LIPOPOLYSACCHARIDE-BINDING POTENTIATORS

TARGETING LIPOPOLYSACCHARIDES FOR THE DEVELOPMENT OF LPS-BINDING POTENTIATORS

By ZAID AHMED SAMEER, H. B. Sc

A Thesis Submitted to the School of Graduate Studies in Partial Fulfilment of the Requirements for the Degree Master of Science

McMaster University
Department of Biochemistry and Biomedical Sciences
Hamilton, Ontario, Canada

MASTER OF SCIENCE (2019)

TITLE: Targeting lipopolysaccharides for the development of LPS-binding potentiators
AUTHOR: Zaid Ahmed Sameer, H. B. Sc. (McMaster University)
SUPERVISOR: Dr. Eric D. Brown

NUMBER OF PAGES: xi, 63

Abstract

The rise of Gram-negative pathogens that are resistant to all currently known antibiotics threatens to create a public health crisis. Gram-negative bacteria, by possessing an outer membrane, are resistant to many classes of antibiotics. Compounds that bind lipopolysaccharides (LPS) at the outer membrane, and permeabilize the cell to those otherwise impermeable antibiotics are termed 'potentiators'. Those LPS-binding potentiators could be used in the clinic as antibiotic adjuvants to help combat Gram-negative resistance. In this project, the LPS-binding characteristics of potentiators are studied. Pentamidine, is a recently discovered LPS-binding potentiator. This work quantifies pentamidine's affinity to LPS and suggests approaches to achieve higher affinity. This knowledge could lead to the development of safer, more effective potentiators. Additionally, this project uncovers currently commercially available drugs for their LPS-binding ability. We discovered that LPS-binding is not a sole predictor of potentiation ability. However, those compounds could be a starting point for the development of novel potentiators. Moreover, in this work, we study the interaction of polymyxins with lipopolysaccharides under mobile colistin resistance (*mcr-1*) conditions. We highlight that, while the *mcr-1* mechanism of resistance is complex, the ability of polymyxins to interact with lipopolysaccharides and permeabilize the cell persists. This makes the development and exploitation of potentiators as tools for treating highly drug-resistant Gram-negative infections of great importance.

Acknowledgements

Dr. Eric Brown – Your ideas, feedback and support made this work possible. I was inspired and motivated each time I had a discussion with you. I am extremely grateful for the opportunity to do my graduate studies at your lab.

Dr. Gerry Wright & Dr. Giuseppe Melacini – Your constructive-criticism and feedback were immensely helpful. Thank you for support.

Dr. Chris Whitfield – Thank you for your great feedback.

Craig MacNair – Thank you for your great feedback.

Rashik Ahmed – You have done an immense job with the NMR experiments. Thank you for a great collaboration.

Jalees Nasir – Your help with MS was incredible. Thank you.

Elna Luckham – Thank you for the MALDI-MS experiment.

Dr. Olga Ovchinnikova – Thank you for teaching me a thing or two about LPS.

All Brown lab members – You are fantastic. I learned many things from you and for that I will always be grateful.

My parents – Nothing that I achieved, or will ever achieve, would be possible without you. Thank you for your support and belief.

My siblings – Thank you for being there for me.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

OM	outer membrane
IM	inner membrane
LPS	lipopolysaccharide
R-LPS	rough type LPS
S-LPS	smooth type LPS
Da	Daltons
MDR	multi-drug resistant
PMB	polymyxin B
DAB	diaminobutyric
PMBN	polymyxin B nonapeptide
BC	BODIPY™ TR Cadaverine
ITC	isothermal titration calorimetry
NMR	nuclear magnetic resonance
STD	saturation transfer difference
FDA	US food and drug administration
MIC	minimum inhibitory concentration
PETN	phosphoethanolamine
MOA	mechanism of action
NPN	N-phenylnaphthalen-1-amine

Declaration of Academic Achievement

Zaid Sameer performed the majority of the experiments, data collection, and analyses presented in this thesis. This report was also prepared by Zaid Sameer.

NMR experiments, and their analysis were performed by Rashik Ahmed (PhD Candidate – Melacini Lab). Rashik also prepared the NMR figures (Figures 2.1 & 2.2) and wrote their corresponding methods.

The NPN permeability assay was designed by Craig MacNair (PhD Candidate – Brown Lab)

Elna Luckham performed the MALDI-TOF experiment and prepared its corresponding figure (Figure 4.4).

Chapter 1 - Introduction

1.1 The Gram-negative resistance crisis

The evolution of Gram-negative bacterial pathogens that are resistant to all currently known antibiotics comprises a serious threat to public health. Antibiotic-resistant Gram-negative bacteria dominate the World Health Organization's list of pathogens for which the discovery and development of new antibiotics are urgently needed[1]. The list contains twelve bacterial pathogens, nine of which are of the Gram-negative type. In fact, the three bacterial pathogens ranked the highest priority by that list (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*) are all Gram-negatives. This urgency stems from the fact that the current antibiotic development pipeline suffers a lack of novel Gram-negative therapeutics and cannot control the Gram-negative crisis[2]. The major scientific challenge to the discovery of novel Gram-negative antibiotics is that Gram-negative cells are very efficient in obstructing the entry of many potential antibiotics. Innovative therapeutics that interfere with that obstruction and allow the entry of antibiotics to the Gram-negative cell, present valuable tools to help face the resistance era.

1.2 The Gram-negative outer membrane (OM) as an antibiotic barrier

Gram-negative bacteria possess an outer membrane (OM) that makes them inherently resistant to many antibiotics. The Gram-negative cell is enclosed by an inner membrane (IM), a thin layer of peptidoglycan and an OM. Several classes of antibiotics (such as rifamycins, macrolides, and aminocoumarins) have an

intracellular target and are effective against the OM-lacking Gram-positive bacteria. Those antibiotic classes are comprised of large hydrophobic chemical structures that are hindered by the OM from gaining access to the cytosol. The impermeability of the OM towards such large hydrophobic molecules drastically reduces the number of chemical matter that is effective in inhibiting the growth of Gram-negatives. In fact, when screening compounds that are effective against *P. aeruginosa*, the hit rate is up to 1000-fold lower when compared to Gram-positive bacteria[3]. In addition to OM impermeability, this difference is attributed to the presence of efflux pumps that force out many potential antibiotics. One strategy for increasing the number of chemical compounds that are active against Gram-negatives is by the use of a 'potentiator' molecule that permeabilizes the cell to such compounds[4]. Potentiators, as antibiotic adjuvants, would be used in combination with a large hydrophobic antibiotic in the clinic for treating multi-drug resistant Gram-negative infections.

1.3 Lipopolysaccharides (LPS) of the OM

1.3.1 Lipopolysaccharide (LPS) structure and properties

Lipopolysaccharides (LPS) are unique to the OM of most Gram-negative bacteria and constitute a permeability barrier to many antibiotics. The OM is an asymmetric lipid bilayer consisting of phospholipids at the inner leaflet and LPS at the outer leaflet[5,6]. LPS is composed of a sugar polymer attached to a lipid component that anchors it in the membrane. The structure of an LPS molecule has three main regions: lipid A, core-oligosaccharides, and the O-antigen (Figure 1.1).

The O-antigen and the core-oligosaccharides are not essential for bacterial growth and are therefore very heterogeneous in structure[5]. LPS containing an O-antigen is termed smooth type LPS (S-LPS), whilst one lacking the O-antigen is termed rough type LPS (R-LPS). The lipid A component is the one most conserved in structure and is composed of a highly acylated glucosamine disaccharide that is bis-monophosphorylated at position 1 and 4'. Lipid A is recognized by the human immune system, triggering an inflammatory response that leads to sepsis or endotoxic shock in Gram-negative infections, and is therefore termed endotoxin[7,8].

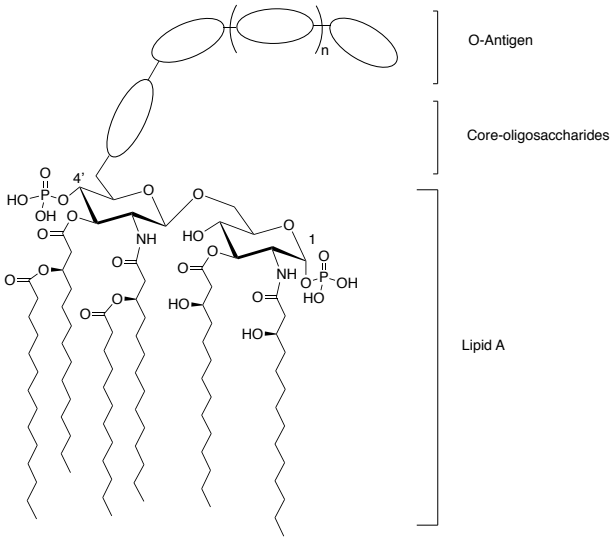


Figure 1.1 – The generalized structure of a lipopolysaccharide (LPS) molecule.

Lipid A carries negatively charged phosphate groups that interact with divalent cations giving rise to the unique permeability properties of the OM. Those divalent cations (such as Ca^{+2} and Mg^{+2}) bridge adjacent LPS molecules[9], and this electrostatic cross-linking is believed to add a polar component to the membrane barrier properties of the surface, making it impermeable to large hydrophobic

molecules[3]. While small hydrophilic and amphiphilic molecules (smaller than 600 Da) are able to transverse the OM by passing through porin proteins that span the OM, larger structures are unable to diffuse effectivly[3]. This dramatically decreases the intracellular concentration of such compounds making the cell essentially resistant to their antibiotic effects. Indeed, strains of Gram-negative bacteria that lack LPS, possessing phospholipids instead at the outer leaflet of the OM, are more permeable and susceptible to antibiotics of such properties[10,11].

1.3.2 LPS biosynthesis and transport in *E. coli*

LPS biosynthesis starts in the cytoplasm, where Lipid X is synthesized from UDP-GlcNAc by *LpxA*, *LpxC*, *LpxD* and *LpxH*[12] (Figure 1.2). Lipid X is then transported to the inner leaflet of the IM where Kdo_2-Lipid A is assembled (Figure 1.2). Several core-oligosaccharides are then added to Kdo_2-Lipid A sequentially by multiple core-oligosaccharide transferases (such as *WaaC*, *WaaF* and *WaaG*)[13]. The resulting R-LPS is transported to the outer leaflet of the IM by *MsbA*[13]. The O-antigen is synthesized separately and is ligated onto R-LPS by *WaaL*. The resulting S-LPS is finally transported to the outer leaflet of the OM by the lipopolysaccharide transport system (Lpt)[14]. While LPS biosynthesis and transport is essential in many Gram-negatives, the continuous discovery of Gram-negative strains lacking LPS[10] suggests that LPS essentiality is strain dependent[15]. Still, LPS biosynthesis, transport and sequestering remain as attractive targets for antibiotic discovery and development.

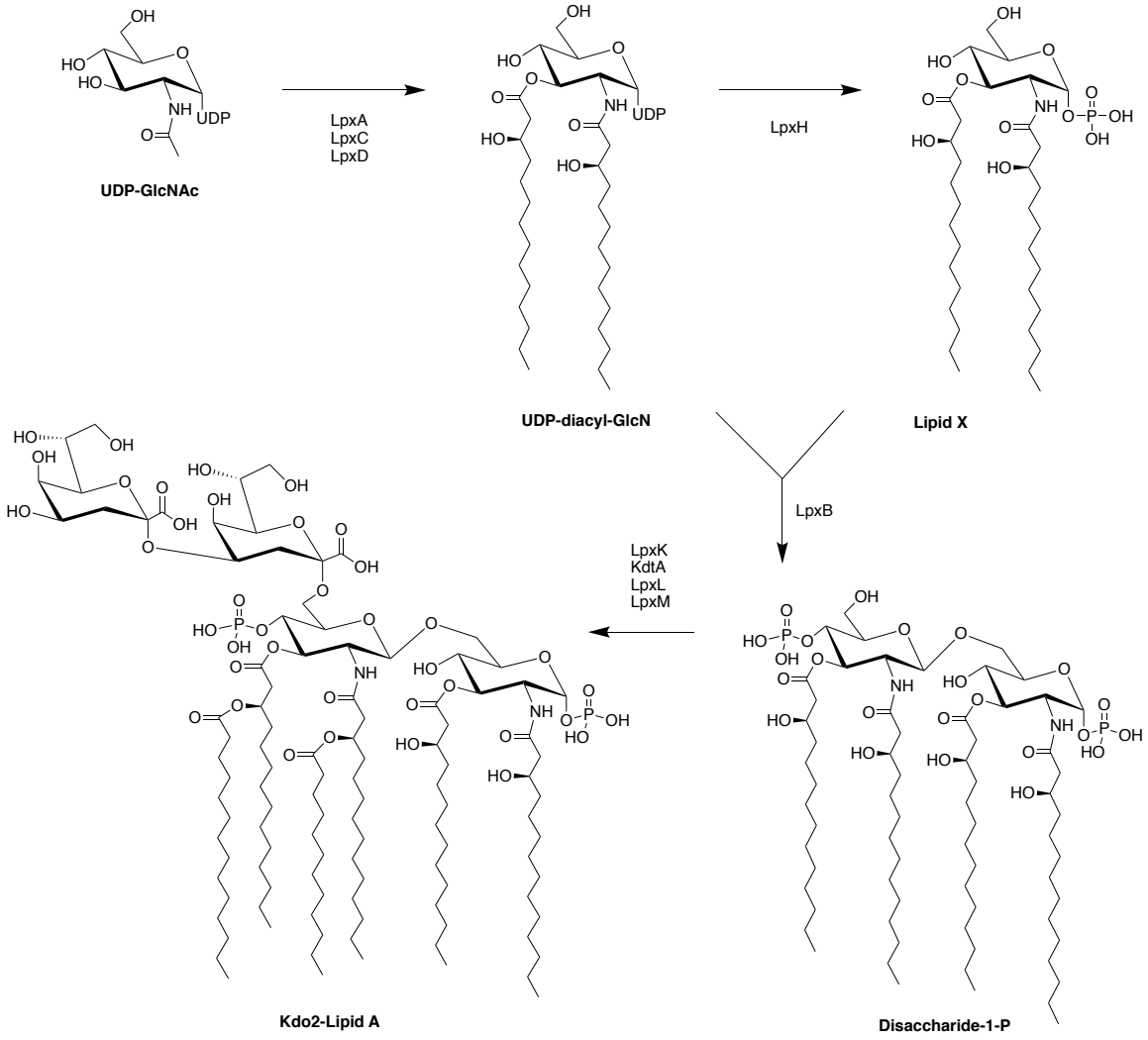


Figure 2.2 – A summary of Kdo_2-Lipid A biosynthesis in *E. Coli* and the genes involved.

