentiation of rifampicin, novobiocin, and clarithromycin by colistin against *mcr-1*-positive *E. coli* (**a**) and a spontaneous mutant of *E. coli*-expressing *mcr-1* (**b**) generated in the presence of rifampicin and colistin. Dark blue regions represent higher cell density. Data in **a** and **b** represent the mean OD (600 nm) of two biological replicates.

Figure 4

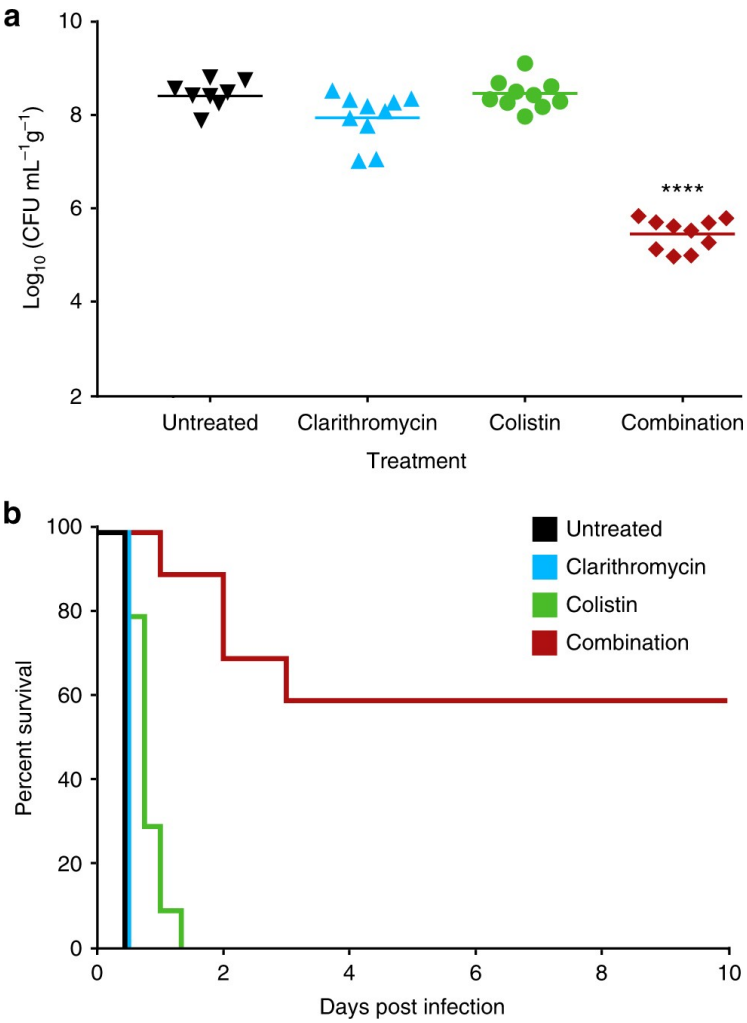
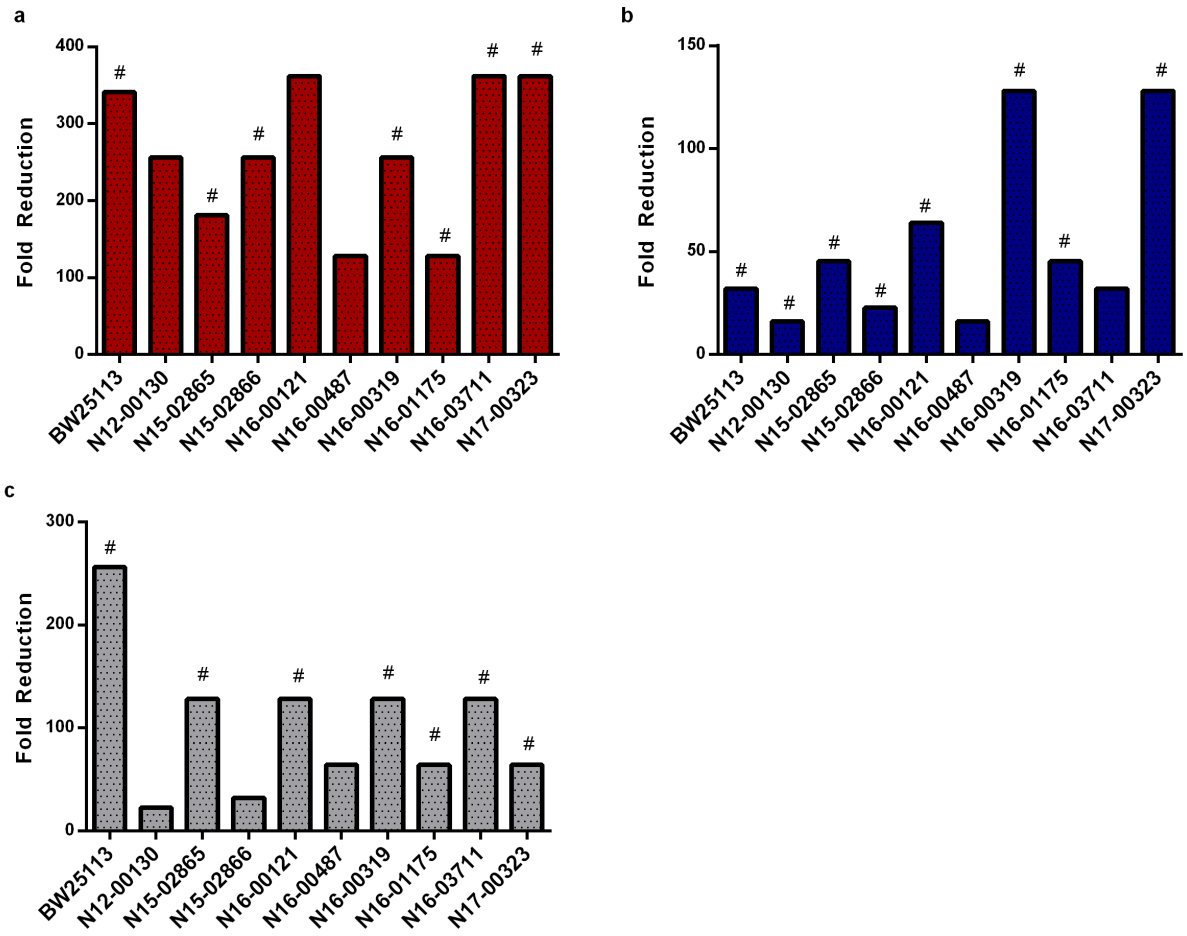


Figure 4. Colistin and clarithromycin combination therapy is efficacious in two mouse models of infection. **a** Single-dose treatment at 1 h post-infection of clarithromycin ($n = 10$, blue, 200 mg/kg, p.o.), colistin ($n = 10$, green, 7.5 mg/kg, i.p.) or the combination ($n = 10$, red) in a neutropenic mouse thigh infection model using *mcr-1*-expressing *K. pneumoniae*. Colony-forming units (CFU) within thigh tissue were enumerated at 8 h post-infection and compared to the untreated group ($n = 8$, black). Horizontal lines represent the geometric mean of the bacterial load for each treatment group. The combination of colistin and clarithromycin resulted in a 2.9-$\log_{10}$ reduction ($p < 0.0001$, Mann–Whitney *U*-test) in CFU when compared to the untreated control 8 h after infection. **b** Survival curve of a *K. pneumoniae*-expressing *mcr-1* bacteremia infection dosed at 1, 24, 48, 72, 96, and 120 h post-infection as outlined above for clarithromycin ($n = 10$), colistin ($n = 10$), untreated ($n = 10$), and the combination ($n = 10$).

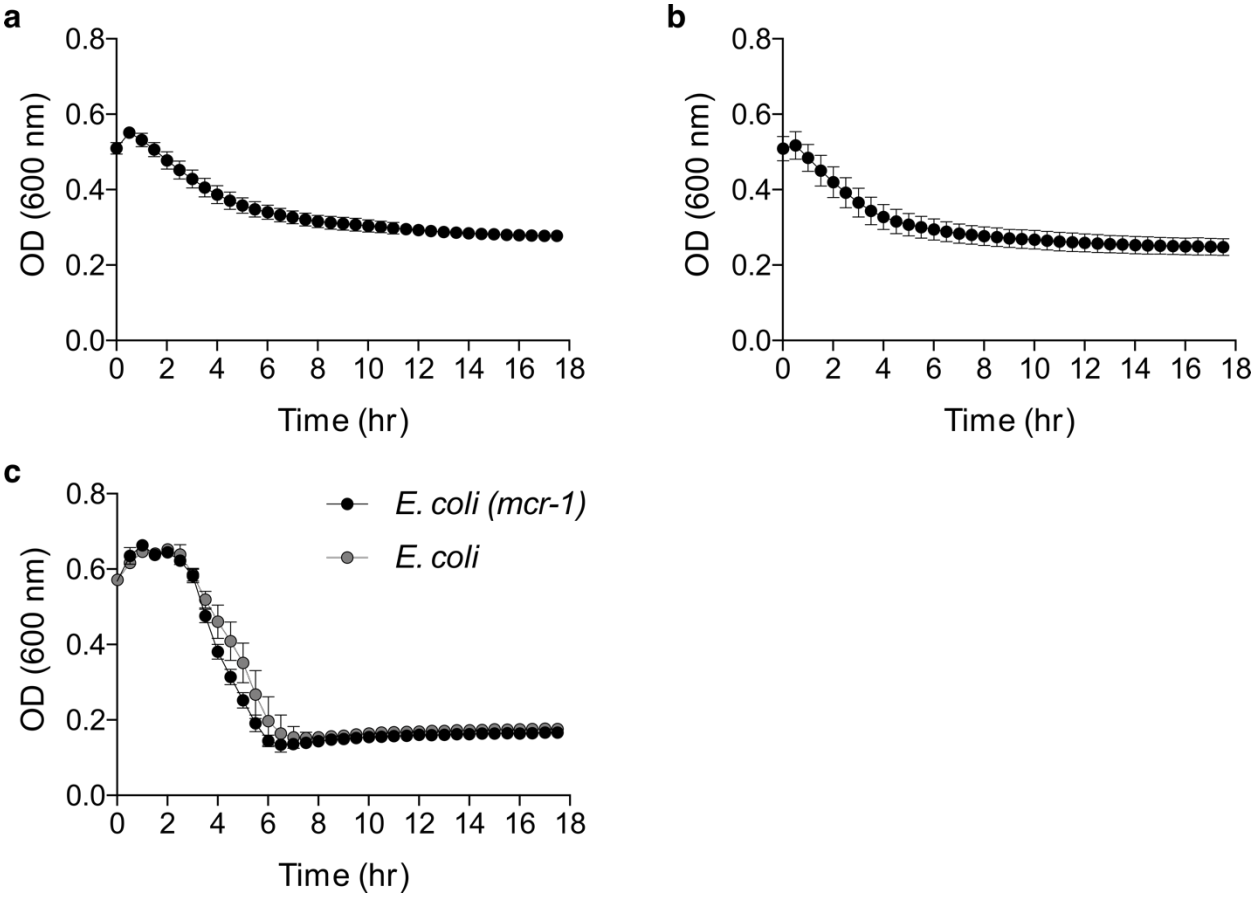
Supplemental Figures and Legends

Supplemental Figure 1



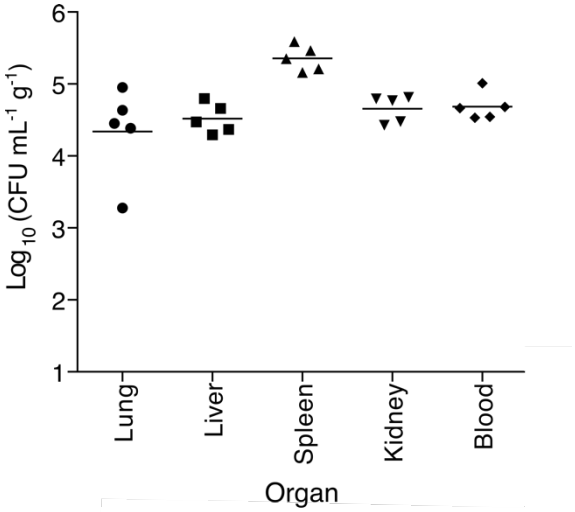
Supplemental Figure 1. Clinical and retail food derived *mcr-1* expressing *E. coli* isolates are susceptible to colistin potentiation. a,b,c, Mean fold reduction of rifampicin (**a**), novobiocin (**b**), and clarithromycin (**c**) in the presence of 2 µg/mL colistin for nine clinical and retail food derived *mcr-1* positive *E. coli* isolates and *E. coli* BW25113 expressing pGDP2:*mcr-1*. Potentiation below clinical breakpoint is indicated with a #. Data in **a, b,** and **c** are representative of two biological replicates.