1.3.3 Targeting LPS in drug discovery

Many antibiotic development efforts have been devoted to targeting LPS biosynthesis, transport and sequestering. Genes involved in Kdo_2-Lipid A biosynthesis are essential in many Gram-negatives. Recent antibiotic discovery efforts have been successful in developing inhibitors of those essential proteins[16] such as *lpxC* inhibition[17,18]. Although, core-oligosaccharide transferases are not

essential for bacterial growth, their inactive genes have an increased OM permeability[19]. This knowledge lead to the study of LPS-binding permeabilizers as antibiotic adjuvants. One such compound (SPR741) is in clinical development[20–22]. Moreover, inhibition of the lipopolysaccharide transport system (Lpt) produced a potent *P. aeruginosa* antibiotic, that is currently in clinical development[23]. Lastly, while, the development of LPS sequestrants for the treatment of sepsis has been unsuccessful, it continues to be investigated[24–26].

1.4 The polymyxins

1.4.1 Polymyxin structure and properties

The polymyxin class of antibiotics is considered a last resort option in treating multi-drug resistant (MDR) Gram-negative infections[27,28]. Polymyxins were first isolated from *Bacillus polymyxa* in the 1940s[29]. However, the use of polymyxins in treating systemic Gram-negative infections was long abandoned due to toxicity concerns[29]. Yet, the recent rise of MDR Gram-negative pathogens has forced clinicians to resort to polymyxins as a last treatment option[28]. The two clinically approved polymyxins are polymyxin B (PMB) and colistin (polymyxin E)[29]. PMB and colistin are non-ribosomal cyclic lipopeptides that differ in one amino acid residue such that colistin's D-phenylalanine is replaced by D-leucine in PMB (Figure 1.3). Both possess five diaminobutyric (DAB) residues, making them highly positively charged (+5) at physiological pH (pH = 7.4). Both, also, possess an acyl chain granting them an amphiphilic structure. Their antimicrobial spectrum involves most aerobic Gram-negative bacilli, such as *A. baumannii*, *P. aeruginosa*, and

Enterobacteriaceae, while Gram-positives are resistant[29]. Toxicity of polymyxins, specifically nephrotoxicity[29], is a major concern when polymyxins are used clinically.

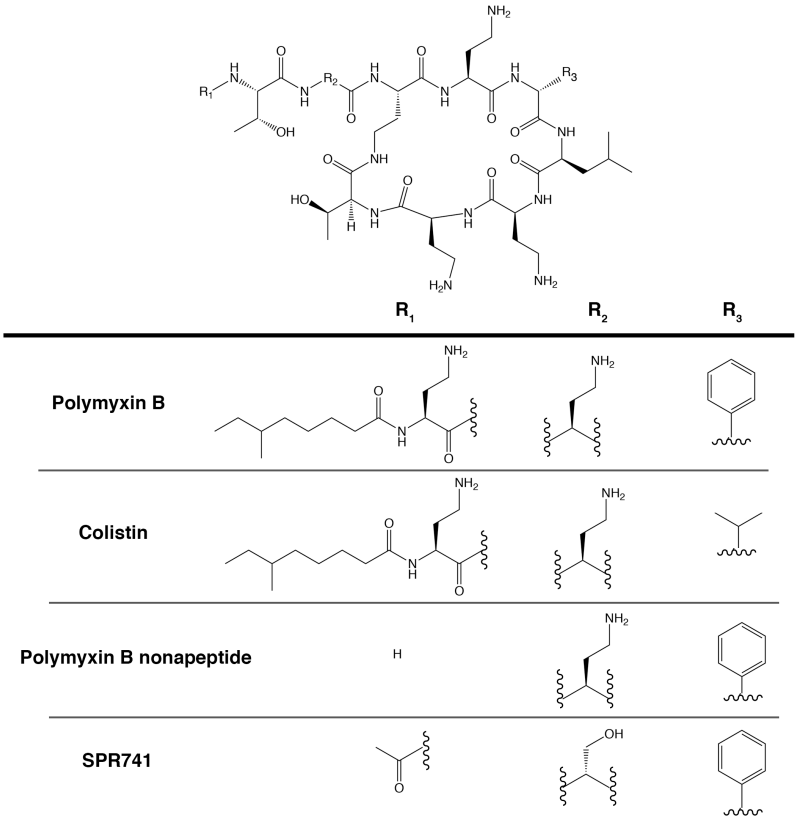


Figure 3.3 – The chemical structure of some polymyxin derivatives.

1.4.2 Polymyxins mechanism of action

The mechanism of action of polymyxins has long been debated and is still being studied to this date[30]. There general consensus is that polymyxins, through their positively charged DAB residues, bind the negatively charged lipid A phosphates of LPS, displacing divalent cations that cross-link adjacent LPS molecules and disrupting the barrier properties of the OM[31]. Taking advantage of

the compromised OM integrity, they transverse to the inner membrane (IM)[32]. This mechanism of transversing the OM is termed self-promoted uptake[33]. Ultimately, they interact with the IM, disrupting proton motive force (PMF) and causing leakage of cytosolic contents and cell death[32]. Other hypotheses for the mechanism of action of polymyxins were suggested[34], however, those seem to be less widely accepted.

1.4.3 Polymyxin resistance

Resistance to polymyxins can be inherent, chromosomally mediated, or transferred through a plasmid. Gram-negative bacteria that lacks LPS at the OM are inherently resistant to polymyxins[11]. When LPS is present, polymyxin resistance is typically a result of structural modifications to LPS, by the addition of positively charged moieties and/or by changing the acylation pattern at the Kdo_2-lipid A portion (Figure 1.4). These modifications are believed to reduce the negative charge of the OM, and the packing of LPS at the OM. Both those outcomes are believed to lower the affinity of polymyxins to LPS interfering with their self-promoted uptake mechanism.

The PhoP-PhoQ and PmrA-PmrB two-component regulatory systems are a chromosomally mediated form of polymyxin resistance that add the positively charged moieties 4-aminoarabinose and phosphoethanolamine respectively to Kdo_2-Lipid A and/or changes its acylation pattern[35] (Figure 1.4). This regulatory system is activated upon environmental stress, such as low pH, low Mg^{+2} and sub-inhibitory concentrations of cationic antimicrobial peptides (CAMPs)[35].

Mobile polymyxin resistance elements, such as mobile colistin resistance (*mcr*-1), are easily transferable and are rapidly disseminating globally, posing a serious threat to the utility of polymyxins as last resort antibiotics[36]. *Mcr-1* is a phosphoethanolamine transferase that adds a phosphoethanolamine to one of the two lipid A phosphates. *Mcr-1* was discovered in 2016[36], and Gram-negative isolates harboring *mcr-1* (or one of its variants) are now routinely detected in hospitals globally[37,38]. Most concerningly, MDR Gram-negative pathogens carrying *mcr-1* have already been isolated in hospitals[39]. Such pathogens could result in potentially untreatable infections[40] and, therefore, the discovery and development of therapy options that overcome *mcr-1* resistance is of crucial importance.

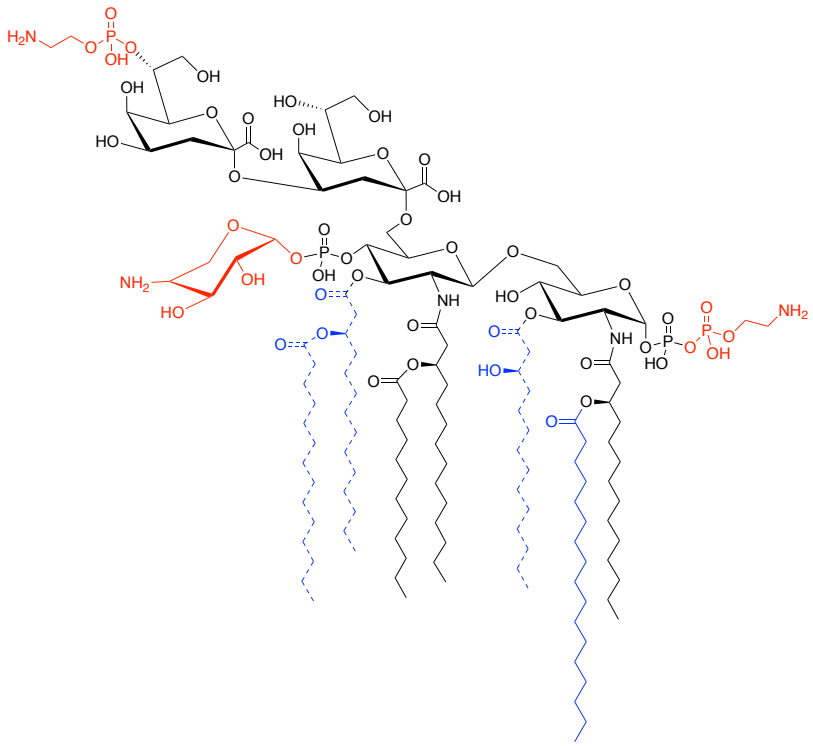


Figure 4.4 – Kdo_2-Lipid A modifications leading to polymyxin resistance. (red) the addition of positively charged moieties. (blue) changes in acylation pattern, where dashed lines indicate deacylation.

1.5 LPS-binding potentiators

It has long been believed that molecules with an ability to disrupt LPS by displacing the cross-bridging divalent cations, permeabilize the Gram-negative cell to large hydrophobic antibiotics[41]. Potentiators that chelate those cations (such as EDTA), because of their indiscriminate chelating ability, have been deemed of reduced value as drug candidates, and the focus has been on polycationic molecules with lipophilic characters that bind the negatively charged LPS[41]. These characteristics make those compounds membrano-philic, and off-target toxicity, by interacting with human host cell membranes, has been a major concern in the development of potentiators[42]. The permeabilizing mechanism of action of potentiators is not totally understood. It is believed that displacing the cross-bridging cations by a chelator (such as EDTA) from the OM results in LPS release into the media[43]. This results in phospholipids migrating to the outer leaflet of the OM, creating phospholipid patches that are more permeable to large hydrophobic compounds[43]. However, so far, there is no evidence that polymyxins cause phospholipid patches at the OM[43]. Another hypothesis is that polymyxins insert in the OM, creating cracks that are utilized by large hydrophobic compounds to transverse the OM[44].

The development of LPS-binding potentiators has been focused on structural analogues of polymyxins. Despite the fact that colistin, itself, can be used as a potentiator[45], toxicity concerns lead to the development of polymyxin B nonapeptide (PMBN) by the removal of PMB's acyl chain (Figure 1.3). PMBN lacks

bactericidal activity but retains the OM-disruption ability, and has been found to have a weaker affinity towards LPS when compared to PMB[41]. Ultimately, PMBN toxicity profile was determined to be similar to that of PMB, and the compound did not enter clinical development[41]. SPR741 is a less toxic polymyxin-derivative LPS-binding potentiator that is currently in the clinical development pipeline[2,20]. SPR741 was developed by decreasing the positive charge of PMB to +3 (Figure 1.3), resulting in a highly attenuated toxicity.

Pentamidine, a commercially available anti-fungal drug, is a non-polymyxin derived LPS-binding potentiator[46]. Pentamidine's potentiation ability persists under *mcr-1* expression making it a valuable tool for overcoming *mcr-1* mediated colistin resistance[46]. Despite having toxic side effects, as an already FDA approved drug, pentamidine is worth a serious consideration for the repurposing in the clinic as an antibiotic adjuvant. Detailed mechanism of action and toxicity studies of pentamidine as a potentiator are necessary however. Other non-polymyxin derived potentiators, such as oligo-acyl-lysyls (OAKs) and cationic steroid antibiotics (CSA) are being developed as well[4].

Potentiators would increase the number of antibiotic classes available to combat the Gram-negative resistance crisis. The ability of some potentiators to permeabilize *mcr-1*-expressing cells[45,46], would be an invaluable tool to the overcoming mobile colistin resistance. However, the discovery and development of safe potentiators, will require careful characterization their mechanism of action. More specifically, describing the LPS-binding properties that would make

potentiators specific to LPS, lowering their off-target toxicity, is needed to help guide potentiator development.

1.6 Project objectives

LPS-binding potentiators have the potential to be invaluable tools for combating the Gram-negative crisis. They would make several classes of antibiotics that are effective against Gram-positive bacteria now active against Gram-negatives. Moreover, they would help combating resistance against the last resort antibiotic colistin. However, the lack of deep understanding of potentiator mechanism of action and the LPS-binding properties required for specific targeting hinder the development of safe, effective potentiators.

The first aim of this project was to highlight the mechanism of action of the LPS-binding potentiator pentamidine. This was done by highlighting the thermodynamic binding parameters of pentamidine to LPS. This knowledge could help guide toxicity studies of pentamidine and repurposing of pentamidine in the clinic as an antibiotic adjuvant. Additionally, this knowledge could help develop safer analogues of pentamidine as potentiators.

Secondly, this project aimed at uncovering non-polymyxin derived LPS-binding potentiators. While the project uncovered several already FDA approved drugs with LPS-binding ability, the ability to bind LPS did not equate to potentiation. Those compounds could be starting points for the development of safe, effective LPS-binding potentiators.

Lastly, the project aimed at examining the interaction of polymyxins with LPS under *mcr-1* modification, to help better understand the potentiation mechanism of action in those conditions. We discover that modification of LPS by *mcr-1* allows the permeabilization ability of some potentiators to persist. This knowledge highlights the importance of exploiting potentiators for overcoming *mcr-1* resistance.

Chapter 2 - Characterizing the thermodynamic binding parameters of pentamidine to LPS

2.1 Introduction

With the rise of multidrug-resistant, extensively drug-resistant, or even pandrug-resistant Gram-negative pathogens, increasing the number of antibiotic classes that are active against these pathogens is critically needed[2]. By having an OM, Gram-negative bacteria are inherently resistant to many classes of large hydrophobic antibiotics that are active against the OM-lacking Gram-positive bacteria[4,43]. Our lab has recently discovered the ability of pentamidine, a commercially available anti-fungal drug, to potentiate large hydrophobic antibiotics, allowing them to inhibit the growth of Gram-negative pathogens[46]. Excitingly, the potentiation ability of pentamidine persists under mobile colistin resistance (*mcr-1)* expression, and a combination therapy of pentamidine and a large hydrophobic antibiotic was found effective in clearing infection in mice infected with *mcr-1* carrying Gram-negative pathogens[46].

Pentamidine is approved for the use as a treatment for multiple fungal such as African trypanosomiasis, leishmaniasis, and babesiosis, and the prevention and treatment of pneumocystis pneumonia[47]. Yet, toxic side effects are common and they include hypotension, pancreatitis, and leukopenia[48]. It is believed that pentamidine interfere with DNA biosynthesis resulting fungicidal activity[49]. Still, the success of pentamidine in treating colistin resistant Gram-negative infections in mouse models make repurposing pentamidine, or the development of safer analogues, an attractive option. However, an understanding of pentamidine's

mechanism of potentiation is important for a careful assessment of pentamidine's toxicity when used as an antibiotic adjuvant or for the development of safer potentiators.

We believe that LPS is pentamidine's direct target. We demonstrated evidence that, similar to polymyxins, pentamidine causes OM disruption[46]. Also, the addition of extracellular LPS into the growth media, abolishes pentamidine's potentiation ability[46]. Pentamidine possess two positively charged amidines that are 14 Å apart. This feature is believed to grant pentamidine efficient binding to the two negatively charged phosphates of the lipid A moiety of LPS[50]. We therefore hypothesize that pentamidine binds LPS, displaces the divalent cations that cross-bridge adjacent LPS molecules, disrupting and permeabilizing the OM. To test this hypothesis, an *in vitro* direct study of pentamidine's interaction with LPS is necessary.

The thermodynamic parameters of pentamidine's interaction with LPS are poorly characterized. This hinders our understanding of pentamidine's potentiation mechanism and impedes the design and development of more effective, less toxic analogues. Pentamidine's interaction with LPS has been studied by David *et al.*[51] and Guo *et al.*[52] in the field of developing LPS-binding sequestrants for treating sepsis. David *et al.*[51] suggest that pentamidine binds LPS with a high affinity (dissociation constant, K_d of ~120 nM), while Guo *et al.*[52] suggest a much lower affinity (K_d predicted to be in the high μM range) . Both studies utilize fluorescence experiments in which an LPS-binding fluorophore is displaced by pentamidine. The

disagreement between those two studies warrants a careful label-free characterization of the thermodynamic parameters of the interaction between pentamidine and LPS.

Here, we determine the thermodynamic binding parameters of pentamidine to LPS and compare it to that of PMB by Isothermal Titration Calorimetry (ITC) and by Saturation Transfer Difference (STD) NMR. Our results indicate a lower affinity of pentamidine (K_d = 43 ± 1 μM) to LPS compared to PMB (K_d = 8 ± 1 μM). Moreover, we examine two pentamidine analogues, and find that a higher affinity to LPS corresponded to an enhanced potentiation ability. This knowledge could help develop a more LPS-specific, less toxic analogue of pentamidine.

2.2 Results

To determine the thermodynamic binding parameters of pentamidine to LPS, and compare it to that of PMB, we used the label-free technique ITC widely regarded as a gold standard experiment for ligand-binding studies. The heat change is measured upon addition of the ligand to a cell containing LPS, and the data are analyzed elucidating the interaction. The results indicate that pentamidine binds LPS with a dissociation constant (K_d) of 41.4 ± 0.5 μM, while PMB binds LPS with a K_d of 7.1 ± 1.3 μM (Figure 2.1). In disagreement with our results, David *et al.*[51] reported that pentamidine, compared to PMB, binds LPS with a higher affinity (K_d ~ 120 nM). We, therefore, found it necessary to confirm our binding affinity results by an orthogonal technique.

To confirm the ITC results by another label-free technique, we used STD NMR. In those experiments, the LPS solution was titrated with increasing concentrations of pentamidine (or PMB) and the interaction was monitored by ^{1}H and STD NMR. In agreement with our ITC results, our NMR results suggest that dissociation constant K_d of pentamidine to LPS is 45 ± 2 μM while that of PMB is 9 ± 1 μM (Figure 2.2). Moreover, we demonstrated that PMB and pentamidine compete for LPS-binding, suggesting that both compounds bind LPS similarly at the lipid A portion (Figure 2.3). With more confidence in the determined dissociation constants, we aimed to compare our results to those obtained by Guo *et al.*[52] which are measured using the LPS-binding fluorophore BODIPY™ TR Cadaverine.

BODIPY™ TR Cadaverine (BC) is a fluorophore reported by the literature[53] to probe LPS binding. LPS binding quenches the fluorescence intensity of BC, while the addition of an LPS binding competitor displaces BC and results in fluorescence enhancement. A plot of fluorescence intensity at 620 nm under different displacer concentrations produces a binding curve. An ED_{50} value is the displacer concentration at which 50% of BC is displaced and is used here as a measure of LPS binding affinity. Carrying the experiment on isolated LPS, we determined the ED_{50} of pentamidine to be 89.0 ± 23.1 μM while that of PMB to be 1.7 ± 0.2 μM (Figure 2.4 a). We also obtained similar results when displacing BC bound to whole *E. coli* cells (Figure 2.4 b). It is important to mention that the ED_{50} values do not compare in magnitude to dissociation constant values, since they are dependent on the concentration of LPS and the saturation level with BC. Since

the BC displacement results are in line with our ITC and NMR results, and because the fluorescence experiment uses less reagents and is less laborious, we opted to use it to study the affinity of two pentamidine analogues to LPS.

To study the affinity to LPS of different pentamidine analogues, with different potentiation ability, we used the BC displacement assay. Compound T10, which has a higher potentiation ability, binds LPS with a higher affinity (Table 2.1). While compound T2, which lacks one of the amidine groups and has a lower potentiation ability, binds LPS with a lower affinity (Table 2.1). It is interesting that a higher affinity corresponded to a better potentiation ability. However, constructing a conclusive correlation requires the examination of a larger number of analogues.

2.3 Discussion

The discovery that pentamidine, a commercially available anti-fungal drug, is able to sensitise Gram-negative pathogens to large hydrophobic antibiotics, and to overcome mobile colistin resistance (*mcr-1*) is very important to developing tools to combat antibiotic resistance. As a potentiator, pentamidine renders a plethora of antibiotics now effective against Gram-negative pathogens. This dramatically increases the diversity of chemical matter that is now active against Gram-negatives. It is believed that when resistance develops against the combination therapy, it is most likely to be against the partner antibiotic, and a choice of another antibiotic partner should restore activity[45]. This makes understanding pentamidine's mechanism of action very necessary as such knowledge could help

guide the repurposing of pentamidine in the clinic and/or the development of other safer potentiators.

The affinity of pentamidine to LPS (K_d = 43 ± 1 μM) could be improved, increasing specificity to LPS, possibly lowering the concentration needed to achieve potentiation, and, as a result, lowering toxicity. The determined affinity agrees with the concentration of pentamidine needed to achieve potentiation, and with its concentration needed to disrupt the OM as evidenced by atomic force microscopy (AFM) (25 μg/mL or 42 μM). The ITC results indicate that pentamidine's interaction with LPS is mainly enthalpically-driven as a product of the interaction between the positively charged amidine's with the negatively charged phosphates of LPS (Figure 2.1). However, it is less enthalpically-driven when compared to PMB's interaction with LPS (Figure 2.1). This is not surprising since, compared to the +2 charge of pentamidine, PMB possesses a +5 charge which would allow the interaction with not only the two negatively charged phosphates of lipid A but with the negatively charged moieties of inner-core sugars (the negatively charged KDO sugars and the inner-core phosphates) as well. Therefore, pentamidine's affinity and specificity to LPS could possibly be improved by the addition of other positively charged moieties, in a manner similar to that of PMB.

By examining two pentamidine analogs, we found that a higher affinity to LPS corresponded to a higher potentiation ability. Compound T10, which has a higher potentiation ability, possesses a phenyl substituent at the alkyl chain of

pentamidine, was found to have a higher affinity to LPS compared to pentamidine (Table 2.1). That finding is expected since it is believed that the hydrophobic alkyl chain of pentamidine interacts with the hydrophobic acyl chains of lipid A and contributing to the interaction[51]. Therefore, increasing the hydrophobicity of the alkyl chain could well result in a better hydrophobic interaction between compound T10 and LPS. On the other hand, compound T2 lacks one of the amidine groups and is expected to bind the lipid A phosphates less efficiently. Indeed, compound T2 had a lower affinity to LPS compared to pentamidine and a lower potentiation ability (Table 2.1). Those results suggest that a careful design of a more LPS-specific analogue of pentamidine could involve increasing the hydrophobicity at the alkyl chain linker.

Here, we elucidate the mechanism of action of the LPS-binding potentiator, pentamidine, by characterizing its thermodynamic binding parameters to LPS. We find that pentamidine's affinity to LPS (dissociation constant, K_d = 43 ± 1 μM) could be improved, increasing specificity to LPS and possibly lowering toxicity. That could possibly be achieved by a careful construction of a structure activity relationship (SAR), which could involve the addition of more positively charged moieties and/or increasing hydrophobicity and rigidity at the alkyl chain linker. This knowledge could help guide the repurposing of pentamidine in the clinic, and/or the development of less toxic potentiators.

2.4 Materials & Methods

S-LPS, R-LPS, PMB and Pentamidine were purchased from Sigma-Aldrich. Pentamidine analogues were purchased from WuXi AppTech. BODIPY™ TR Cadaverine was purchased from ThermoFischer Scientific.

Fluorescence displacement assay. Concentrated solutions of LPS binding compounds were prepared and serially diluted in Tris-HCl buffer (50 mM, pH= 7.4). Compounds with limited water solubility were prepared and serially diluted in buffer containing 25% DMSO. A solution containing BC (2 μM) and R-LPS (3 μg/mL) was prepared in buffer, and then, 18 μL of that solution was added to a Corning Nonbinding Surface Black 384 micro-well plate containing 2 μL of the serially diluted compound. Fluorescence intensity was read by a Tecan Infinite Pro m1000 plate reader using Magellan v7.2 software. The excitation and the emission wavelengths were set to wavelength 580 and 620 nm respectively, with both bandwidths set to 5 nm. Where cells were used, the quenched BC-LPS solution was replaced with a solution of equal volumes of cells (at 0.5 OD) and BC (2 μM).

Isothermal titration calorimetry. Solutions of S-LPS 250 μg/mL (~50 μM) and the ligand (PMB or pentamidine) (500 μM) in Tris-HCl buffer (50 mM, pH= 7.4) were prepared and degassed for 15 minutes. Nano ITC machine (TA Instruments) was used to carry out the titration. 19 injections of 2.5 μL ligand solution were added into the LPS solution (cell volume 170 μL) at 240 second intervals at 37 ºC. NanoAnalyze (TA Instruments) software was used for data analysis. Control

experiments of injecting buffer into LPS solution and ligand into buffer were performed.