Supplemental Figure 2



Supplemental Figure 2. Expression of *mcr-1* reduces susceptibility to the lytic activity of colistin. a, b, Kinetic analysis of lysis in *mcr-1* expressing *E. coli* at 100 µg/mL (**a**) and 200 µg/mL (**b**) of colistin. **c,** Kinetic analysis of lysis in *mcr-1* expressing (black) and wild-type (grey) *E. coli* at 500 µg/mL ampicillin. **a**, **b**, **c**, OD (600 nm) was monitored every 30 min for 18 hours in the presence of antibiotic and data represents the means with standard deviation from two biological replicates.

Supplemental Figure 3



Supplemental Figure 3. ***K. pneumoniae* expressing *mcr-1* demonstrates rapid dissemination in a murine bacteremia model**. Female CD-1 mice (n=5) were infected with *K. pneumoniae* (~1 x 10^6 CFU, i.p.) in PBS and 5% porcine mucin. Mice were euthanized one hour after infection, and a variety of organ tissue aseptically collected weighed, homogenized, serially diluted in PBS and plated onto solid LB supplemented with kanamycin (50 µg/mL). Plates were incubated overnight at 37°C and colonies were quantified to determine bacterial load. Horizontal lines represent the geometric mean of the bacterial load for each organ.

Supplemental Tables

Supplemental Table 1

	E.coli				*E. cloacae*				*E. aerogenes*				*S.* Typhimurium				*K. pneumoniae*			
Antibiotic	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**	**1**	**2**	**3**	**4**
Azithromycin	12.5	6.25	0.03	181	35.36	25	0.2	128	12.5	6.25	0.2	32	12.5	6.25	0.2	32	6.25	12.5	0.20	64
Clarithromycin	50	50	0.2	256	150	100	0.2	512	150	50	0.1	512	100	50	0.39	128	100	50	0.10	512
Erythromycin	50	50	0.78	64	>200	200	1	200	>200	100	0.28	362	100	100	1.56	64	50	100	0.78	128
Minocycline	2.21	3.13	0.2	16	6.25	3.13	0.2	16	6.25	3.13	0.39	8	4.42	6.25	0.20	32	3.13	3.13	0.39	8
Mupirocin	50	50	0.78	64	100	50	0.39	128	100	50	0.78	64	100	100	0.29	341	50	100	0.39	256
Novobiocin	25	25	0.59	43	>200	>200	1.56	256	25	25	0.59	43	100	>200	1.56	256	50	50	1.56	32
Rifabutin	4.42	4.69	0.01	384	8.84	12.5	0.01	1365	8.84	12.5	0.01	1024	6.25	4.69	0.02	256	6.25	12.5	0.01	1024
Rifampicin	6.25	6.25	0.02	342	12.5	25	0.01	2048	12.5	12.5	0.02	512	12.5	12.5	0.05	256	12.5	12.5	0.02	512
Rifaximin	12.5	12.5	0.05	256	12.5	12.5	0.02	512	12.5	12.5	0.05	256	12.5	6.25	0.10	64	25	12.5	0.05	256
Roxithromycin	>200	200	0.78	256	>200	>200	0.78	512	>200	>200	0.78	512	>200	200	3.13	64	200	>200	0.78	384

Supplemental Table 1. **MIC of Enterobacteriaceae strains transformed with pGDP2:*mcr-1* in the presence and absence of 2 µg/mL colistin.** Columns 1, wild-type MIC. Column 2, *mcr-1* expressing MIC. Column 3, MIC of the *mcr-1* expressing strain in the presence of 2 µg/mL colistin. Column 4, fold reduction determined by dividing column 2 by column 3. Data represent the mean of at least two biological replicates.

Supplemental Table 2

Mutant Strain	MIC		MIC (Colistin 2 µg/mL)		
	Colistin (µg/mL)	Rifampicin (µg/mL)	Rifampicin (µg/mL)	Clarithromycin (µg/mL)	Novobiocin (µg/mL)
1	6.25	>200	>200	0.1	3
2	6.25	>200	37.5	0.1	3
3	6.25	>200	>200	0.1	3
4	6.25	>200	>200	0.1	2.25
5	6.25	>200	>200	0.1	1.5
6	6.25	>200	>200	0.1	3
7	6.25	100	50	0.1	1.5
8	6.25	>200	>200	0.4	1.5
9	6.25	>200	>200	0.1	1.5
BW (*mcr-1*)	6.25	6.25	0.02	0.1	1.5

Supplemental Table 2. Characterization of spontaneous colistin and rifampicin combination suppressor mutants in *E. coli* BW25113 transformed with pGDP2*:mcr-1*.

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Chapter III – Outer membrane disruption overcomes intrinsic, acquired and spontaneous antibiotic resistance

Preface

The work presented in this chapter is in preparation for submission, as of June 3, 2020:

MacNair CR and Brown ED, Outer membrane disruption overcomes intrinsic, acquired and spontaneous antibiotic resistance*. In preparation.*

CRM performed all experiments, CRM and EDB wrote the manuscript.

Abstract

Disruption of the outer membrane (OM) barrier allows for the entry of otherwise inactive antimicrobials into Gram-negative pathogens. Numerous efforts to implement this approach have identified a large number of OM perturbants that sensitize Gram-negative bacteria to many clinically available Gram-positive active antibiotics. However, there is a dearth of investigation into the strengths and limitations of this therapeutic strategy, with an overwhelming focus on the characterization of individual potentiator molecules. Herein, we look to explore the utility of exploiting OM perturbation to sensitize Gram-negative pathogens to otherwise inactive antimicrobials. We identify the ability of OM disruption to change the rules of Gram-negative entry, overcome pre-existing and spontaneous resistance, and impact biofilm formation. Disruption of the OM expands the threshold of hydrophobicity compatible with Gram-negative activity to include hydrophobic molecules. We demonstrate that while resistance to Gram-positive active antibiotics is surprisingly common in Gram-negative pathogens, OM perturbation overcomes many antibiotic inactivation determinants. Further, we find that OM perturbation reduces the rate of spontaneous resistance to rifampicin and impairs biofilm formation. Together, these data suggest that OM disruption overcomes many of the traditional hurdles encountered during antibiotic treatment and is a high priority approach for further development.

Introduction

Increasing the arsenal of available antibiotics is paramount to addressing the growing resistance crisis[1,2]. Encouraging progress has been made in the treatment of Gram-positive pathogens[3], with three new antibiotic classes, cyclic lipopeptides (daptomycin)[4], oxazolidinones (linezolid)[5], and the pleuromutilins (retapamulin)[6] introduced within the last 20 years. Additionally, two novel Gram-positive active antibiotics are currently in clinical trials[3]. Unfortunately, antibiotic development for Gram-negative bacteria has remained stagnant. The last novel class of Gram-negative active antibiotics, the quinolones, were introduced into the clinic over 50 years ago[7] and none are currently in the development pipeline[3].

The failure to develop antibiotics with Gram-negative activity can largely be attributed to the inability of small molecules to accumulate within these bacteria[8]. All Gram-negative bacteria are protected from toxic stressors by an outer membrane (OM) that reduces compound influx into the cell[9]. The OM is an asymmetric bilayer composed of lipopolysaccharides (LPS) in the outer leaflet and phospholipids in the inner leaflet[10], and is found uniquely within Gram-negative bacteria. Tight packing of LPS and an overall negative charge act to exclude most large and hydrophobic molecules[11]. Permeability has mostly limited Gram-negative active antimicrobials to those capable of traversing through porins[12].

Although the intracellular target of many Gram-positive active antibiotics is present in Gram-negative pathogens, these molecules are unable to traverse the

OM. Antibiotic activity against Gram-negative bacteria is primarily restricted to compounds with high polarity and a molecular weight (MW) less than 600 Da[8]. Recent work has expanded these “rules” of Gram-negative entry identifying molecules that are rigid, flat and contain positive charge to be more compatible with passage through porins[13,14]. These concepts have been applied in medicinal chemistry efforts to alter Gram-positive active antibiotics for Gram-negative activity, by developing analogues that better adhere to the parameters of Gram-negative entry. This approach, while promising, is limited to scaffolds amenable to modification without losing affinity for their intracellular target and may have a detrimental impact on otherwise favourable pharmacological properties of these drugs. An alternative approach is the direct perturbation of the OM barrier, which facilitates the entry of many Gram-positive active antibiotics into Gram-negative pathogens[15]. Indeed, an approved OM perturbant used alongside clinically proven Gram-positive active antibiotics would immediately expand the arsenal of available treatments for Gram-negative pathogens[16].

The unique properties of LPS make the OM distinct from eukaryotic membranes and an exploitable bacterial target. OM perturbing peptides[17,18], small molecules[19] and chelators[20,21] disrupt the divalent cation bridges that stabilize LPS. Sensitizing Gram-negative bacteria to Gram-positive active antibiotics by OM perturbation has been reported for decades[15]. However, the recent rise of antibiotic resistance in Gram-negative pathogens has resulted in a resurgence of effort in this area. Indeed, a wealth of recent work shows OM

perturbants in combination with traditionally Gram-positive active antibiotics can successfully treat murine infection models of *Acinetobacter baumannii*[19,22], *Klebsiella pneumoniae*[23], and *Escherichia coli*[24]. In 2017 the first OM perturbant SPR741 completed Phase Ia and Ib clinical trials with a promising pharmacokinetic and tolerability profile[25].

Despite the growing number of promising OM perturbants, questions remain on the potential of this antibiotic strategy[26]. As a combination approach, growth-inhibition relies on the activity of both the OM disruptor and partner antibiotic, which may increase susceptibility to resistance development[27]. Additionally, a high abundance of resistant elements for Gram-positive active antibiotics are present in Gram-negative bacteria[28]. Therefore, while OM perturbation may allow entry of many Gram-positive active antibiotics into Gram-negative pathogens, growth-inhibition might be ineffective due to pre-existing resistance. In addition to horizontally acquired resistance, OM perturbation is likely to encounter many of the same challenges that plague Gram-negative antibiotic treatment, including spontaneous resistance development and biofilms.

Herein, we look to interrogate the potential of OM perturbation as an approach in antibiotic combination treatment. We first investigate how OM disruption changes the rules of Gram-negative entry, identifying a significant expansion to the threshold of hydrophobicity compatible with Gram-negative activity. We next uncover the ability for OM perturbation to render many antibiotic inactivation resistance elements ineffective, as well as decrease the development

of spontaneous resistance. Finally, we explore the ability of OM disruption to attenuate biofilm formation. Overall, we find that OM perturbation overcomes many of the perceived hurdles to its clinical implementation, warranting increased attention towards this highly rewarding approach.

Results

OM perturbation increases the range of hydrophobicity compatible with Gram-negative entry

Several diverse stressors are known to permeabilize the OM, including magnesium limitation[29], chelators[20], peptides[18], small organic compounds[19], and genetic perturbations[30]. We first sought to investigate if antibiotic sensitivity in *E. coli* varies with the type of OM perturbant used, focusing our efforts on five potentiators: the chelator (ethylenediaminetetraacetic acid (EDTA)), small molecule (pentamidine), peptides (colistin and SPR741) and deletion of the gene *waaC*. While structurally distinct, the perturbants EDTA, pentamidine, colistin, and SPR741 all increase OM permeability by disrupting the cation bridging between LPS molecules. Deleting *waaC* in *E. coli* compromises the OM by ablating the heptosyltransferase that adds the first heptose sugar onto the Kdo_2 moiety of LPS inner core, truncating LPS structure[31].

We screened a panel of 43 antibiotics to measure their degree of potentiation alongside these five OM perturbants. Compounds were considered potentiated if the minimum inhibitory concentration (MIC) was reduced >4-fold when compared to a no-treatment control (Figure 1a). SPR741 potentiated the

highest number of antibiotics, followed by EDTA, Δ*waaC*, colistin and pentamidine. Of the 43 antibiotics tested, 22 were potentiated by at least one type of OM perturbant. As previously reported, hydrophobic antibiotics were highly compatible with potentiation[15]. Nine hydrophobic, traditionally Gram-positive antibiotics (novobiocin, fusidic acid, mupirocin, clarithromycin, erythromycin, roxithromycin, clindamycin, rifampicin, and rifaximin) were potentiated by all five potentiators tested. We found that potentiation was often conserved between OM perturbants with potentiation in three or more conditions observed for 16 of 22 drugs, with some exceptions. For example, the MIC of vancomycin is reduced 32-fold by EDTA but ≤4-fold for all other probes. Additionally, we noted a complete absence of potentiation for 21 of 43 drugs, a subset that mostly comprised Gram-negative active antibiotics, such as the fluoroquinolones, tetracyclines aminoglycosides, and β-lactams. Notably, potentiation of β-lactams appears compound-specific as we observed moderate potentiation (<10-fold) in at least one OM perturbant with four of seven β-lactams tested. Taken together, these data indicate moderate variability in antibiotic potentiation with respect to the OM perturbant. However, we observe striking conservation in the potentiation of macrolides, rifamycins and other hydrophobic Gram-positive active antibiotics, irrespective of the source of OM disruption.