Preparation of NMR samples. S-LPS was resuspended in 20 mM Sodium Phosphate Buffer pH 7.4, 100% D_2O, 0.05% NaN_3 to a final concentration of 1 µM. Similarly, Pentamidine and Polymyxin B were dissolved in the same buffer to a stock concentration of 1.5 mM. Pentamidine or Polymyxin B were titrated into the lipopolysaccharide NMR samples to achieve the desired concentrations.

NMR Spectroscopy. All NMR spectra were recorded at 25 °C, using a Bruker AV 700 spectrometer equipped with a TCI cryo-probe. All spectra were analyzed with TopSpin 3.2.1. Additional details are discussed below.

^{1}H NMR. ^{1}H NMR spectra were acquired with 128 scans, 32K complex points (t_2) and a spectral width of 11.98 ppm. The spectra were processed with a lb factor 5.00 and exponential multiplier window function.

STD NMR. STD spectra were recorded with 1024 scans, 32K complex points and a spectral width of 11.98 ppm. Selective saturation of lipopolysaccharide was implemented through methyl irradiation, using a train of twenty Gaussian-shaped pulses of 50 ms, separated by a 1 ms inter-pulse delay. In the case where selective saturation of lipopolysaccharide was not achieved, control spectra of ligand alone were acquired to account for leak-through arising from non-selective saturation. A 30 ms spin lock was also employed to suppress lipopolysaccharide signals that overlap with ligand resonances. Subtraction of on-resonance intensities from off-resonance was completed through phase cycling. Saturation transfer reference

(STR) spectra were recorded with 128 scans, 32K complex points and a spectral width of 11.98 ppm. A binding isotherm was created through modeling the STD amplification factors (STD_{af}) at each ligand concentration using the ligand aromatic proton peaks. The STD_{af} was calculated as:

$$STD_{af} \sim \frac{I_0 - I_{sat}}{I_0} L_{Tot.} \quad (1)$$

where I_0 - I_{sat} represents the signal intensity in the STD spectrum, I_0 is the intensity in STR spectrum and $L_{Tot.}$ is the total ligand concentration.

The STD_{af} were modeled through a Scatchard-like binding isotherm:

$$STD_{af} / STD_{af,max} = [Ligand] / ([Ligand] + K_{d,eff.}) \quad (2)$$

where [Ligand] is the concentration of free ligand and $K_{d,eff.}$ is an effective site-specific dissociation constant for the binding of Pentamidine or Polymyxin B to lipopolysaccharide.

Fractional Inhibitory Concentration index (FIC_i). FIC_i values were obtained from Stokes *et. al*.[46] FIC_i is calculated according to the formula:

$$FIC_i = MIC_{ac}/MIC_a + MIC_{bc}/ MIC_b = FIC_a + FIC_b$$

Where MIC_a is the minimum inhibitory concentration (MIC) of compound A alone; MIC_{ac} is the MIC of compound A in combination with compound B; MIC_b is the MIC of compound B alone; MICbc is the MIC of compound B in combination with compound A. FIC_a is the FIC of compound A; FIC_b is the FIC of compound B.

Synergy is defined as an FIC index of ≤0.5. Antagonism is defined as an FIC index of ≥4. Rifampicin was the partner antibiotic in analogue studies.

2.5 Tables, Figures & Captions

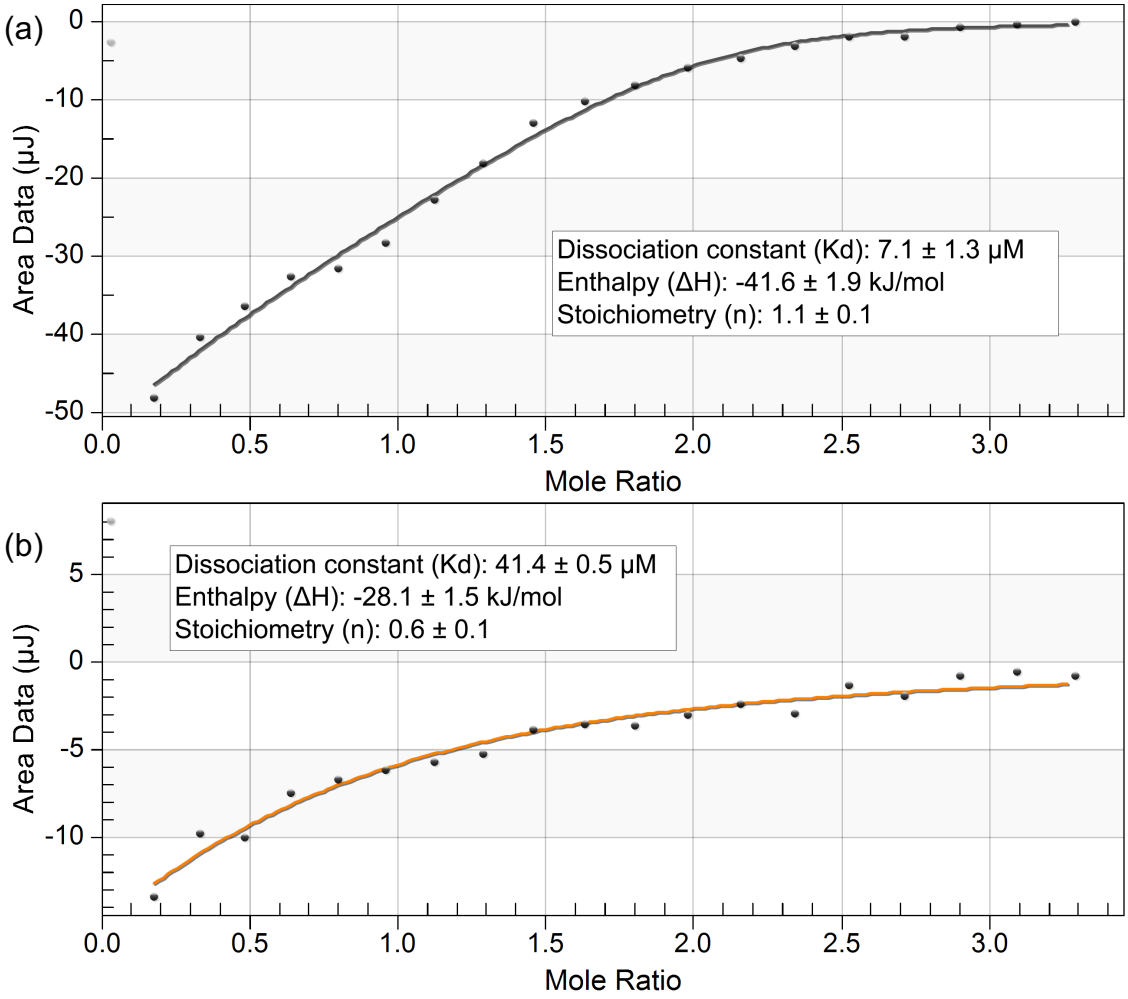


Figure 2.1 – Isothermal titration calorimetry (ITC) indicates a lower affinity of pentamidine to LPS compared to PMB. The binding curves of (a) PMB and (b) pentamidine with LPS. Insets: the thermodynamic parameters of each interaction as calculated by data fitting. The parameters represent the mean values of three independent replicates. The experiments were performed at 37 °C, using 50 µM smooth-LPS (S-LPS) in Tris-HCl buffer (50 mM, pH= 7.4).

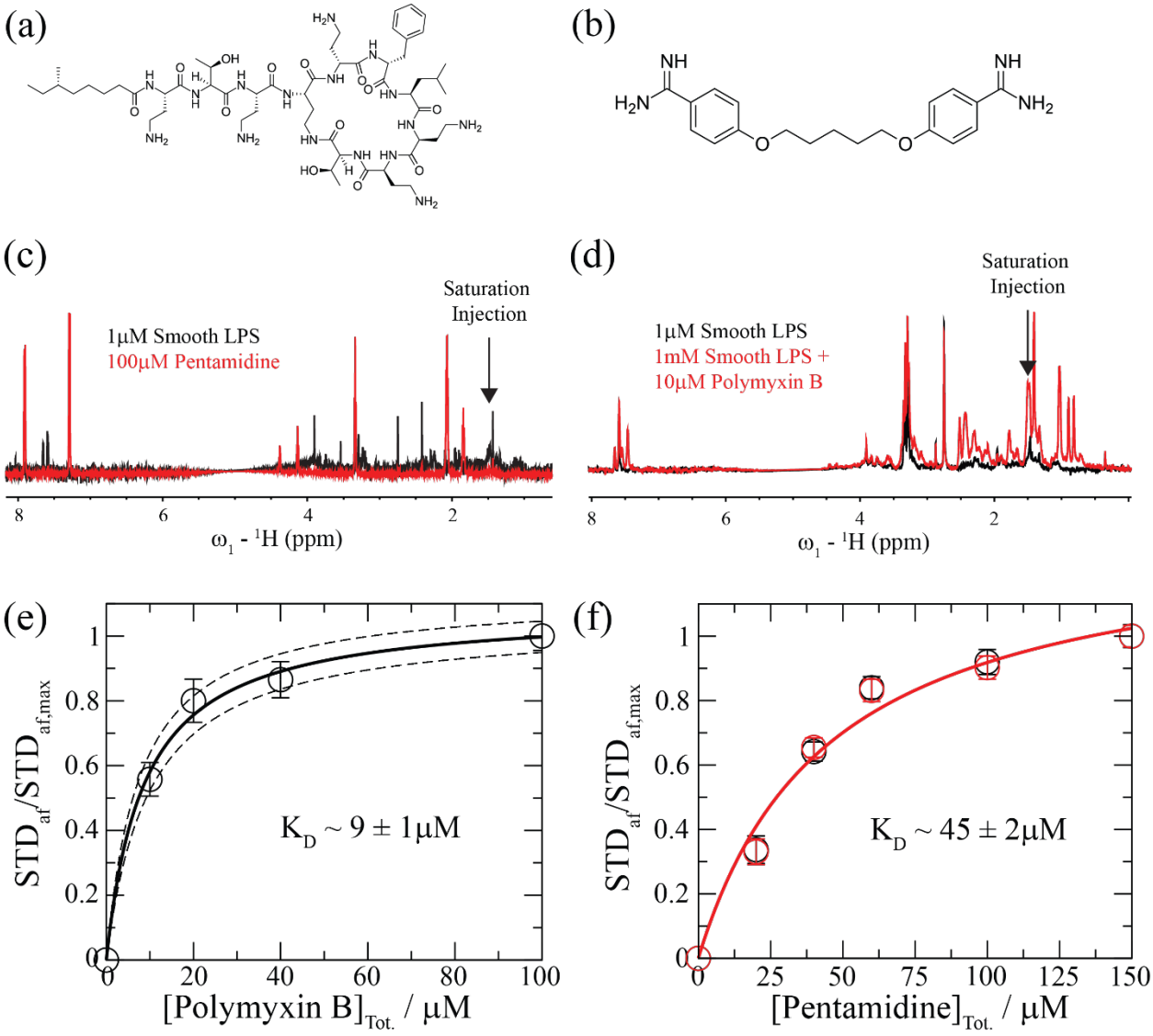


Figure 2.2 – Pentamidine binds LPS with lower affinity than PMB as determined by STD NMR. Molecular structures of (a) Polymyxin B and (b) Pentamidine. (c) ^{1}H NMR comparison of LPS (black) and Pentamidine (red). (d) ^{1}H NMR comparison of LPS without (black) and with (red) Polymyxin B. (e) STD-based binding isotherm for the interaction of LPS with Polymyxin B. Solid line indicates fitting with Scatchard-like binding model. Dashed lines indicate error of

fitting, where fitting error was determined through modeling experimental points plus and minus the error on each individual point. The range of K_ds from these fits were used to determine the error on the K_d. (f) STD-based binding isotherm for the interaction of LPS with Pentamidine. Solid black line and overlapping solid red line indicate fitting of experimental points derived from the two aromatic pentamidine signals between 7 – 8 ppm. The range of K_ds from these two fits were used to determine the error on the K_d.