Next, we looked to investigate how OM perturbation may expand the thresholds of molecular weight and hydrophobicity compatible with Gram-negative activity, as entry through OM porins is typically restricted to small,

hydrophilic compounds with a MW less than 600 Da[12]. To this end, we screened a library of 3645 known bioactive compounds that included off-patent drugs, natural products, and other biologically active compounds in four conditions: *E. coli*, *E. coli* with SPR741, *E. coli* Δ*waaC* and methicillin-resistant *Staphylococcus aureus* (MRSA). SPR741 was selected from the four chemical probes because it potentiated the highest number of antibiotics (Figure 1a) and is currently the closest OM perturbant to clinical implementation. We anticipate that potentiation by SPR741 would highly correlate with other OM perturbants.

We calculated MW and lipophilicity (cLogD at pH 7.4) for all 3645 screening compounds (Figure 1b-d) and classified those that reduced normalized growth below 50% as growth-inhibitory (Supplemental Figure 1). From this, we found that OM perturbation increased the number of growth-inhibitory compounds from 85 in *E. coli* alone to 203 in *E. coli* with SPR741, 78 of which overlap between the two conditions (Figure 1e). Compounds with growth-inhibitory activity against *E. coli* alone largely adhered to the previously established rules of MW compatible with Gram-negative permeability with a mean MW of 406.6, and 92% of compounds less than 600 Da (Figure 1c). Comparatively, an analysis of the 203 inhibitors with growth-inhibitory activity against *E. coli* with SPR741 revealed a trend towards a larger MW (mean of 437.9, but not statistically different from 406.6), with 87% of inhibitors less than 600 Da (Figure 1c). Nevertheless, the addition of SPR741 significantly expanded the range of cLogD compatible with antimicrobial activity towards more hydrophilic compounds

(Figure 1d). The average cLogD of compounds inhibiting *E. coli* growth was -1.34 compared to 1.57 in the presence of SPR741, an approximately 800-fold increase. Indeed, of the 125 compounds with growth-inhibitory activity dependent upon the presence of SPR741, 87% are considered hydrophobic (cLogD >0).

The use of a Δ*waaC* strain of *E. coli* phenocopied the expansion of growth-inhibitory compounds in the presence of SPR741. Growth-inhibition against Δ*waaC* was observed in 108 compounds, which show a non-significant increase in average MW but a significant increase in lipophilicity, compared to those compounds active against *E. coli* (Supplemental Figure 2).

Finally, we looked to compare if OM perturbation recapitulated the range of physicochemical properties compatible with activity against the Gram-positive pathogen MRSA. Growth inhibition was observed in 177 compounds, of which 122 overlap with those active in the presence of *E. coli* with SPR741 (Supplemental Figure 3). Compounds inhibitory to *S. aureus* had an average MW of 479.6 and cLogD of 1.32. There was no significant difference in MW or hydrophobicity of actives when comparing MRSA and *E. coli* in the presence of SPR741 or the deletion of Δ*waaC* (Supplemental Figure 3,4). Together, these results indicate the ability for OM disruption to increase the range of hydrophobicity compatible with growth-inhibition, similar to that observed for Gram-positive bacteria.

OM disruption overcomes antibiotic inactivation

Perturbation of the OM sensitizes Gram-negative bacteria to a wide range of Gram-positive active antibiotics. Previous work has focused on a limited number of antibiotic classes compatible with OM perturbation – primarily rifamycins[22], aminocoumarins[19] and macrolides[23]. Antibiotics in these classes are highly potentiated by all OM disrupting probes (Figure 1a) and are efficacious alongside OM perturbants in murine models of infection[19,22,23]. Notably, aminocoumarin antibiotics are not currently available for clinical use, making macrolide and rifamycins the most readily available partners for a clinically approved OM perturbant. Given this, we aimed to investigate how resistance to macrolide and rifamycin antibiotics impacts potentiation by OM disruption.

We first transformed individual plasmids constitutively expressing the macrolide resistance elements *mphA* and *ermC* into *E. coli,* then determined the MIC of these strains to erythromycin in the presence and absence of SPR741. The perturbation of a control strain (*E. coli* transformed with an empty vector) by SPR741 reduces the MIC for erythromycin 64-fold from 25 μg/mL to 0.39 μg/mL (Figure 2a). Introduction of the macrolide resistance phosphatase MphA increases the MIC of erythromycin to 200 μg/mL (Figure 2b). In this strain, OM perturbation by SPR741 reduced the MIC of erythromycin to 3.125 μg/mL (Figure 2b), maintaining the same level of reduction (64-fold) observed in the empty vector control. Conversely, expression of *ermC*, a 23S ribosomal RNA methylation enzyme[32,33] increases the MIC of erythromycin in *E. coli* to above 200 μg/mL, irrespective of the addition of SPR741 (Figure 2c).

We next extended this analysis to several additional macrolide (*mphB, ereA*) and rifampicin (*arr, rph-Lm, rpoB*) resistance elements. Using the same constitutive expression plasmid system, we determined the MIC of these strains to erythromycin, clarithromycin or rifampicin in the presence and absence of SPR741. Here, we found that *E. coli* harbouring the macrolide-inactivating enzymes MphB, a phosphatase, and EreA, an esterase, are susceptible to erythromycin potentiation by SPR741 with an average reduction in MIC of 48 and 24-fold, respectively (Figure 2d). Similar results were observed for the potentiation of clarithromycin by SPR741 against *E. coli* expressing *mphA* and *mphB*, with a reduction in the MIC of clarithromycin similar to that observed in the empty vector control strain (Figure 2d). Expression of the target-modifying resistance gene *ermC* limited potentiation of clarithromycin by SPR741, consistent with results observed for erythromycin.

Rifampicin is highly potentiated by SPR741, such that its MIC in a control *E. coli* strain (containing empty vector) is reduced 1024-fold from 6.25 μg/mL to 0.006 μg/mL (Figure 2d). We observed that *E. coli* strains harbouring the rifampicin inactivation enzymes Arr or Rph-Lm are significantly less susceptible to rifampicin (MIC 400 μg/mL) but are sensitized in the presence of SPR741 (*arr,* 128-fold reduction in MIC; *rph-Lm,* 256-fold reduction in MIC) (Figure 2d). Conversely, the introduction of a mutation in *rpoB,* which reduces the binding of rifampicin to its target, increases the MIC of rifampicin to 400 μg/mL and is mostly unaffected by SPR741 (4-fold reduction in MIC).

Lastly, we queried whether OM perturbation alters the efficacy of resistance elements to Gram-negative active antibiotics not highly potentiated by OM disruption. We speculated OM perturbation might impact the function or activity of resistance enzymes beyond increasing antibiotic influx. Ten additional resistance elements were tested, covering a wide range of antibiotic classes (Supplemental Figure 5). We observed no significant reduction of MIC in strains harbouring resistance with perturbation by SPR741, suggesting that these resistance elements continue to operate irrespective of OM disruption. We predict that the use of OM perturbants may overcome resistance elements but only for antibiotics where compound accumulation is limiting. Additionally, the mechanism of antibiotic resistance is vital in determining if OM perturbation will be efficacious as we observe susceptibility in strains expressing antibiotic inactivation but not target modification.

OM perturbation is efficacious against clinical *E. coli* isolates

OM perturbation reduces the MIC of potentiated antibiotics in a lab strain of *E. coli* harbouring various antibiotic inactivation resistance elements. We looked to investigate this phenotype using a collection of 120 *E. coli* isolates from a diverse range of tissues (blood, urine, rectal and sputum) collected from patients in Hamilton, Canada. We examined the impact of OM perturbation by SPR741 on the MIC of rifampicin, clarithromycin, and novobiocin. Each isolate was sequenced and analyzed for genes conferring resistance to rifamycin, aminocoumarin and macrolide antibiotics (Figure 3a) using the Resistance Gene

Identifier (RGI) software of the Comprehensive Antibiotic Resistance Database (CARD), which predicts the presence of resistance genes based on homology and SNP models[28]. This analysis indicated three mechanistic subtypes of resistance elements across isolates: efflux, antibiotic inactivation, and target modification. When classified with respect to antibiotic class, we found that RGI predicted solely non-specific efflux pumps to be putatively linked to rifamycin and aminocoumarin resistance. In contrast, inactivation and target modification resistance elements appeared to be macrolide-specific (Figure 3a).

To determine if OM perturbation could sensitize these strains to concentrations of the partner antibiotic theoretically obtainable during standard antibiotic treatment, we looked to assign a cut-off value similar to a traditional clinical breakpoint. Clinical breakpoint is conventionally defined as the concentration of antibiotic that defines a species of bacteria as susceptible or resistant. Breakpoint values for Gram-negative pathogens are not available for the traditionally Gram-positive active antibiotics used alongside OM perturbants. Therefore, we assigned a value deemed “potentiation breakpoint” to our antibiotic partners using the CLSI breakpoint value for the treatment of all *Staphylococcus* species. The selected potentiation breakpoint for rifampicin and clarithromycin is 1 μg/mL, and 2 μg/mL respectively. With the removal of novobiocin from the market in 2011, there is no currently listed clinical breakpoint. However, we considered a concentration of novobiocin as below potentiation breakpoint when the MIC is less than steady-state serum levels (5 μg/mL)[34].

We first determined the MIC_{50} and MIC_{90} values for rifampicin, clarithromycin, and novobiocin in all 120 *E. coli* clinical isolates (Table 1). Without OM perturbation, MICs are above the potentiation breakpoint in all strains, while the addition of SPR741 reduced MIC_{90} values to below our potentiation breakpoint for rifampicin, clarithromycin and novobiocin. The average reduction in MIC by the addition of SPR741 for rifampicin, novobiocin and clarithromycin was 561, 162, and 551, respectively (Figure 3b). Potentiation below our selected breakpoint was observed for 118 of 120 strains in both rifampicin and novobiocin. Notably, the two resistant strains, C0004 and C0244, resisted potentiation by SPR741 for both novobiocin and rifampicin. Upon further investigation, these two strains were found to be resistant to OM perturbation by SPR741. The strain C0244 was highly resistant to polymyxin B, which is known to confer cross-resistance to the OM-disruption by polymyxin derivatives similar to SPR741[19]. However, C0004 was sensitive to polymyxin B, and the mechanism behind the observed resistance to potentiation by SPR741 is currently unknown. Outside of strains resistant to OM perturbation, the MICs of novobiocin and rifampicin were reduced to clinically obtainable levels in all remaining isolates. Altogether we would predict 118 of 120 strains to be susceptible to treatment by an OM perturbant combined with rifampicin or novobiocin, making these antibiotics highly attractive partners.

Given the large quantity of macrolide-specific resistant elements within our *E. coli* isolates, we aimed to examine their impact on the potentiation of

clarithromycin in depth. Forty-eight strains were predicted to harbour at least one of the following macrolide resistance genes: *mphA, mphE, msrE* or *ermB.* We divided strains into two categories based on the presence or absence of macrolide specific resistance elements. Strains harbouring macrolide resistance (*mphA, mphE, msrE* or *ermB*) were deemed “resistant” and all other strains “sensitive” (Figure 4). We then monitored growth in the presence of clarithromycin with and without SPR741, finding a significant difference in MIC of “resistant” compared to “sensitive” isolates in both conditions (Figure 4a). However, we observed no significant change in the range of fold reduction in MIC when comparing “resistant” and “sensitive” isolates (Figure 4b). Importantly, SPR741 reduced the clarithromycin MIC to below potentiation breakpoint in 113 of 120 clinical isolates (Figure 3b, Figure 4a).

We took particular interest in the seven strains where we were unable to reach the potentiation breakpoint of clarithromycin. Two strains in this group, C0004 and C0244, were not predicted to be macrolide-resistant. However, we previously identified these strains as having reduced susceptibility to OM disruption by SPR741. Three of the five remaining strains were predicted to contain *ermB*, a 23S ribosomal RNA methyltransferase similar to *ermC,* and were not potentiated below breakpoint (Figure 4b): C0012, C0013 and C0452. Strains, C0012 and C0452, contained both *mphA* and *ermB,* which may also contribute to the observed high level of resistance. Two strains, C0240 and C0008, were predicted to harbour *mphA* but no other macrolide specific resistance elements.

Despite the high frequency of predicted broad-spectrum and macrolide specific resistance present in Gram-negative pathogens, the degree of potentiation is mostly unaffected (Figure 4b), and the majority of MICs are reduced below the potentiation breakpoint (Table 1, Figure 3b).

The three mechanistic subtypes of resistance proved to each uniquely influence potentiation by OM perturbation. Broad-spectrum efflux pumps did not provide a barrier to potentiation below breakpoint for rifampicin, novobiocin or clarithromycin. Macrolide inactivation by phosphatases was common within our isolates, predicted in forty-seven strains. Inactivation by *mphA* or *mphE* proved mostly surmountable by OM perturbation, with 87% of harbouring strains potentiated below breakpoint. Resistance by *ermB* proved challenging, with 60% of strains (3 of 5) remaining above potentiation breakpoint. Notably, *msrE*, which protects the ribosome from inhibition by physically removing macrolides from their binding site[35,36] was overcome in the one strain harbouring this resistance (Figure 4a). Overall, these results are in concordance with the constitutively expressed resistance elements in a wild-type strain of *E. coli* (Figure 2d), where antibiotic inactivation proved largely surmountable to OM perturbation, and target modification was difficult to overcome.

We note here that the clinical strains used in this study do not cover a diverse geographic range, and regional differences in resistance prevalence may be encountered. For a more global perspective, we looked at the occurrence of macrolide resistance genes *mphA* and *ermB* in *E. coli* using 15757 whole-

genome sequence assemblies available from NCBI using CARD RGI software[28]. *E. coli* is predicted to harbour m*phA* and *ermB* in 12.95% and 1.59% of strains, respectively. The relatively low incidence of *ermB* is encouraging, and we anticipate the combination of an OM perturbant and clarithromycin to be highly efficacious irrespective of geographic location.

OM perturbation reduces spontaneous resistance and biofilm formation

The combination of an OM perturbant and an otherwise inactive antibiotic partner requires the efficacy of both components to inhibit bacterial growth[23]. As such, resistance may develop more rapidly than traditional monotherapy approaches[26,27]. Rifampicin was highly efficacious against our clinical *E. coli* strains (Table 1), providing significant therapeutic potential as a partner antibiotic. However, spontaneous resistance to rifampicin develops rapidly by mutations in *rpoB,* which reduce rifampicin binding to the ribosome[37]. Indeed, as previously reported[23], this target modifying resistance was not overcome by OM perturbation (Figure 2d). We determined the frequency of resistance (FOR) for rifampicin in the presence of OM perturbation by SPR741 and the deletion of *waaC.* After 24 h of incubation, *E. coli* displayed a resistance frequency of 2.13×10^{-9} (Figure 5a). The addition of SPR741 significantly reduced the FOR to 3.42×10^{-10} compared to the *E. coli* control. Conversely, genetic perturbation (Δ*waaC*) did not significantly reduce the FOR with a mean FOR of 9.38×10^{-10}. After 48 hours of incubation, the FOR of *E. coli* increased 84-fold to 1.77×10^{-7}. Comparatively, we observed only a ~9-fold increase after 48 h with both SPR741 and Δ*waaC*,

increasing FOR to 2.97.0 x 10^{-9} and 8.09 x $10^{-9,}$ respectively. We speculate resistance at 24 h might represent the pre-existing resistance in the population, while resistance after 48 h requires colonies to adapt to the antibiotic stress and develop resistance. Alternatively, this phenotype may simply be concentration-dependent as we did not normalize rifampicin concentrations to the MIC of each condition.

We next examined the development of resistance by serial passaging, which controls for concentration-dependence. Bacteria in three conditions, *E. coli, E. coli* with SPR741, and *E. coli* Δ*waaC,* were passaged for 21 days in rifampicin. Bacterial culture for each sequential passage was selected from the ¼ MIC concentration of the previous passage. In the control condition, *E. coli* rapidly gained resistance to rifampicin, with a 64-fold increase in MIC observed in just 12 passages to 800 μg/mL (Figure 5b). However, passages in the presence of OM perturbation by SPR741 or Δ*waaC* showed only a four-fold and a two-fold increase in MIC, respectively, after 21 passages. The MIC of rifampicin remained below the potentiation breakpoint throughout the entire experiment. Overall, perturbation of the OM reduces spontaneous resistance development to rifampicin.

Biofilm formation poses a significant challenge in the treatment of bacterial infections. Gram-negative bacteria form biofilms composed predominantly of an extracellular polymeric substance (EPS) that contains anionic charge allowing association with divalent cations to provide stability[38]. Noting the parallels

between this interaction and the cation bridging of LPS, we speculated that OM disruption might impede biofilm formation. To address this, we assessed biofilm formation in *E. coli* alongside ¼ MIC of the five OM perturbants EDTA, SPR741, colistin, pentamidine, and genetic deletion of *waaC.* The ablation of biofilm formation with EDTA[39,40] and in *E. coli* Δ*waaC*[41] is consistent with previous reports. We did not identify a conserved impact on biofilm formation across OM perturbants; while both colistin and pentamidine trended towards an increase in biofilm formation, a significant reduction was observed for SPR741, EDTA, and Δ*waaC* (Figure 5c). Despite the specificity of biofilm formation to individual perturbants, these results indicate that OM disruption by specific agents can reduce biofilm formation.

Discussion

Antibiotic development has failed to keep pace with the rapid dissemination of resistance. The impermeability of Gram-negative pathogens presents a unique challenge to discovery efforts. Disruption of the OM barrier through chemical or genetic perturbation can increase the susceptibility of Gram-negative bacteria to many traditionally Gram-positive active antibiotics. Several groups have published proof of principle studies for this approach using peptide[26], small molecule[19], chelator[20], and genetic perturbants[30]. Despite a resurgence of effort in this area, previous work overwhelmingly focuses on the characterization of individual potentiator molecules, and the field lacks a thorough investigation of the strengths and limitations of this approach.

To better understand the changes in permeability conferred by OM perturbation, we screened a diverse library of bioactive molecules for potentiation with SPR741 and the deletion of *waaC*. In concordance with other reports[15,18,19], we found OM disruption to significantly increase *E. coli* susceptibility to hydrophobic compounds. In the presence of OM perturbation, hydrophobicity compatible with antimicrobial activity better correlated with MRSA than untreated *E. coli*. Expanding the range of physicochemical properties amenable to Gram-negative entry through OM perturbation has remarkable implications for antibiotic development. Indeed, accounts of biochemical screening and hit optimization efforts typical of modern target-based antibacterial drug discovery show that these efforts often produce potent inhibitors. However, the resulting compounds are invariably too hydrophobic and incompatible with Gram-negative entry[42]. Our results suggest that a clinically viable OM disruptor would allow not only the immediate use of many Gram-positive active antibiotics but could also bring new life to previously abandoned drug leads.

The combination of an OM perturbant and Gram-positive active antibiotic requires the activity of both components to inhibit growth in Gram-negative bacteria. As such, resistance elements to antibiotics commonly partnered with OM perturbants have the potential to reduce combination efficacy. We discovered that the expression of antibiotic inactivation enzymes had minimal impact on the potentiation of erythromycin, clarithromycin and rifampicin. However, resistance by target modification rendered potentiation by OM disruption largely ineffective.

We speculate that inactivation enzymes may have a limited turnover rate that is being overwhelmed by increased antibiotic influx. However, target modifications, such as *rpoB,* can reduce the affinity of an antibiotic for its target by >1000-fold[43] rendering increased influx ineffective at overcoming resistance.

Antibiotic inactivation and target modification resistance elements for Gram-positive active antibiotics are frequently harboured within Gram-negative pathogens. Looking to determine how this may impede the therapeutic potential of an OM perturbant, we investigated 120 clinical *E. coli* isolates for SPR741 potentiation of rifampicin, novobiocin and clarithromycin. Genome analysis predicted non-specific resistance by efflux machinery in all strains and no rifampicin or novobiocin specific resistance elements. MIC_{90} values for rifampicin and novobiocin were below the potentiation breakpoint in the presence of SPR741. Macrolide specific resistance was predicted in 40% of the *E. coli* strains. Despite this, 113 of 120 strains were brought below the potentiation breakpoint, and typically, the presence of macrolide inactivation genes *mphA* or *mphE* did not render OM perturbation ineffective. Although target modification by *ermB* proved challenging to overcome, this resistance gene is predicted in less than 2% of global *E. coli* isolates, so we anticipate the use of clarithromycin alongside a potent OM perturbant to be efficacious against the overwhelming majority of *E. coli* strains.

Spontaneous resistance often reduces activity by modifying the antibiotic target, which, as we have shown, can be difficult to overcome with OM

perturbation. Additionally, bacteria have the opportunity to develop resistance to the OM disrupting activity of the perturbant, potentially increasing the frequency of resistance. Therefore, we looked to determine if OM perturbation alters the rate of spontaneous resistance. Rifampicin is particularly susceptible to spontaneous resistance, seemingly limiting its clinical potential for use alongside an OM perturbant. Direct plating of bacteria onto rifampicin with OM perturbation showed a reduced FOR that became more prominent over time. Serial passage experiments also showed a reduction in resistance development. The presence of SPR741 or the deletion of *waaC* was sufficient to maintain the MIC of rifampicin below potentiation breakpoint for all 21 passages tested. We speculate OM perturbation does not reduce pre-existing resistance within a population but may impair the bacteria's ability to adapt to antibiotic stress. Increased influx may reduce the time available to induce the SOS response, which is known to be essential for the development of rifampicin resistance[44].

Development of biofilms can severely impede antibiotic treatment for otherwise susceptible bacterial infections. Biofilms formed by Gram-negative pathogens are predominantly composed of EPS, which has many structural similarities to LPS. Therefore, we looked to determine if chemical and genetic perturbations of the OM also impact biofilm formation. We observed variability in this phenotype across OM perturbants – EDTA, SPR741 and the genetic deletion of *waaC* reduced biofilm formation while pentamidine and colistin increased biofilm formation. We reason that this increase may be attributed to the known

ability for sub-inhibitory concentrations of antibiotics to stimulate biofilm formation[45,46]. That some OM perturbants impair biofilm formation is encouraging, particularly for guidelines that may be implemented to prioritize OM perturbants for further development.

In this work, we focused primarily on the impact of OM perturbation in *E. coli.* Previous efforts have indicated that the potentiation activity of OM perturbants is often conserved within *Enterobacteriaceae* species and *A. baumannii*[16]. As such, we anticipate our results may be relevant to these pathogens also. Nevertheless, *Pseudomonas aeruginosa* is uniquely resistant to many OM perturbants, so we suggest further work may be in order to identify and investigate OM perturbants in this pathogen. We also note that our studies were conducted using high concentrations of each OM perturbant to ensure potent OM disruption. As it may be difficult to reliably reach comparable concentrations in patients using currently available OM perturbants, identifying more potent, non-toxic OM disruptors will surely be important to the therapeutic potential of this approach.

Targeting the OM is a unique and potentially revolutionary strategy for antibiotic discovery. OM perturbation sensitizes Gram-negative pathogens to a range of clinically approved Gram-positive active antibiotics and expands the chemical space compatible with novel antibiotic discovery efforts. Horizontally acquired resistance genes, spontaneous resistance development and biofilms are all significant hurdles to successful antibiotic treatment. In this work, we

uncover the capacity for OM disruption to overcome many of these challenges, uniquely positioning this approach amongst discovery efforts in the Gram-negative resistance crisis.

Methods

Reagents

SPR741 was provided by Spero Therapeutics. All other chemicals were purchased from Sigma-Aldrich. Compounds were routinely dissolved in DMSO or H_2O and stored at -20°C.

Bacterial strains and culture conditions

E. coli strain K-12 BW25113 or *E. coli* K-12 BW25113 (Δ*waaC*) were used for all experiments except those using the clinical isolate collection. The *S. aureus* USA300 JE2 strain was also used in compound screening. *E. coli* K-12 BW25113 was transformed with plasmids containing constitutively expressed resistant elements[47] (Supplemental Table 1). Clinical isolates of *E. coli* were collected from patients at Hamilton Health Sciences (HHS) hospital (Hamilton, Canada). Bacterial growth was in cation-adjusted MHB at 37°C unless stated otherwise. Resistance gene prediction was conducted using CARD, RGI software using paradigm “Strict”[28].

Potentiation assays

All MICs were conducted in at least two biological replicates following CLSI protocol[48]. Fold reduction of MIC was determined by dividing the MIC of the

antibiotic alone by its MIC in the treatment condition. OM probes in Figure 1a were used at the following concentrations: EDTA (2 mM), colistin (0.05 μg/mL), pentamidine (75 μg/mL), and SPR741 (6.25 μg/mL). SPR741 was used at 6.25 μg/mL for all assays using laboratory *E. coli*. Potentiation assays for clinical *E. coli* isolates were conducted at ¼ MIC SPR741.

High-throughput compound screening

All chemical screening was performed at the Centre for Microbial Chemical Biology (McMaster University). Overnight cultures of *E. coli*, *E. coli* ΔwaaC, and *S. aureus* were brought to an OD600 of 0.1, diluted 1/200 into MHB for each condition tested and dispensed into 384-well plates to a final volume of 30 μL per well. 60 nL of each compound (5 mM stocks) was added for a final concentration of 10 μM per well. OD600 was read immediately after compound addition and again after 18-20 h. Data were normalized by interquartile-mean based methods[49] and compounds reducing growth >50% were considered actives. Screening was performed in duplicate.

Physicochemical property calculations

Structure analysis was conducted using MarvinSuite 20.9.0, ChemAxon (http://www.chemaxon.com). Initial structure preparation was performed using the Standardizer tool to strip salts/solutes and verified with StructureChecker. Molecular weight and logD at pH 7.4 (cLogD) were then calculated using cxcalc.

Biofilm formation assays

Biofilm formation was determined in polystyrene 96-well plates as previously described[41] with minor changes. Briefly, bacteria were inoculated 1/500 from an overnight culture and plates prepared as in a standard MIC assay. After 48 h of incubation at 30°C, growth was measured by absorbance at OD_{600}. Plates were then washed, dried at 37°C for 30 min, and crystal violet added to the plates. After 30 min of incubation at room temperature, excess crystal violet was washed away, and the residual solubilized with 30% acetic acid. Crystal violet was quantified by measuring $OD_{570,}$ and relative biofilm formed calculated by Crystal Violet (OD_{570})/ Growth(OD_{600}). Concentrations of potentiators used are the same as in Figure 1a.