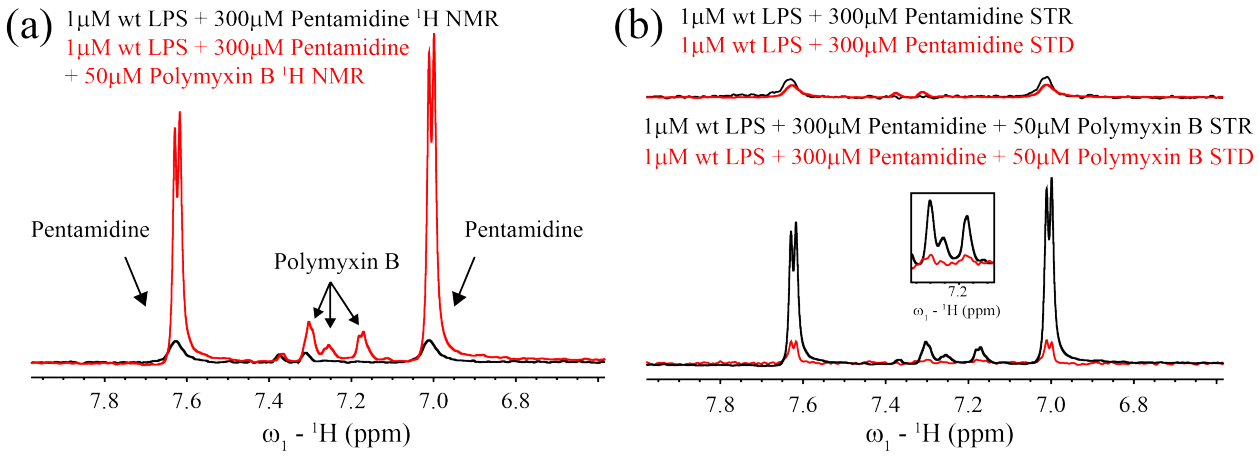


Figure 2.3 – Polymyxin B and Pentamidine binding sites in LPS overlap. (a) ^{1}H-NMR spectra of wild-type LPS and Pentamidine in the absence (black) and presence (red) of Polymyxin B. ^{1}H-NMR resonances of Pentamidine and Polymyxin B are indicated with black arrows. (b) STD (red) and STR (black) spectra of wild-type LPS in the presence of Pentamidine are shown on the top half of the panel, while STD (red) and STR (black) spectra of the same mixture in the presence of Polymyxin B are shown on the bottom. Resonance assignment as shown in (a) follows. Panel inset shows a zoomed-in snapshot of the polymyxin B resonances in the bottom STD and STR spectra.

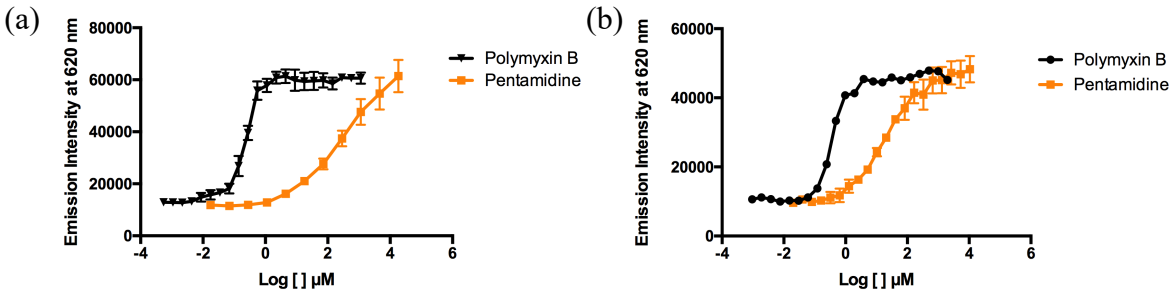


Figure 2.4 – **Pentamidine binds LPS *in vitro* and *in vivo* with a lower affinity than PMB as determined by BODIPY-cadaverine displacement assay.** BODIPY™-TR-Cadaverine (BC) displacement assay was previously used to quantify the binding affinity of compounds to LPS. The fluorescence of BC is quenched upon LPS binding. The displacement of BC by a test compound results in dequenching of fluorescence intensity. BC is displaced from (a) purified rough LPS (R-LPS) and (b) from wild-type *E. coli* cells.

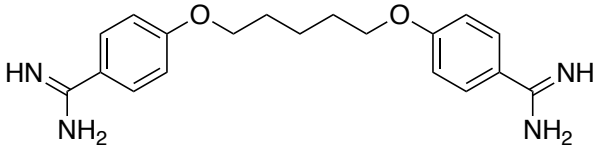
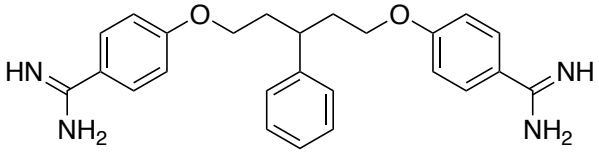
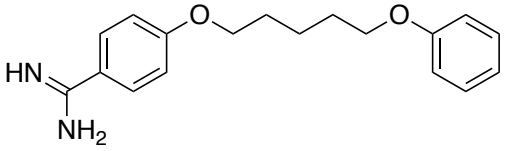
Compound	Structure	ED_{50}[a]	FIC_i[b]
Pentamidine		193 ± 30 μM	0.25
T10		32.8 ± 10 μM	<0.047
T2		239 ± 20 μM	0.517

Table 2.1 – Summary of the LPS-binding and potentiation characteristics of pentamidine analogues. [a]ED_{50} is the concentration of compound that is able to displace 50% of the LPS-binding probe BODIPY™-TR-Cadaverine (BC) and is used here as a measure of affinity to LPS. [b]FIC_i is a measure of potentiation ability where a lower FIC_i value indicates a lower concentration of potentiator compound required to achieve potentiation (see methods section). FIC_is were calculated using rifampicin as a partner antibiotic.

Chapter 3 - Examining LPS-binding drugs for potentiation ability

3.1 Introduction

Antibiotic adjuvants that permeabilize the Gram-negative cell to otherwise impermeable antibiotics would be important tools for combating resistance[4,45,46]. It has long been believed that molecules with an ability to disrupt lipopolysaccharides (LPS) in the outer membrane (OM) by displacing the divalent cations that cross-bridge LPS, permeabilize the cell to large hydrophobic antibiotics[41]. Since potentiators that chelate those cations (such as EDTA) have been deemed of reduced value as drug candidates, the focus has been on polycationic molecules with lipophilic characters that bind the negatively charged LPS[41]. Those characters make such molecules able to interact with human cell membranes and, therefore, toxicity has been a major concern in the development of potentiators[42]. Only one potentiator (SPR741), a polymyxin derivative, is currently in the current clinical development pipeline[2,20]. To discovery and development of safe potentiators, to add to the pipeline, will require careful characterization of the LPS-binding properties that makes those molecules effective and specific to LPS.

Potentiator development has been focused on structures derived from polymyxins and/or other cationic antimicrobial peptides (CAMPS)[4]. However, the recent discovery that pentamidine, a commercially available anti-fungal drug, is an LPS-binding potentiator[46] suggests that there is an overlooked chemical diversity in potentiator discovery. Here, we examine commercially available FDA approved drugs for the ability to bind LPS. 38 out of the 1600 drugs exhibited LPS-binding

ability. However, the majority of those compounds are disinfectants that would be toxic systemically. We investigated amiodarone, an antiarrhythmic medication, spermine, a bare-bones LPS-binding molecule, and neomycin, an aminoglycoside for the ability to potentiate large hydrophobic antibiotics. However, those compounds did not exhibit potentiation suggesting that LPS-binding is not a sole predictor of potentiation ability.

3.2 Results

To identify LPS-binding compounds, we used the BODIPY-cadaverine (BC) displacement assay to screen a library of 1600 FDA approved drugs. An already approved drug that is an LPS-binding potentiator, could be repurposed as a tool to combat resistance[46,54]. BC is believed to bind lipid A phosphates, and, upon binding, its fluorescence emission is quenched[53]. Compounds that bind LPS, displace BC and result in fluorescence enhancement. Moreover, since all compounds screened are at 10 μM, the screening results do not only indicate LPS binding but also quantify the binding (i.e. higher LPS affinity results in higher fluorescence intensity). 38 out of the 1600 drugs exhibited significant fluorescence intensity increase (as determined by a cut-off of two standard deviations above the mean). A full list of those 38 compounds is in appendix I. Most of those hits were antimicrobials and detergent that act by binding LPS and have a considerable toxicity upon systemic use (Figure 3.1 B). Our aim was to uncover a less toxic LPS-binding potentiator and we, therefore, picked amiodarone, an antiarrhythmic medication with an acceptable safety profile[55], for potentiation studies.

To study the potentiation ability of amiodarone, we tested whether it synergizes with novobiocin (a large hydrophobic antibiotic) against *E. coli* using a microbroth dilution chequerboard assay. We found no significant synergy between amiodarone and novobiocin (Figure 3.2). Synergy with other large hydrophobic antibiotics was tested and similar results were obtained (data not shown). This lead us to hypothesize that LPS-binding (as indicated by the BC displacement assay) does not equates to potentiation.

To test the hypothesis that LPS-binding (as indicated by the BC displacement assay), does not equate to potentiation, we studied the potentiation ability of spermine (a bare-bones LPS-binding scaffold[56]). Similar to amiodarone, spermine did not synergize with novobiocin (Figure 3.2). To test the hypothesis further, we examined neomycin, an aminoglycoside known by the literature to bind LPS[56]. Just as amiodarone and spermine, neomycin did exhibit LPS-binding ability (as suggested by the BC displacement assay) but did not potentiate novobiocin effectively (Figure 3.2). We, therefore, concluded that LPS-binding is not a sole predictor of the ability to potentiate.

3.3 Discussion

The discovery and development of LPS-binding potentiators has been fraught with toxicity concerns. While the development of potentiators has been focused on the synthesis of analogs of the toxic class of antibiotics, the polymyxins. Here, we attempted to uncover LPS-binding ability of already approved drugs. The BC displacement assay was developed to the use for discovery of LPS-binding

sequestrants for the treatment of sepsis[53]. While, the assay was successful in pointing out LPS-binding antimicrobials, the ability to bind LPS did not equate to the ability to potentiate hydrophobic antibiotics.

Amiodarone, an anti-arrhythmic medication, and spermine (an LPS-binding polyamine) and neomycin do not potentiate novobiocin (a large hydrophobic antibiotic). Amiodarone possesses a polar portion composed of a positively charged amine and a hydrophobic portion. Such characteristics are believed to give a compound an ability to bind LPS. Spermine possesses four amines, two of them presumably bind the two lipid A phosphates, while the other two bind the two negatively charged KDO moieties. Neomycin possesses multiple positively charged amines and is believed to bind LPS and allow itself access to its intracellular target by the so-called self-promoted uptake pathway[56]. Yet, these molecules did not potentiate novobiocin. In, fact Balakrishna *et. al.*[42] acylated spermine to achieve OM permeabilizing activity. This indicates, while LPS-binding alone does not predict the ability to potentiate, such compounds could be modified to achieve potentiation. Still, the exact LPS-binding characteristics required for OM-permeabilization must be unraveled to help guide the development of potentiators.

3.4 Materials & Methods

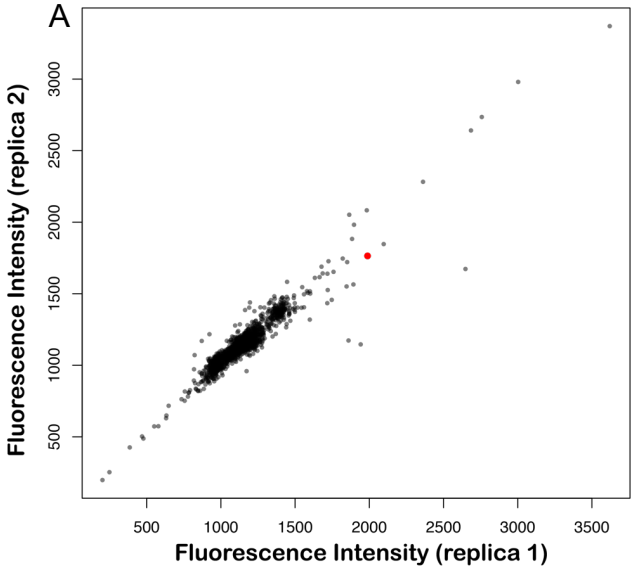
Fluorescence displacement assay (high-throughput screening format). A solution of BC (2 μM) and R-LPS (3 μg/mL) is prepared in Tris-HCl buffer (50 mM, pH= 7.4). 49.5 μL of that solution is added to a Corning Nonbinding Surface Black

384 micro-well plate followed by the addition of 0.5 µL of the test compound (1 mM).

Microbroth chequerboard assay. Chequerboard analyses were conducted in LB media in accordance with CLSI guidelines[57]. At least two biological replicates were done for each combination.

3.5 Figures & Captions

A



B

FI	Compound
3495	CETRIMONIUM BROMIDE
2991.5	CETYLPYRIDINIUM CHLORIDE
2746.5	CHLORHEXIDINE HYDROCHLORIDE
2663	COLISTIN SULFATE
2322	POLYMYXIN B SULFATE
2160.5	TRICHLORMETHINE HYDROCHLORIDE
1972	ALEXIDINE HYDROCHLORIDE
1877	PENTAMIDINE ISETHIONATE
1726	BENZETHONIUM CHLORIDE

Figure 3.1 – High-throughput screening reveals 38 LPS-binding compounds. A) a replica plot displaying the result of a high-throughput screen of 1600 compounds using BC displacement assay. Red dot is pentamidine. B) the top 9 hits of the screen are all antimicrobials and detergent that act by binding LPS (FI = fluorescence intensity).