Resistant mutant development

To conduct frequency of resistance (FOR) plating assays, an overnight culture of *E. coli* was diluted 1/500 into MHB and grown to mid-log (2-3 h), concentrated in PBS and 200 µL of cells transferred onto solid MHB in 100 mm Petri dishes supplemented with rifampicin (100 µg/mL) or rifampicin (100 µg/mL) and SPR741 (6.25 µg/mL). *E. coli* Δ*waaC* was plated only on rifampicin (100 µg/mL). Plating was also conducted on an SPR741 (6.25 µg/mL) control resulting in a lawn of bacteria after 24 h of incubation. Approximately 2 x 10^{10} CFU were deposited on each plate as determined by serial plating on non-selective MHB. Plates were incubated at 37°C, and resistant colonies counted 24 h and 48 h post-incubation. The frequency of resistance was calculated by dividing the number of resistant colonies by total CFUs plated. A subset of approximately ten

colonies per plate was selected and re-streaked onto rifampicin or rifampicin with SPR741 to reconfirm resistance. All assays were conducted in biological duplicate, each composed of at least two technical replicates.

For passaging experiments, MICs of rifampicin were performed daily in three conditions, *E. coli* control, *E. coli* and SPR741 (6.25 µg/mL) or *E. coli* Δ*waaC*. MIC assays were performed as outlined above with the following modification: a 1/1000 dilution of bacteria from the ¼ MIC concentration of the previous day's passage was used to inoculate the subsequent passage. This process was continued for 21 passages in biological duplicate.

Acknowledgements

We thank Spero Therapeutics for providing SPR741. Kristina Klobucar for providing the *E. coli* Δ*waaC* strain. Shawn French for assistance in physicochemical property calculations. Dr. Andrew McArthur and Brian Alcock for assistance with RGI and CARD. Caressa Tsai for valuable discussions and manuscript review. We also thank Dr. Gerry Wright for providing the *E. coli* clinical isolate collection and resistance plasmids. This work was supported by a salary award to E.D.B from the Canada Research Chairs program and operating funds to E.D.B from the Ontario Research Fund (ORF-RE09-047). C.R.M. was supported by a Canadian Institutes for Health Research scholarship.

Author contributions

C.R.M. and E.D.B. conceived and designed the research. C.R.M. performed all experiments and analyzed data. C.R.M. and E.D.B. wrote the manuscript.

Figures and Legends

Figure 1

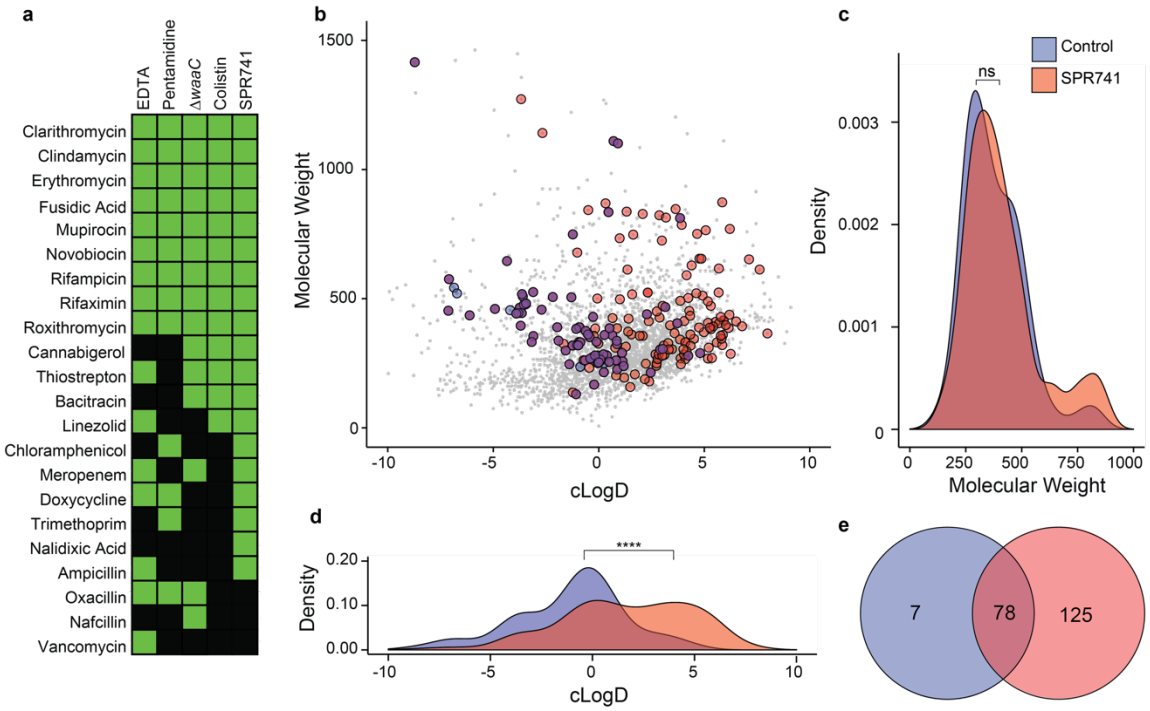


Figure 1. Identifying changes in permeability by outer membrane perturbation. (a) Heat map showing antimicrobials potentiated (reduction in MIC >4-fold, green) or unaffected (reduction in MIC ≤ 4-fold, black) by five OM perturbing conditions. (b) Physicochemical space of 3645 compounds screened for bacterial growth inhibition, visualized by molecular weight and calculated logD (cLogD) at pH 7.4. Compounds are coloured by growth-inhibitory activity, *E. coli* control (blue), *E. coli* with SPR741 (red), no activity in either condition (grey) and activity in both conditions (purple). (c,d) Density plots of molecular weight and cLogD for growth-inhibitory compounds in the *E. coli* control (blue) and SPR741 condition (red). SPR741 significantly alters the hydrophobicity (d) of compounds compatible with growth-inhibition ($p<0.0001$, Kolmogorov-Smirnov test) but not molecular weight (c) ($p>0.05$, Kolmogorov-Smirnov test). (e) Venn diagram showing the number and overlap of compounds with growth-inhibitory activity in the *E. coli* control (blue) and SPR741 condition (red).

Figure 2

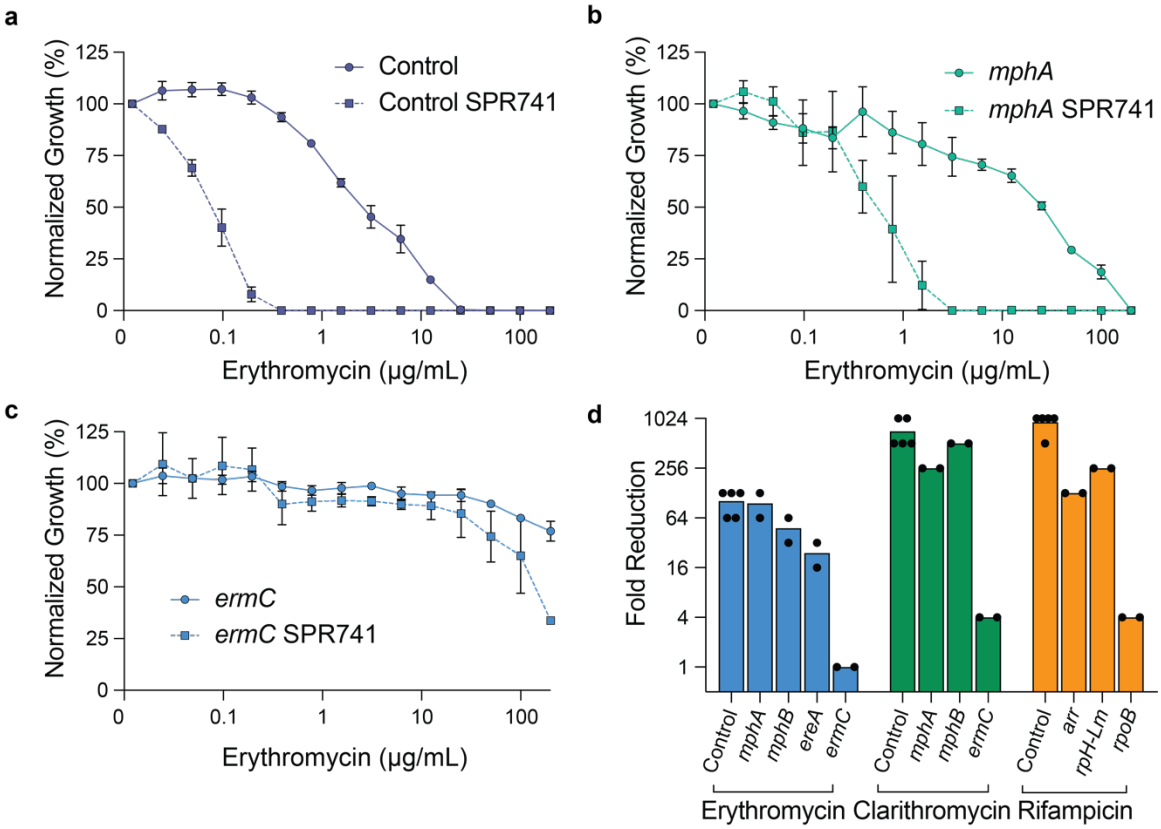


Figure 2. Perturbation of the outer membrane overcomes resistance by antibiotic inactivation. (a,b,c) Potency analysis of erythromycin in *E. coli* harbouring (a) plasmid control, (b), *mphA* or (c) *ermC* in the presence and absence of SPR741. Data shown represent the mean of at least two biological replicates with SEM. (d) Fold-reduction of MIC by SPR741 for erythromycin, clarithromycin and rifampicin in the presence of various resistance elements. Fold-reduction is calculated by dividing the MIC of an antibiotic alone by its MIC in the presence of an OM perturbant.

Figure 3

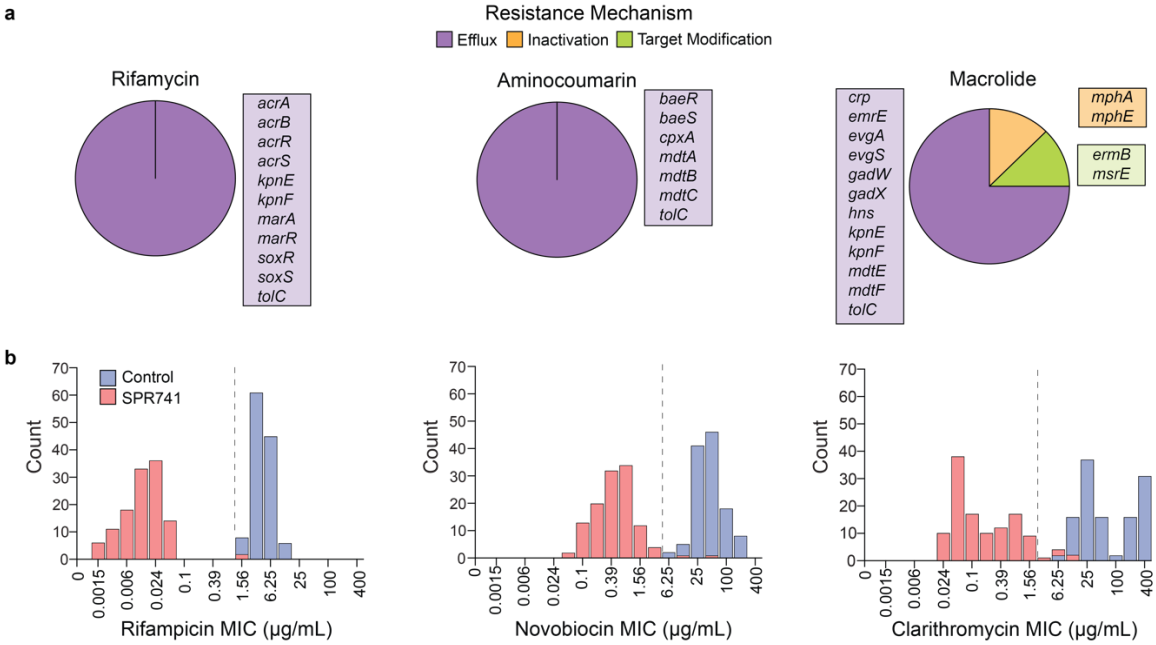


Figure 3. Gram-positive antibiotics are potentiated to therapeutic levels in clinical *E. coli* isolates by OM perturbation. (a) Resistance genes for rifamycin, aminocoumarin and macrolide antibiotics predicted in 120 clinical *E. coli* isolates. Genes are sorted by mechanism into antibiotic efflux (purple), inactivation (orange) and target modification (green). Pie charts represent the total number of unique resistance genes predicted in the strains separated by their corresponding resistance mechanisms. (b) Histograms showing the distribution of rifampicin, novobiocin, and clarithromycin MIC in the presence and absence of SPR741. Dotted lines mark the potentiation breakpoint concentration for each antibiotic.

Figure 4

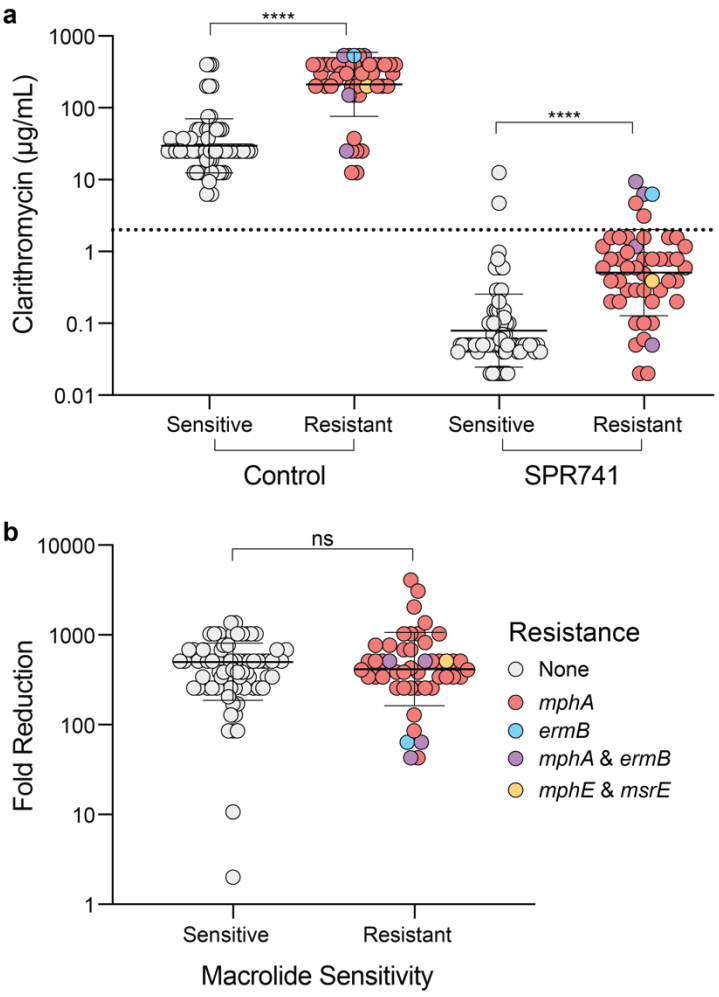


Figure 4. OM perturbation overcomes horizontally acquired macrolide resistance. (a,b) *E. coli* strains were divided into two groups. Strains predicted to contain macrolide specific resistance elements (Resistant, red, blue, purple, orange) and no macrolide specific resistance (Sensitive, grey). Resistant strains are sub-divided by colour into their predicted resistance genes. MIC values of clarithromycin (a) were significantly increased ($P<0.0001$, Mann-Whitney U-test) in strains predicted to harbour macrolide resistance (Resistant) in the presence and absence of SPR741. The dotted line marks the potentiation breakpoint value of 2 µg/mL. (b) Fold-reduction of clarithromycin MIC was not significantly different between the sensitive and resistant strains ($P>0.05$, Mann-Whitney U-test).

Figure 5

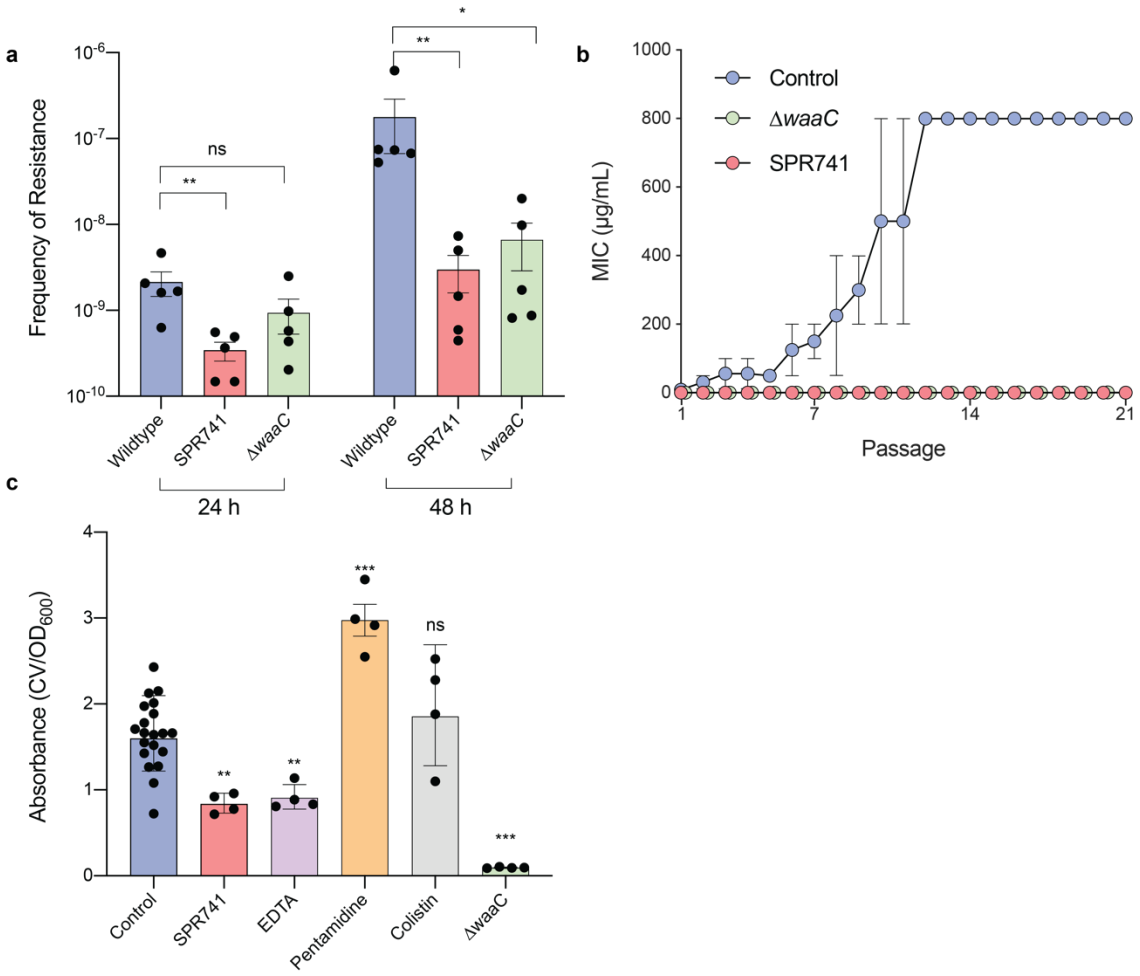


Figure 5. Disruption of the OM reduces spontaneous resistance to rifampicin and attenuates biofilm formation. (a) Frequency of resistance to rifampicin after 24 h and 48 h for *E. coli*, *E. coli* and SPR741, and *E. coli* Δ*waaC.* (b) Rifampicin MIC during serial passage of *E. coli* (blue), *E. coli* Δ*waaC* (green), and *E. coli* with SPR741 (red). (c) Crystal violet (CV) biofilm assay. Absorbance is calculated by Crystal Violet (OD_{570})/ Growth(OD_{600}). All data is shown with SEM and representative of at least two biological replicates. (c) Conditions are compared to the untreated control and significance calculated using the Mann-Whitney U-test (ns=P ≥0.05, *=P<0.05, **= P<0.01, ***=P<0.001).

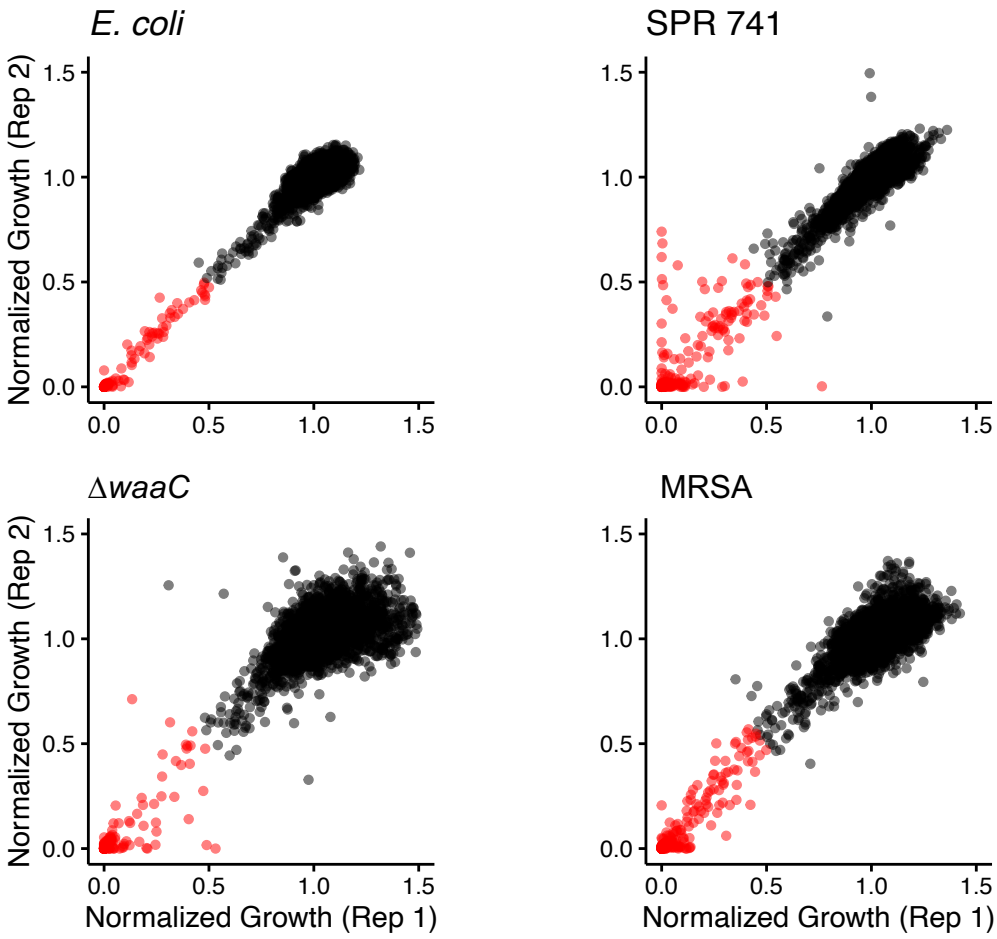
Tables

Table 1. MIC_{50} and MIC_{90} values for rifampicin, clarithromycin, and novobiocin in the presence and absence of SPR741 against *E. coli* (n=120).

	E. coli		***E. coli* + SPR741**	
	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)
Rifampicin	3.125	6.25	0.01	0.04
Clarithromycin	50	400	0.1	1.56
Novobiocin	37.5	100	0.39	1.56