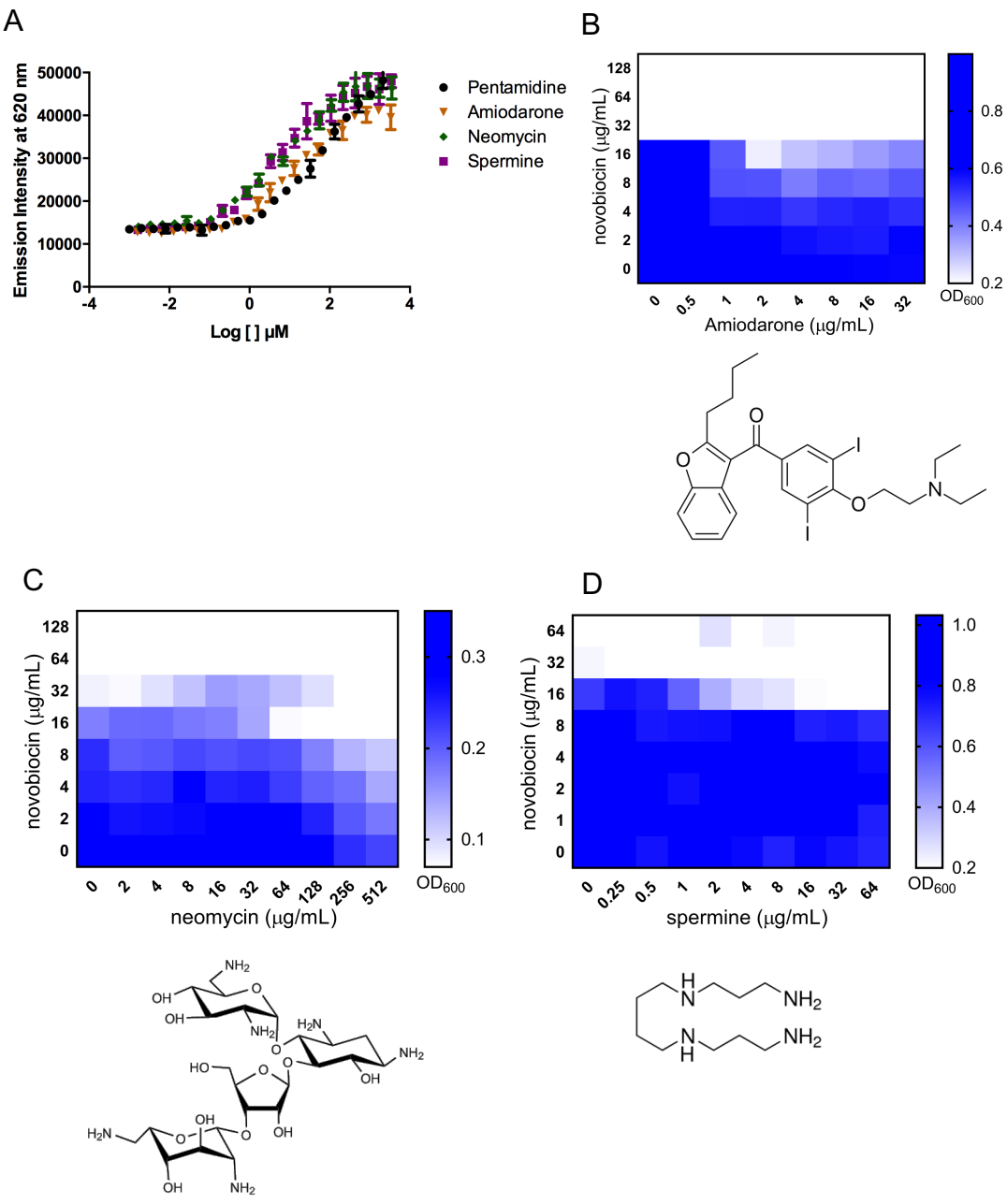


Figure 3.2 – Amiodarone, neomycin and spermine bind LPS but do not potentiate novobiocin. (A) BC displacement assay shows that amiodarone, neomycin and spermine bind LPS. (B) (C) and (D) are microbroth chequerboard assays showing that amiodarone, neomycin and spermine, respectively,

do not potentiate novobiocin effectively. (C) is obtained using an aminoglycoside resistant *E. coli* strain, while (B) and (D) using wild-type *E. coli.* The chemical structure of amiodarone, spermine and neomycin is indicated below each corresponding chequerboard. Synergy with other large hydrophobic antibiotics was tested and similar results were obtained (data not shown).

Chapter 4 - Studying the affinity of PMB to LPS under *mcr-1* modification

4.1 Introduction

Mobile colistin resistance (*mcr*-1) threatens the utility of the widely regarded last resort antibiotic class the polymyxins[58]. The carbapenem resistant Gram-negative pathogens *A. baumannii*, *P. aeruginosa*, and *Enterobacteriaceae* are ranked critical priority pathogens by the WHO[1]. The polymyxin class of antibiotics is a last resort treatment option that is used in treating carbapenem resistant Gram-negative infections[27]. However, the discovery of a plasmid-borne colistin resistance gene (*mcr-1*) that is rapidly disseminating, greatly challenges the use of polymyxins[36,58]. The loss of polymyxins from the already depleted Gram-negative antibiotic arsenal threatens the rise of pan-resistant pathogens. Fortunately, our group recently discovered that polymyxins themselves can be used, in combination with a partner large hydrophobic antibiotic, to treat *mcr-1*-expressing Gram-negative pathogens.

The polymyxin class of antibiotics—polymyxin B (PMB) and E (also known as colistin)—interact with lipopolysaccharides (LPS) in the outer leaflet of the outer membrane (OM) of Gram-negative cells. Polymyxins' mechanism of action is debatable and is still being studied to this date. However, there seems to be a consensus that, polymyxins through their positively charged diaminobutyrate (DAB), displace Ca^{+2} and Mg^{+2} ions that cross-bridge adjacent LPS molecules and perturb the OM. Doing so, they gain access to the inner-membrane (IM) where they disrupt proton motive force (PMF) and cause cell death[32].

Mcr-1 is a phosphoethanolamine (PETN) transferase that adds a PETN residue to a lipid A phosphate of LPS (Figure 4.1). That modification is believed to significantly lower polymyxins' affinity to LPS and result in resistance[58]. However, we have previously demonstrated that, although *mcr-1* expression results in a large (~16- to 32- fold) increase in the minimum inhibitory concentration (MIC) of polymyxins (Figure 4.1), polymyxins' ability to disrupt the OM is only slightly (~2- to 3- fold) decreased[45]. This finding allowed us to take advantage of colistin's persistent ability to disrupt the OM by using it to permeabilize the cell to large hydrophobic antibiotics and inhibit the growth of *mcr-1*-carrying pathogens[45]. Indeed, a combination therapy of colistin and rifampicin was found effective in treating mice infected with *mcr-1* positive *Klebsiella pneumoniae*[45]. Those results warrant a careful examination of the mechanism of *mcr-1* for a better understanding of using membrane permeabilizers as tools for overcoming *mcr-1* resistance.

Here, we study the interaction of PMB with LPS purified from *mcr-1*-expressing *Salmonella* Typhimurium and compare it to that of wild-type. Our results indicate that, the affinity of PMB to LPS is only reduced by ~4-fold upon *mcr-1* expression, which is consistent with our earlier discovery of the persistence of polymyxins' permeabilizing ability[45]. These results do not explain the large (~16-fold) increase in PMB's MIC and lead us to believe that the mechanism of *mcr-1* is more complex than previously thought to be, probably having other effects than simply reducing PMB's affinity to LPS.

4.2 Results

It is widely accepted that a phosphoethanolamine (PETN) addition to the lipid A phosphate of LPS decreases PMB affinity to LPS[36]. To quantify that affinity and to highlight additional thermodynamic binding parameter in the context of *mcr-1* expression, we purified LPS from wild-type and *mcr-1*-expressing *Salmonella* Typhimurium and studied their interaction with PMB using isothermal titration calorimetry (ITC). The dissociation constant (Kd) of PMB to *mcr-1*-modified LPS was determined to be 12.6 ± 1.5 μM, while that of PMB to wild-type LPS was determined to be 3.1 ± 0.4 μM (Figure 4.2). Both interactions were found enthalpically-driven, however, *mcr-1* modification of LPS resulted in a less enthalpic drive (Figure 4.2). The slight (~4-fold) decrease in PMB's affinity to LPS upon *mcr-1* expression was found surprising since it does not fully explain the large (~16-fold) change in PMB's MIC of those two strains. That unexpected finding lead us to explore other methods to measure PMB affinity to LPS.

BODIPY-cadaverine (BC) (Figure 4.3 a) is an LPS binding fluorophore used previously by literature to measure affinity to LPS[53]. BC is believed to bind lipid A phosphates, and, upon binding, its fluorescence emission is quenched. To assess the difference *mcr-1* makes to BC's ability to bind LPS we studied BC's interaction with wild-type cells, *mcr-1*-expressing cells, and LPS purified from those two strains (Figure 4.3 b & c). We found no difference in BC's affinity to LPS upon *mcr-1* modification. Following that, we used PMB to compete for LPS binding and displace LPS-bound BC. The results show no difference in PMB ability to displace

BC from wild-type LPS and *mcr-1*-modified LPS (Figure 4.3 d). We were interested in examining how those results compare to the difference in PMB's ability to disrupt and permeabilize the OM upon *mcr-1* expression.

To examine PMB's ability to perturb the OM under *mcr-1* expression, we used the hydrophobic probe N-phenyl-1-naphthylamine (NPN). An intact OM is impermeable to NPN, however under OM disruption, NPN binds the phospholipid layer and fluoresces. When *mcr-1* is expressed, it only takes ~3-folds the amount of PMB to achieve the same OM permeability as wild-type (Figure 4.4). The results that PMB's affinity to LPS and its ability to disrupt the OM is only ~3- to 4-fold lower under *mcr-1* influence lead us to hypothesize that only a small proportion of LPS molecules are modified under *mcr*-1 expression.

To investigate the proportion of modified molecules in our LPS sample that is purified from *mcr-1*-expressing cells, we hydrolyzed LPS to lipid A and examined its chemical composition using MALDI-TOF mass spectrometry. The results show that the peak which corresponds to the modified species is of a much lower intensity when compared to the peak of the unmodified species (Figure 4.5). Assuming that LPS hydrolysis to lipid A, and the soft ionization technique (MALDI) leave the PETN decoration intact, this result suggests that only a small proportion of lipid A molecules carry a PETN modification.

4.3 Discussion

Despite its current importance in combating MDR Gram-negative infections, much remains unclear about polymyxin's mechanism of action (MOA), especially

under *mcr-1* expression. There is a general belief that polymyxins' MOA involves three steps. First, through their positively charged diaminobutyrate (DAB), they bind the negatively charged lipid A phosphates of LPS, displacing divalent cations that cross-link adjacent LPS molecules and disrupting the barrier properties of the OM. Secondly, taking advantage of the altered OM integrity, polymyxins transverse to the inner membrane (IM). Ultimately, they interact with the IM, disrupting proton motive force (PMF) and causing leakage of cytosolic contents and cell death. In the case of *mcr-1* resistance, because *mcr-1* is a PETN transferase that adds a PETN to a lipid A phosphate, replacing one of lipid A's negative charges with a positive charge, it is expected that the affinity of polymyxins to LPS to be reduced, affecting the first step mentioned earlier. Additionally, because the expression of *mcr-1* in Gram-negative bacteria (such as *E. coli*, *K. pneumoniae*, *A. baumannii* and *S.* Typhimurium) generally results in a large ~16-fold increase of polymyxin's MIC[59,60], it is expected that the modification of LPS by *mcr-1* to result in a comparable dramatic reduction in polymyxin's affinity to LPS and consequently its ability to interact with, disrupt and permeabilize the OM. However, we have demonstrated previously that polymyxin's ability to permeabilize the cell is only very slightly (~2- to 3- folds) decreased upon *mcr-1* expression. Moreover, here we describe that polymyxin's affinity to LPS extracted from *mcr-1* expressing *S*. Typhimurium cells is only slightly (~4-fold) reduced when compared to wild-type. Those results are most probably a result of *mcr-1* modifying only a small proportion

of LPS molecules. Yet, those results indicate that the mechanism of *mcr-1* resistance involves more than just interfering with the first step in polymyxin's MOA.

Those previous findings are consistent with the belief that the direct target for PMB is not LPS but the inner-membrane (IM)[32]. One possible explanation to how a small change in LPS affinity and OM permeability could lead to a large change in MIC is that, since PMB disrupts the proton motive force (PMF) across the inner membrane, when a small amount of PMB makes it to the IM and disrupts PMF, the periplasmic space becomes less acidic and drives even more of the highly positively-charged PMB molecules to that periplasmic space and results in an amplified outcome.

A more comprehensive understanding of how *mcr-1* grants Gram-negative bacteria resistance against polymyxins is needed. It seems that the mechanism of *mcr-1* resistance is more complex than previously thought, and it appears that the affinity argument is too simplistic. Evidence suggests that only a small proportion of LPS molecules is modified under *mcr-1* expression. Indeed, Yang *et al.*[61] discovered that there is a balance that bacteria must achieve when expressing *mcr-1*, because bacteria that over-expresses *mcr-1* have growth and viability impairment and are less fit. Moreover, in a pre-print, Li *et al.*[62] demonstrate that upon *mcr-1* expression, there is an accumulation of PETN in the membranes, and notice a protein expression pattern that is consistent with colistin efflux. Undoubtedly, under *mcr-1* expression, such effects could interfere with the second and the third steps of polymyxin's MOA mentioned above. However, more

comprehensive studies of the mechanism of *mcr-1* at the cellular and molecular levels are necessary for a more comprehensive understanding.

Although challenging, an isolation, or an *in-vitro* production, of a pure PETN-modified LPS or lipid A species could assist in understanding *mcr-1* at the molecular level. Although it is unlikely that all LPS molecules at the OM to be PETN-modified for fitness costs[61]. Studying the interaction between polymyxins and pure PETN-modified LPS or lipid A species could help shed more light on the mechanism of *mcr-1*. It is possible that PMB, utilizing its +5 charges, is still able to interact with, and bind, an PETN-modified LPS molecule since LPS still possesses various negative charges at the other lipid A phosphate, the KDO sugars and the phosphate of the core-oligosaccharide region. Combining different proportions of modified and unmodified LPS species, and probing PMB's interaction with such samples, could help test hypothesis regarding *mcr-1* mechanism as well.

Much remains unclear regarding polymyxins' mechanism of action, and the effect *mcr*-1 has on the mechanism thereof. Here, we highlight the binding parameters of PMB to LPS purified from *mcr*-1 expressing *Salmonella* Typhimurium. We measure a slight (~4-fold) reduction in PMB's ability to bind LPS upon *mcr-1* modification. This leads us to hypothesize that *mcr-1* mechanism of resistance is more complex than previously thought to be. It could involve more than just reducing polymyxin's affinity to LPS. Fortunately, the mechanism of *mcr-1* allows for the use of OM-perturbants to inhibit the growth of *mcr*-1 carrying Gram-negative pathogens, and that strategy must be exploited in drug discovery efforts.

4.4 Materials & Methods

LPS and lipid A isolation. LPS was extracted using an LPS extraction kit (Intron Biotechnology) and following the modified protocol described previously[63]. LPS samples were further purified by removing contaminating proteins, nucleic acids and phospholipids as previously described[64]. LPS was hydrolyzed to lipid A by mild acid hydrolysis as described previously[63].

KDO assay. Molar concentrations of LPS were quantified following the KDO assay as described previously[65].

Isothermal titration calorimetry (ITC). Solutions of LPS (~50 μM as determined by the KDO assay) and PMB (500 μM) in HEPES buffer (10 mM, pH=7.4) were prepared and degassed for 15 minutes. 24 injections of 2 μL PMB solution were added into the LPS solution (cell volume 170 μL) at 240 second intervals at 37 ºC. The first injection (0.48 μL) was excluded from analysis. Nano ITC machine (TA Instruments) was used to carry out the titration and NanoAnalyze (TA Instruments) software was used for data analysis. Control experiments of injecting buffer into the LPS solution and PMB into buffer were performed.

BODIPY-cadaverine LPS binding and displacement assays. BODIPY-cadaverine (BC; Fisher Scientific), at a concentration of 1 μM, was added to a serial dilution of wild-type cells, *mcr-1*-expressing cells, and LPS purified from those two strains in a non-binding surface (NBS) Black 384 microwell plate (Corning) (final volume 40 μL, in 10 mM HEPES, pH=7.4 buffer). To displace BC from LPS, 10 μL of a serial dilution of PMB was added to 30 μL of the quenched

solution in the microtiter plate. Fluorescence intensity was read by a Tecan Infinite Pro m10 (excitation 580 nm, emission 620 nm), with bandwidths set to 5 nm.

NPN permeability assay. The NPN assays were conducted as previously established[66]. Cells from an overnight culture were diluted 1/100 and incubated until mid-log (~ 0.5 OD 600 nm), centrifuged, washed in 5mM HEPES buffer (pH 7.2), spun down and resuspended in the same buffer to an OD (600 nm) of 0.5. NPN solution (40 μM) was prepared in buffer. A volume of 100 μL of cells was added to 90 μL of the NPN solution in black 96-well plates and the fluorescence intensity was recorded. Following that, 10 μL of a serial dilution of PMB was added and the florescence intensity was recorded at various time points. 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 as described previously[67], by dividing each data point by the fluorescence intensity at a concentration of 100 μgmL−1 of PMB, which is beyond the observed plateau in fluorescence for both strains.

MALDI TOF mass spectrometry. Lipid A samples (1 mg/mL dissolved in 1:1 chloroform:methanol) were mixed with a 9-aminoacridine methanol solution at a ratio of 1:1, and then 1 μL of that mixture was spotted on the plate. Bruker UltrafleXtreme MALDI TOF/TOF instrument was used to record the spectra in the negative ion mode. Peptide solution was used as an external standard.

4.5 Figures & Captions

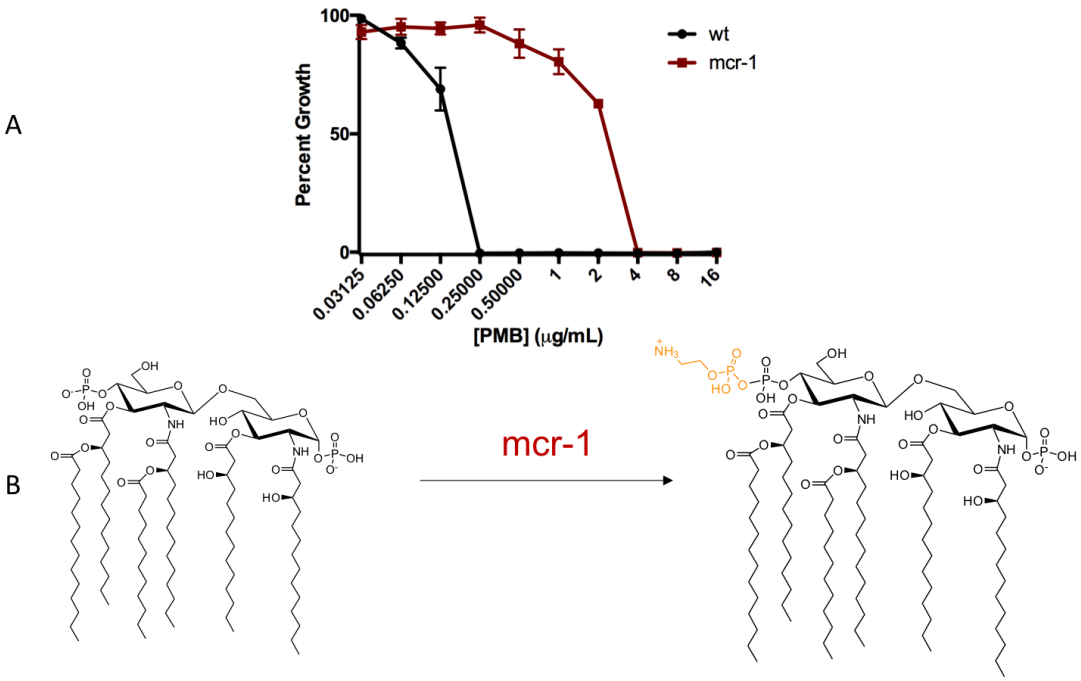


Figure 4.1 – ***mcr-1*** **confers *S.* Typhimurium resistance to PMB.** (A) Growth curves of wild-type *S.* Typhimurium (black) and *S.* Typhimurium pGDP2:*mcr*-1 under different PMB concentrations, showing a ~16-fold shift in MIC. (B) *mcr-1* adds a phosphoethanolamine (PETN) to lipid A phosphate.

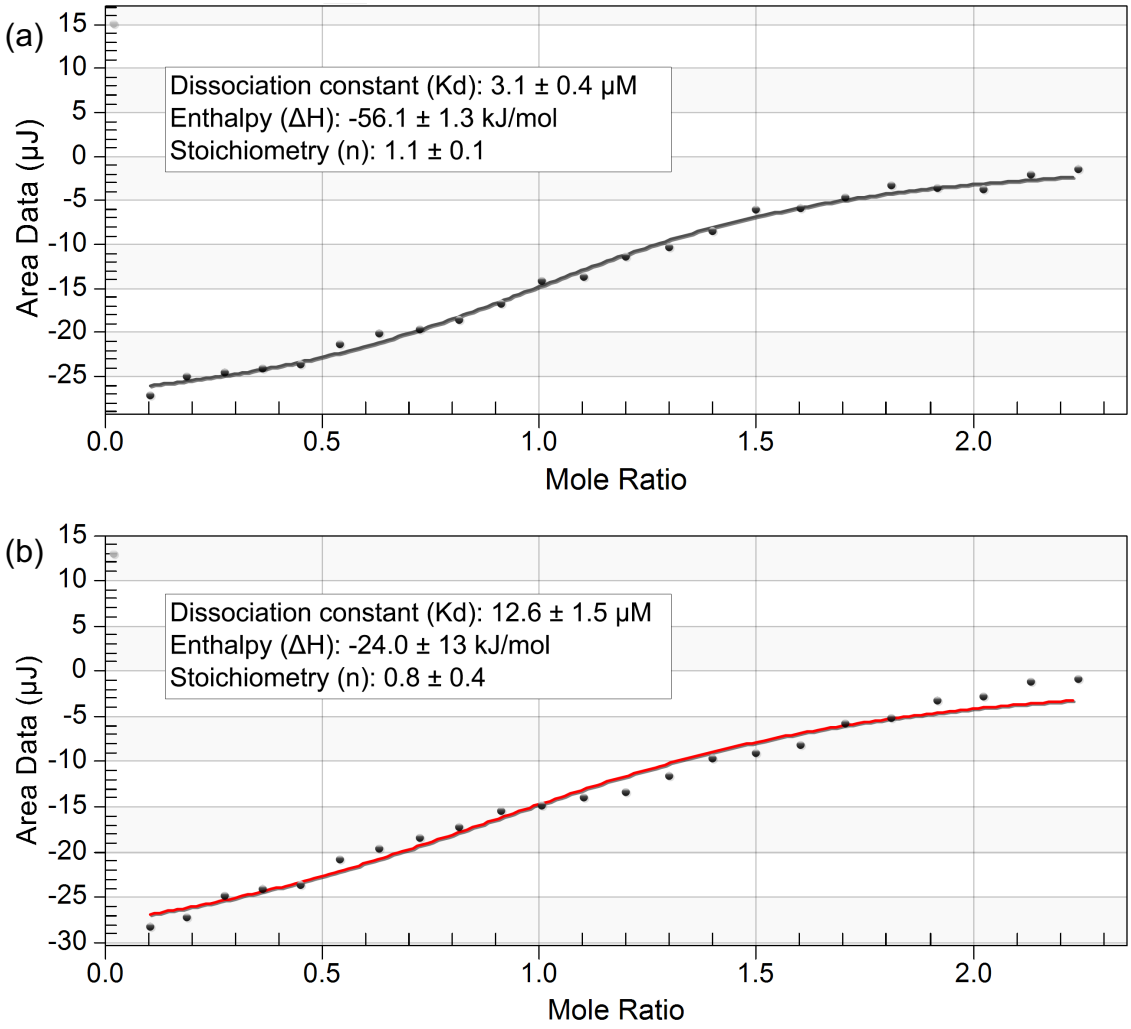


Figure 4.2 – Isothermal titration calorimetry (ITC) indicates a slightly lower affinity of PMB to LPS upon *mcr-1* modification as compared to wild-type LPS. The binding curves PMB to (a) LPS extracted from wild-type *S.* Typhimurium and (b) LPS extracted from *mcr-1*-expressing *S.* Typhimurium. Insets: the thermodynamic parameters of each interaction as calculated by data fitting. The parameters represent the mean values of three independent replicates. The experiments were performed at 37 °C, using 50 μM LPS (LPS) in HEPES buffer (50 mM, pH= 7.4).

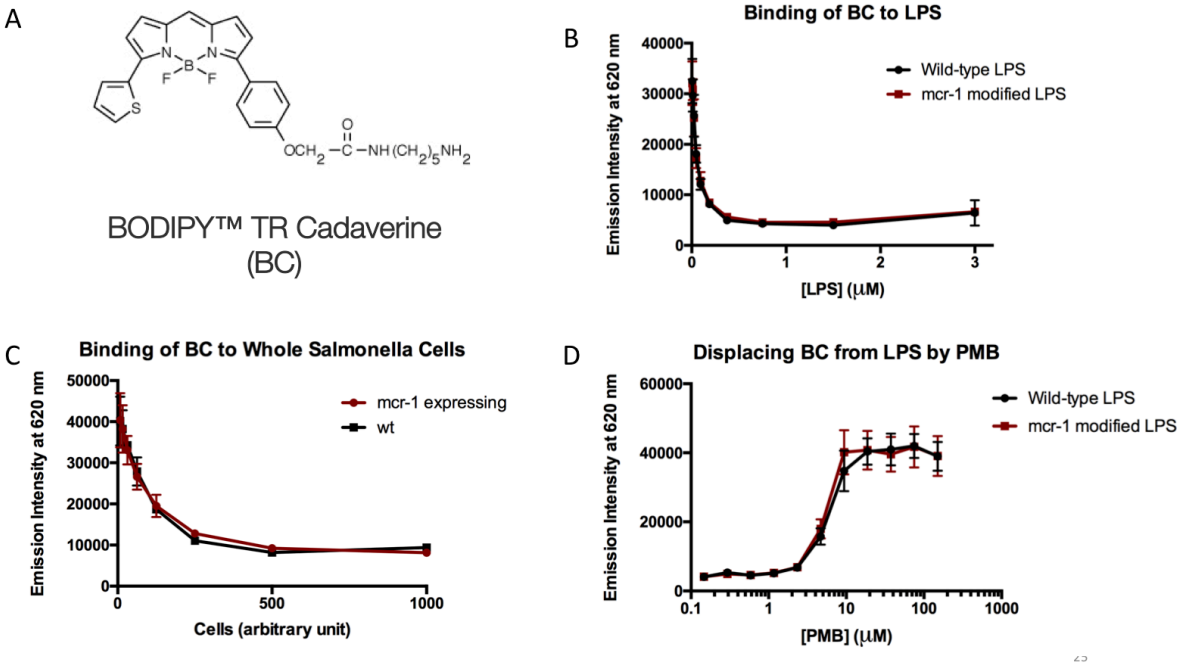


Figure 4.3 – BODIPY-Cadaverine assay indicates no difference in LPS binding upon *mcr-1* modification. The BODIPY-cadaverine probe (A) binds to the lipid A phosphates of LPS, where its fluorescence is quenched. wild-type cells, *mcr-1*-expressing cells (C), and LPS purified from those two strains (B) bind BC with no difference in affinity. (D) The ability of PMB to displace BC from LPS is unaltered upon *mcr-1* modification of LPS.