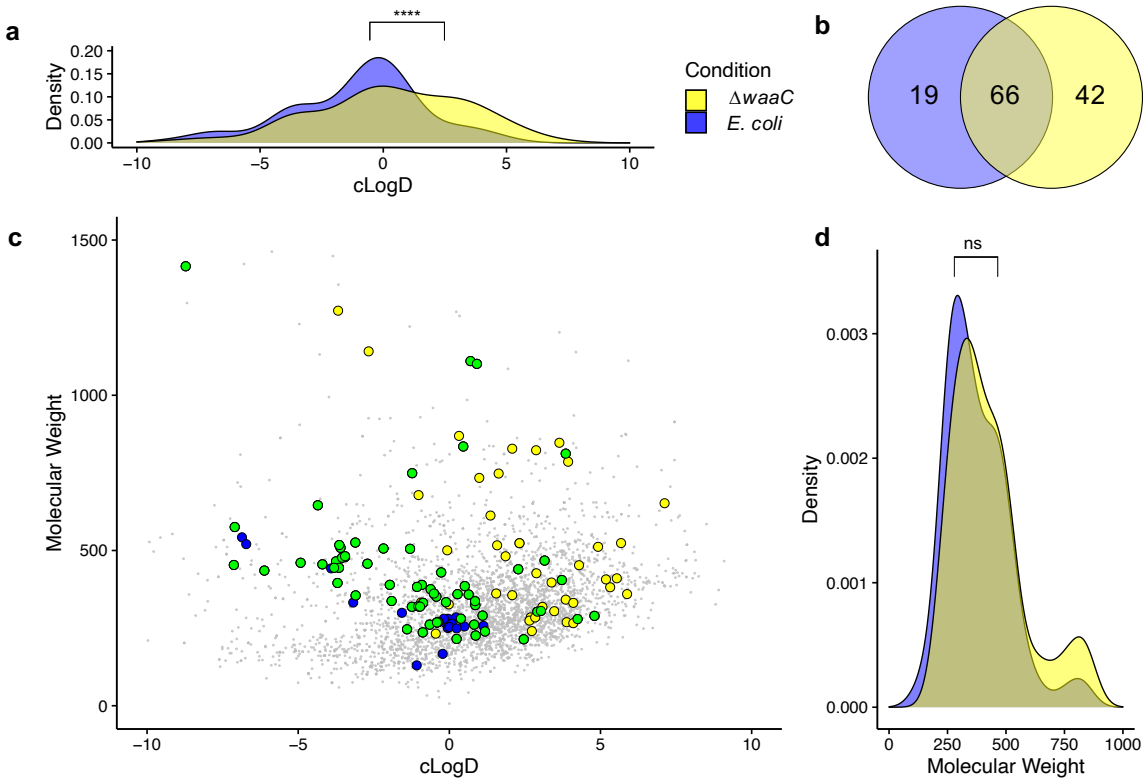
Supplemental Figures

Supplemental Figure 1



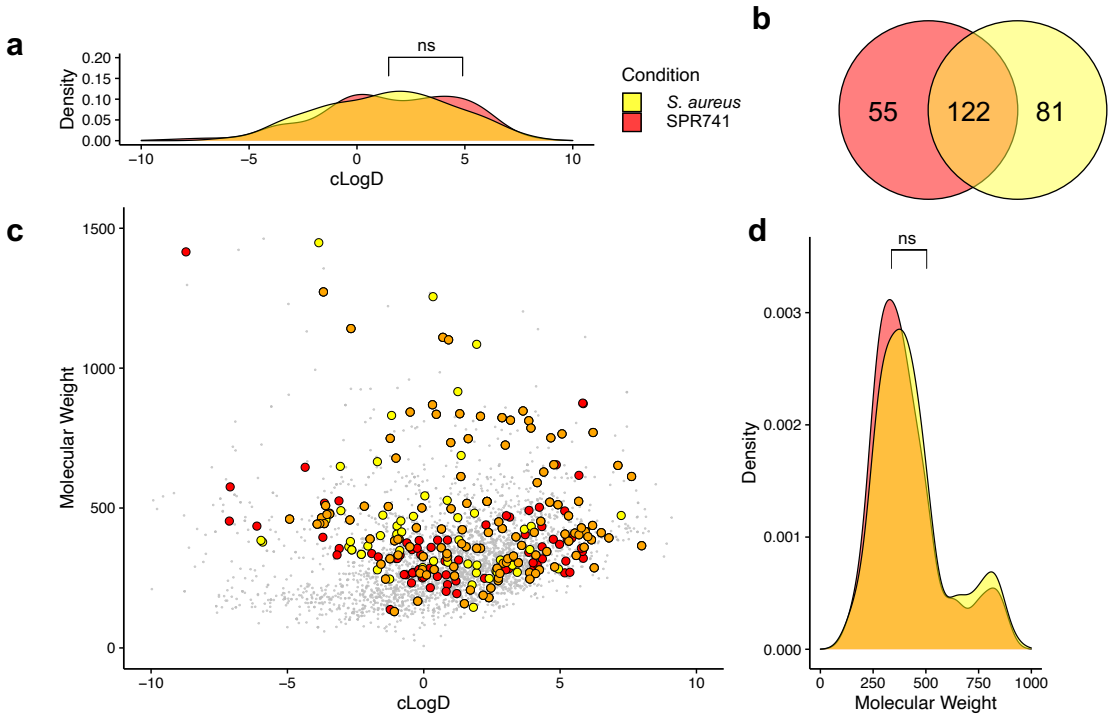
Supplemental Figure 1. Replicate plots of normalized growth for the primary screen of 3645 compounds in four conditions: *E. coli*, *E. coli* with SPR741, *E. coli* Δ*waaC* and methicillin-resistant *Staphylococcus aureus* (MRSA). Compounds inhibiting growth below 0.5 normalized growth in either replicate are considered active (red).

Supplemental Figure 2



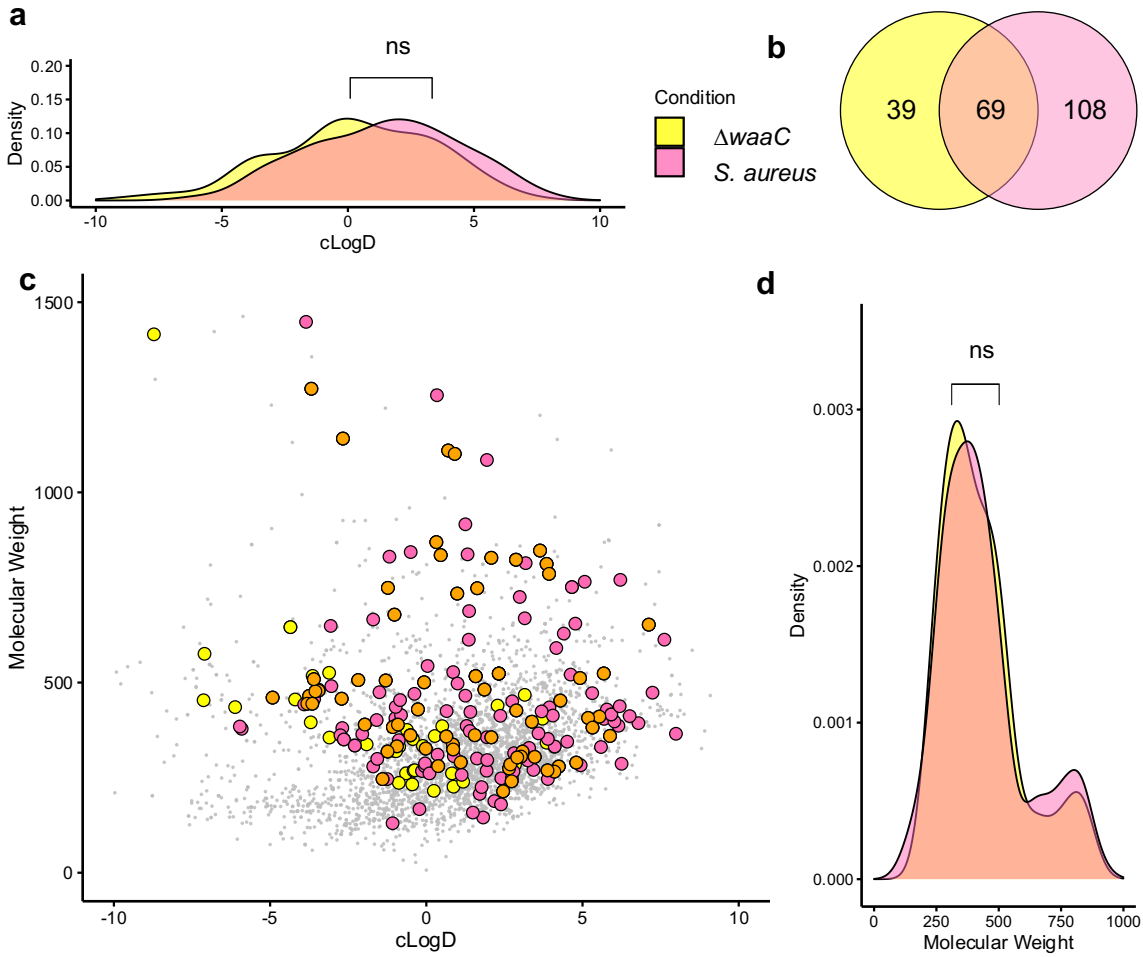
Supplemental Figure 2. Physicochemical space of compounds screened for bacterial growth inhibition, visualized by molecular weight and calculated logD (cLogD) at pH 7.4 (a,c,d). Compounds are coloured by growth-inhibitory activity, no activity (grey), activity in only the *E. coli* control (blue), activity in *E. coli* Δ*waaC* only (yellow) and activity in both conditions (green). (a,d) Density plots of molecular weight and cLogD for growth-inhibitory compounds. Δ*waaC* significantly alters the hydrophobicity of compounds compatible with growth-inhibition ($p<0.0001$, Kolmogorov-Smirnov test) but not molecular weight ($p>0.05$, Kolmogorov-Smirnov test). (b) Venn diagram showing the number and overlap of compounds with growth-inhibitory activity in the *E. coli* control (blue) and Δ*waaC* condition (yellow).

Supplemental Figure 3



Supplemental Figure 3. Physicochemical space of compounds screened for bacterial growth inhibition, visualized by molecular weight and calculated logD (cLogD) at pH 7.4 (a,c,d). Compounds are coloured by growth-inhibitory activity, no activity (grey), activity in *S. aureus* (yellow), activity in *E. coli* with SPR741 only (red) and activity in both conditions (orange). (a,d) Density plots of molecular weight and cLogD for growth-inhibitory compounds. No significant difference in hydrophobicity or molecular weight ($p>0.05$, Kolmogorov-Smirnov test) was observed. (b) Venn diagram showing the number and overlap of compounds with growth-inhibitory activity in *E. coli* with SPR741 (red) and *S. aureus* (yellow).

Supplemental Figure 4



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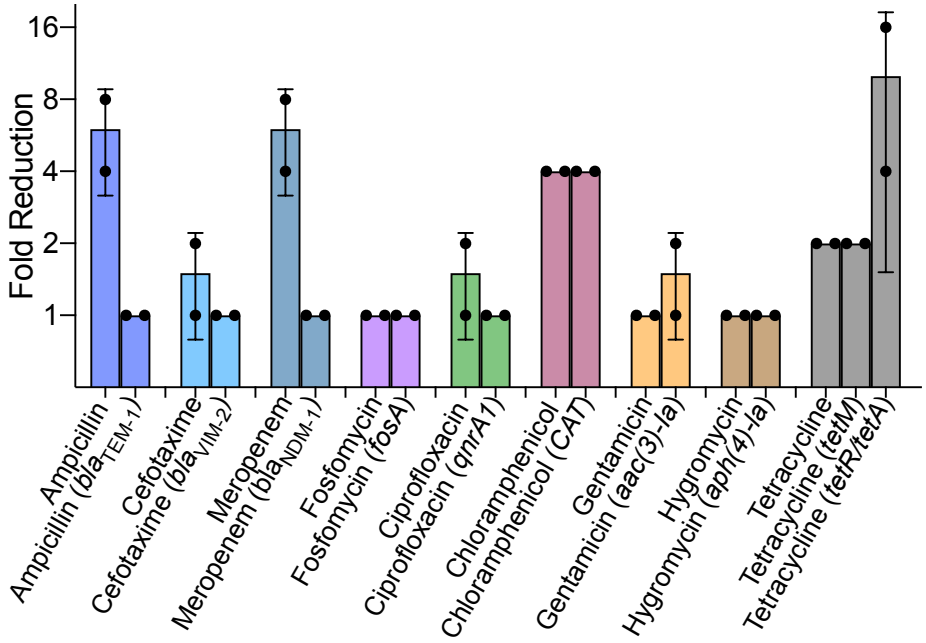
Supplemental Figure 5



Supplemental Figure 5. Fold-reduction of MIC for non-potentiated antibiotics. Each antibiotic was tested in an *E. coli* vector control, and *E. coli* harbouring the specified resistance gene. Fold-reduction is calculated by dividing the MIC of the antibiotic alone by its MIC in the presence of SPR741.

Supplemental Tables

Supplemental Table 1. Plasmids used to overexpress resistance elements in *E. coli*. Additional plasmid information can be found in Cox *et al* [47].

Antibiotic Class	Resistance Gene	Plasmid
Macrolide	*mphA*	pGDP3
	mphB	pGDP3
	ermC	pGDP4
	ereA	pGDP2
Rifamycin	*arr*	pGDP3
	rph-Lm	pGDP3
	rpoB	None
β-lactam	bla_{TEM-1}	pGDP2
	bla_{VIM-2}	pGDP1
	bla_{NDM-1}	pGDP1
Aminoglycoside	*aac(3)-Ia*	pGDP4
	aph(4)-Ia	pGDP3
Tetracycline	*tetM*	pGDP2
	tetR/tetA	pGDP4
Chloramphenicol	*CAT*	pGDP3
Fosfomycin	*fosA*	pGDP1
Fluoroquinolone	*qnrA1*	pGDP2

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Chapter IV- Conclusions

Summary

The work presented in this thesis highlights the promise of exploiting OM perturbation to sensitize Gram-negative pathogens to otherwise inactive antimicrobials. In chapter 2, we employ this strategy to overcome the troubling acquired resistance mechanism, *mcr-1,* using colistin in combination with clarithromycin. This work also points to a mechanism of *mcr-1*-mediated resistance extending beyond the predicted reduction in binding affinity of polymyxins to the outer membrane. We continue to investigate OM perturbation in chapter 3, identifying the strengths and weaknesses of this antibiotic combination approach. Together, these studies raise several questions regarding the potential of OM perturbation. Here, I look to address some of these questions and propose avenues for future investigation in this area.

How does *mcr-1* confer polymyxin resistance?

In chapter 2, we report that bacteria expressing *mcr-1* remain susceptible to the OM-disrupting but not lytic activity of colistin. This research was initiated as an observational study on how we might exploit this sensitivity as a potential treatment for bacteria harbouring *mcr-1*. Indeed, the combination of colistin and clarithromycin is efficacious in murine models of *K. pneumoniae* expressing *mcr-1*. However, the inability of *mcr-1* to protect from OM disruption by colistin motivates a line of inquiry into the mechanistic details of polymyxin resistance from *mcr-1* expression.

The proposed mechanism of *mcr-1* and other genotypes that confer polymyxin resistance is that the addition of cationic groups to the phosphates of lipid A reduces electrostatic attraction between colistin and the OM, which prevents localized OM disruption, colistin uptake, and lysis[1,2]. Indeed, expression of *mcr-1* increases the concentrations of colistin required for growth-inhibition and reduces the rate at which lysis occurs. However, in chapter 2, we report that the OM-disrupting activity of colistin appears largely unaffected by *mcr-1* expression. The relationship between lysis and the ability of polymyxins to interact with and disrupt the OM is not well understood. A growing body of work, including our own, suggests these two processes may not be tightly linked[3-5].

Despite the monogenetic nature of *mcr-1*, we propose that resistance extends beyond a simple reduction in affinity of colistin for the OM. It has been previously shown that the addition of pEtN to LPS reduces repulsion between neighbouring LPS molecules, causing a strengthening of OM packing[2]. The lytic activity of colistin relies on weakening LPS packing by insertion of the fatty-acyl chain into the OM to facilitate self-promoted uptake and access to the IM[6]. Therefore, in chapter 2, we hypothesize that strengthened LPS packing by *mcr-1* may play an important role in reducing self-promoted uptake and the lytic activity of colistin. However, since the publication of this work, several groups have reported additional research that offers an alternative hypothesis to our observations in chapter 2.

Work by Yang *et al.* shows that MCR-1 expression confers a fitness cost to the harbouring bacteria[7]. Both high levels of the protein and the LPS modification are toxic to the cell. In line with this, *mcr-1* is predominantly expressed from low-copy number plasmids. Accordingly, strains expressing *mcr-1* are sensitive to colistin at 2-8 μg/mL[1] (just above its clinical breakpoint of 2 μg/mL), while strains with constitutively activated PmrA-PmrB or PhoP-PhoQ regulatory systems require colistin concentrations of 8-256 μg/mL for growth inhibition[8]. The discrepancy between *mcr*-1- and chromosomally-mediated forms of colistin resistance revealed in this study suggests that a balance is reached between the fitness cost of MCR-1 expression and the extent of colistin resistance, resulting in a heterogenous OM composed of both pEtN-decorated and undecorated LPS molecules. These findings offer an alternative explanation to our observation in chapter 2, in which the concentration of undecorated LPS in the OM may be sufficient for colistin to bind and increase permeability, yet insufficient to produce the large areas of membrane destabilization required for self-promoted uptake and cellular lysis. In this model, it is possible for pEtN-decorated LPS to never interact with colistin, which is more in line with the traditional mechanism of polymyxin resistance.

Further research into the mechanism by which polymyxins kill bacteria has also provided a possible explanation for the reduction in susceptibility to lysis observed in chapter 2. Recent work in a preprint by Sabnis *et al.* proposes that polymyxin antibiotics inhibit growth by targeting LPS in the outer leaflet of the IM,

as opposed to the traditional model of polymyxin disrupting phospholipids in the IM[9]. After being flipped to the outer leaflet of the IM, but before transport to the OM via Lpt machinery, LPS is present in relatively large quantities, exposing it to colistin that has passed through the OM. This model is supported by polymyxin resistant *A. baumannii* isolates that are LPS-deficient with an OM composed of a phospholipid bilayer[10]. Similar to Yang *et al.,* this group reports an incomplete decoration of pEtN in the OM, which is insufficient to prevent OM disruption by colistin. Additionally, Sabnis *et al.* show that even a limited number of pEtN-decorated LPS molecules within a phospholipid bilayer such as the IM can significantly reduce lysis by colistin. Taken together, these observations can offer an alternative explanation to our observation in chapter 2, where *mcr-1* confers resistance to the IM lytic but not OM disrupting activity of colistin. We can hypothesize that due to fitness constraints, *mcr-1* decorates only a sufficient level of LPS in the outer leaflet of the IM to reduce the lytic activity of colistin. This heterogeneity of LPS, while sufficient for IM protection, is inadequate to prevent disruption of the OM.

In parallel with an increasing understanding of the mechanism by which colistin kills bacteria, the field is slowly unravelling the unexpected complexities of *mcr-1* resistance. Current data support the hypothesis that susceptibility of *mcr-1*-expressing bacteria to OM perturbation by colistin is conferred by the heterogeneous decoration of LPS in the OM. Ongoing work in the Brown lab suggests that the fitness cost of *mcr-1* is limited by nutritional requirements,

which may be exploited in experiments to titrate the levels of LPS decoration and further test this hypothesis. While many questions remain, the work presented in chapter 2, is part of a growing body of research contributing to unravelling *mcr-1* resistance and the mechanism of an antibiotic discovered over 70 years ago.

Is outer membrane disruption a viable antibiotic approach?

Using an OM perturbant alongside a traditionally Gram-positive active antibiotic has incredible potential as an antibiotic approach. As reported in chapters 1, 2, and 3, this strategy has been successfully exploited using a wide range of OM perturbants, many of which demonstrate efficacy in animal infection models[3,11,12]. However, this approach has yet to be clinically validated. One of the foremost impediments to such a translation is the high incidence of resistance elements for Gram-positive active antibiotics found in Gram-negative pathogens. This is something we overlooked in chapter 2, where we state, “Gram-negative pathogens are unlikely to be harbouring the appropriate intrinsic resistance mechanisms that would render combination treatment with such Gram-positive active antibiotics ineffective, due to a lack of selective pressure.” As described in chapter 3, macrolide resistance is particularly common in Gram-negative pathogens, which I suspect is related to the sensitivity of Gram-negative pathogens to the macrolide azithromycin. While predominantly used to treat Gram-positive infections, azithromycin is relatively active against many Gram-negative pathogens[13], suggesting a fitness benefit conferred by the presence of macrolide resistance elements.

The presence of resistance elements specific to Gram-positive active antibiotics in Gram-negative bacteria may also be explained by the co-occurrence of multiple resistance elements on horizontally transferred plasmids. Plasmids of this nature can be maintained by selective pressure from any one of several antibiotics, which may be conducive to the hitchhiking of macrolide, and other, resistance elements[14,15]. Work in the Brown lab has also identified that in growth-conditions containing the physiological buffer bicarbonate, which more closely approximates the host environment than standard laboratory growth media, Gram-negatives are sensitized to macrolide antibiotics[16]. Together, these suggestions support a benefit to harbouring macrolide resistance in Gram-negative pathogens beyond what is immediately apparent.

As reported in chapter 3, we were able to overcome resistance conferred by most macrolide-inactivation enzymes with OM perturbation; however, target-modifying mechanisms of resistance posed a greater challenge. Encouragingly, we found that OM perturbation can reduce the MIC of clarithromycin to clinically obtainable concentrations even in the presence of macrolide phosphotransferases, the most common form of macrolide resistance in Gram-negative bacteria[17]. Notably, we also observed that strains harbouring genes for antibiotic inactivation are not potentiated to MICs as low as non-resistant strains. As such, I speculate resistance elements are still operating in the presence of OM perturbation, but are unable to compensate for the rapid antibiotic accumulation. The inability to overcome target modification is disappointing but

not unexpected, as these modifications can drastically reduce antibiotic affinity. Additionally, after target modification, the affinity for the antibiotic is often reduced throughout the lifespan of the bacteria. Further expansion on the range of antibiotic resistance elements that are amenable or recalcitrant to OM perturbation should be explored.

One of the most surprising observations in chapter 3 was the reduction in the frequency of resistance observed by the combination of SPR741 and rifampicin. We were initially concerned by the potential for increased spontaneous resistance when using an OM disruptor and Gram-positive active antibiotic combination, as resistance to either component could render the combination ineffective. The observed reduction in spontaneous resistance alleviates much of this concern, particularly for the use of rifamycins as the partner antibiotic, as such antibiotics are known for their high susceptibility to spontaneous resistance development. It is difficult to speculate on the mechanism behind this observation. Experiments must first be conducted to determine if OM perturbation reduces spontaneous resistance for antibiotics not potentiated by OM disruption, although early work with fosfomycin has indicated that this is not the case. If OM perturbation causes a reduction in resistance frequency for all antibiotics, there may be an unexpected interaction with DNA repair mechanisms, as was recently identified for AcrAB efflux[18]. Alternatively, should the reduction in spontaneous resistance be observed only for antibiotics potentiated by OM perturbation, this phenotype could be attributed to the

increased or rapid antibiotic influx. In chapter 3, we suggest that rapid antibiotic accumulation may impair the ability of bacteria to adapt through mechanisms like the SOS response. Testing frequency of resistance of both potentiated and non-potentiated inducers of the SOS response alongside outer membrane perturbants could shed light on this hypothesis.

Of the potentiators investigated in chapter 3, I consider SPR741 to have the highest clinical potential. SPR741 potently disrupts the OM at concentrations obtainable during human dosing, reduces biofilm formation, spontaneous resistance and overcomes inactivating resistance mechanisms. Moreover, SPR741 has shown encouraging Phase I toxicology and pharmacology[19]. However, I believe SPR741 has several limitations that will impede future development, most notably, a lack of *P. aeruginosa* activity and its susceptibility to polymyxin resistance elements.

Increasing the number of available OM perturbants will be critical to advancing this approach as many currently available molecules suffer from toxicity concerns due to off-target disruption of eukaryotic membranes and lack *P. aeruginosa* activity. The unique characteristics of LPS in the OM should theoretically be targetable without disrupting phospholipid membranes. However, work in the Brown lab looking to identify OM perturbants that uniquely disrupt the OM and not IM has proven difficult. It is interesting to consider testing for IM disruption in the context of the work by Sabnis *et al.* reported above. It is possible traditional assays for IM disruption such as $DiSC_3(5)$ may be confounded by

molecules interacting with LPS in the outer leaflet of the IM leading to compounds inactive against phospholipid membranes being improperly discarded.

Impressive advances have recently been made in our understanding of LPS biogenesis, particularly in the elucidation of LPS transport. Many inhibitors targeting these processes also impair OM integrity at sub-inhibitory concentrations[20]. If physical perturbation of the OM proves too difficult to dissociate from the disruption of eukaryotic membranes, the future of OM disruption as an antibiotic approach may rely on enzyme inhibitors. In chapter 3, we report on the genetic deletion of *waaC* in *E. coli* and identify how this non-lethal perturbation largely phenocopies the OM disruption observed with physical perturbants. The development of a potent, broad-spectrum OM disruptor, be it an enzyme inhibitor or physical perturbant, remains elusive. However, the potential to immediately sensitize Gram-negative bacteria to dozens of novel antibiotic classes, considered with the encouraging results outlined in chapter 3, together suggest that this antibiotic approach has immense potential.

In addition to the identification of better OM perturbants, an area I feel that warrants further investigation is the optimization of the antibiotic partner. In chapter 3, we show that OM perturbation expands the hydrophobicity of molecules compatible with Gram-negative activity. However, the level of potentiation is highly variable between antibiotics, even for those within the same class. Therefore, it is likely that unique physicochemical properties make molecules more or less compatible with potentiation. It may be possible to

develop “rules” of potentiation that could be used to optimize chemical scaffolds for improved synergy alongside OM perturbants. This experiment would require a diverse training set of molecules and a high-throughput method to measure compound influx. However, it is worth noting that traditional potentiation analysis (which quantifies fold reduction in MIC) may be ineffective in this case, as modifications to compounds could simultaneously alter affinity for their intracellular target, confounding possible results. If a thorough understanding of the physicochemical properties compatible with potentiation was to be developed, current antibiotic scaffolds could be explicitly modified for combination use. This may also enable us to prioritize amongst previously abandoned drug leads for reinvigoration as partners for OM perturbants.

The work presented in chapter 3 sought to increase our understanding of OM perturbation as an antibiotic approach. The preclinical data for leveraging OM perturbants alongside Gram-positive active antibiotic combinations are encouraging. OM perturbants showcased the ability to overcome intrinsic, acquired and spontaneous resistance in Gram-negative bacteria. However, many hurdles remain before this approach can be successfully implemented in the clinic. The selection or development of the correct OM perturbant and antibiotic partner combination will be instrumental in determining the success or failure of this approach. As with any combination therapy, optimization of dosing for sufficient overlap in bioavailability can prove difficult and clinical trials are increased in complexity[21]. Nevertheless, I hope that our findings, alongside a

growing body of supportive research in the field, help to push this high-potential antibiotic approach into clinical development.

Concluding remarks

Widespread resistance and a lean drug pipeline threaten a post-antibiotic era. Nevertheless, the scientific community is rising to this formidable challenge. OM perturbation is one of many promising strategies being explored to help tackle the antibiotic resistance crisis. Researchers are leveraging innovations in DNA sequencing, artificial intelligence, and an ever-evolving understanding of bacterial physiology for the development of exciting new approaches. There is much to be hopeful for as the field looks to learn from past mistakes and make up for lost time against an ever-adapting foe.

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