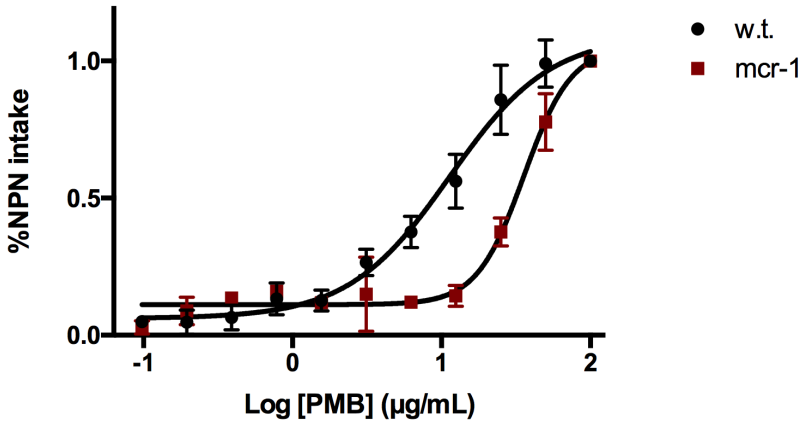


Figure 4.4 – **PMB ability to permeabilize the OM is ~3-folds lower under *mcr-1* expression.** N-Phenyl-1-naphthylamine (NPN) uptake of wild-type (black) and *mcr-1*-expressing *S.* Typhimurium (red) induced by PMB. NPN uptake (%) represents the background subtracted fluorescence divided by the fluorescence observed at 100 µg/mL of PMB. The IC_{50} values of PMB is the concentration required for 50% permeabilization, are 11 ± 2 µg/mL and 36 ± 3 µg/mL for the wild-type and the *mcr-1* expressing cells respectively.

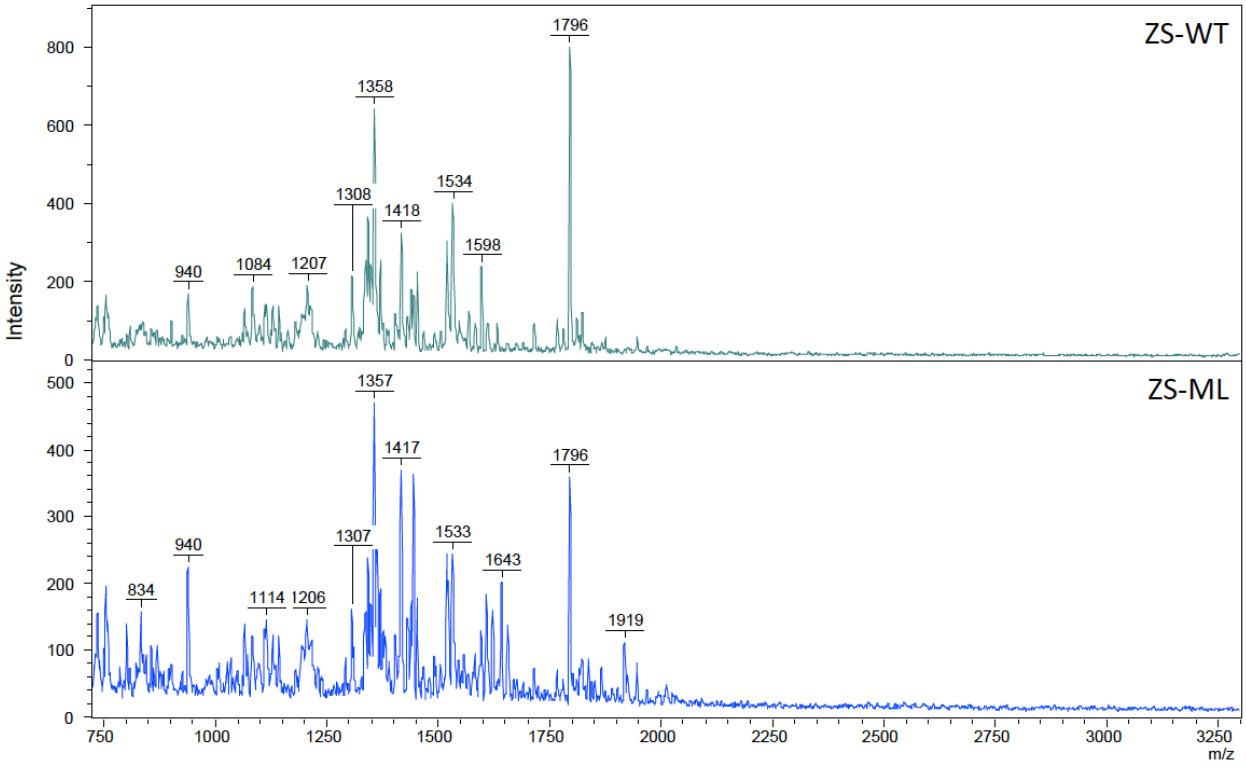


Figure 4.5 – **MALDI-TOF MS analysis of lipid A indicate that *mcr-1* modifies a small proportion of LPS.** (Top) *S.* Typhimurium wild-type lipid A with *m/z* = 1796. (Bottom) *mcr-1*-expressing *S.* Typhimurium lipid A at *m/z* = 1796, and lipid A + PETN at 1919 *m/z*. The peak at 1919 *m/z* has a lower intensity indicating a small proportion of modification. Spectra were obtained in negative ion mode in this 9-aminoacridine matrix.

Chapter 5 - Conclusion

The evolution of Gram-negative pathogens that are resistant to all currently known antibiotics makes the discovery of new therapeutic strategies urgent. This project examined LPS-binding potentiators for their ability to permeabilize the Gram-negative cell to otherwise ineffective antibiotics. This strategy can overcome *mcr-1* mediated colistin resistance, and, therefore is very powerful. LPS is believed to be potentiators' direct target. However, the specific LPS-binding characteristics for LPS-specific targeting is not understood.

This work elucidated the mechanism of action of the LPS-binding potentiator pentamidine. Pentamidine's affinity to LPS agreed with its concentration required for potentiation and OM-disruption. It is proposed that pentamidine's affinity to LPS could be improved, potentially increasing specificity and lowering off-target toxicity. The project, also, uncovered currently approved drugs with LPS-binding ability. While LPS-binding did not equate to potentiation, those structures have a well characterized safety profiles and their structures could be a starting point for potentiator development. Lastly, this work elucidated PMB interaction with LPS under *mcr-1* conditions. It was determined that, while the *mcr-1* mechanism of action is complex, it allows for the use of potentiators to permeabilize *mcr-1* carrying cells and overcome colistin resistance. This strategy must be exploited by drug discovery efforts, to combat mobile colistin resistance.

Much remains to be studied in the design and development of potentiators. Ideally, a description of an LPS-binding pharmacophore for an effective

potentiation would be invaluable. However, that pharmacophore must address specificity and toxicity concerns. The development of such pharmacophore would require comprehensive *in vivo* and *in vitro* efficacy and toxicity studies. Pentamidine, for its relatively simple chemical structure, could serve as tool for such studies. Using pentamidine analogues, a structure-activity relationship (SAR) could be established to construct correlations between thermodynamic LPS-binding parameters, permeabilization ability (using the NPN assay), *in vitro* efficacy (by chequerboard assays), *in vitro* efficacy (using mouse infection models) and toxicity (as reported by various assays such as the blood cell lysis assay).

The molecular mechanism of action (MOA) of LPS-binding potentiators and their effects on the OM are not fully understood. Determining whether LPS-binding potentiators cause LPS release from the OM, phospholipid patches at the OM, and/or cracks at the OM would help highlight the MOA. Biophysical and/or genomic approaches could provide useful information in such studies. This deep understanding of LPS-binding potentiators' MOA would help develop and introduce potentiators as powerful tools for the use in the clinic to combating the Gram-negative resistance crisis.

References

1. World Health Organisation. WHO publishes list of bacteria for which new antibiotics are urgently needed. *Who* (2017). doi:/entity/mediacentre/news/releases/2017/bacteria-antibiotics-needed/en/index.html
2. WHO, W. H. O. *Antibacterial Agents in Clinical Development*. *ANTIBACTERIAL AGENTS IN CLINICAL DEVELOPMENT An analysis of the antibacterial clinical development pipeline, including tuberculosis* (2017). doi:10.1016/S0065-7743(08)60924-0
3. Zgurskaya, H. I., López, C. A. & Gnanakaran, S. Permeability Barrier of Gram-Negative Cell Envelopes and Approaches To Bypass It. *ACS Infect. Dis.* **1,** 512–522 (2015).
4. Zabawa, T. P., Pucci, M. J., Parr, T. R. & Lister, T. Treatment of Gram-negative bacterial infections by potentiation of antibiotics. *Curr. Opin. Microbiol.* **33,** 7–12 (2016).
5. Raetz, C. R. H. & Whitfield, C. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71,** 635–700 (2002).
6. Silhavy, T. J., Kahne, D. & Walker, S. The bacterial cell envelope. *Cold Spring Harb. Perspect. Biol.* **2,** a000414 (2010).
7. Raetz, C. R. *et al.* Gram-negative endotoxin: an extraordinary lipid with profound effects on eukaryotic signal transduction. *FASEB J.* **5,** 2652–60 (1991).
8. Raetz, C. R. H. *et al.* Kdo2-Lipid A of *Escherichia coli*, a defined endotoxin that activates macrophages via TLR-4. *J. Lipid Res.* **47,** 1097–111 (2006).

9. Hancock, R. E. W. Alterations in Outer Membrane Permeability. *Annu. Rev. Microbiol.* **38,** 237–264 (1984).

10. Powers, M. J. & Trent, M. S. Expanding the paradigm for the outer membrane: *Acinetobacter baumannii* in the absence of endotoxin. *Mol. Microbiol.* **107,** 47–56 (2018).

11. Moffatt, J. H. *et al.* Colistin resistance in *Acinetobacter baumannii* is mediated by complete loss of lipopolysaccharide production. *Antimicrob. Agents Chemother.* **54,** 4971–7 (2010).

12. Raetz, C. R. H. *et al.* Discovery of new biosynthetic pathways: the lipid A story. *J. Lipid Res.* **50 Suppl,** S103-8 (2009).

13. Wang, X. & Quinn, P. J. Lipopolysaccharide: Biosynthetic pathway and structure modification. *Prog. Lipid Res.* **49,** 97–107 (2010).

14. Bowyer, A., Baardsnes, J., Ajamian, E., Zhang, L. & Cygler, M. Characterization of interactions between LPS transport proteins of the Lpt system. *Biochem. Biophys. Res. Commun.* **404,** 1093–8 (2011).

15. Zhang, G., Meredith, T. C. & Kahne, D. On the essentiality of lipopolysaccharide to Gram-negative bacteria. *Curr. Opin. Microbiol.* **16,** 779–85 (2013).

16. Onishi, H. R. *et al.* Antibacterial agents that inhibit lipid A biosynthesis. *Science* **274,** 980–2 (1996).

17. Tan, J. H. *et al.* In Vitro and In Vivo Efficacy of an LpxC Inhibitor, CHIR-090, Alone or Combined with Colistin against *Pseudomonas aeruginosa* Biofilm. *Antimicrob. Agents Chemother.* **61,** (2017).

18. Caughlan, R. E. *et al.* Mechanisms decreasing i*n vitro* susceptibility to the LpxC

inhibitor CHIR-090 in the gram-negative pathogen Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **56,** 17–27 (2012).

19. Wang, Z., Wang, J., Ren, G., Li, Y. & Wang, X. Influence of Core Oligosaccharide of Lipopolysaccharide to Outer Membrane Behavior of Escherichia coli. *Mar. Drugs* **13,** 3325–39 (2015).
20. Corbett, D. *et al.* Potentiation of Antibiotic Activity by a Novel Cationic Peptide: Potency and Spectrum of Activity of SPR741. *Antimicrob. Agents Chemother.* **61,** (2017).
21. Zurawski, D. V. *et al.* SPR741, an antibiotic adjuvant, potentiates the in vitro and in vivo activity of rifampin against clinically relevant extensively drug-resistant *Acinetobacter baumannii*. *Antimicrob. Agents Chemother.* (2017). doi:10.1128/AAC.01239-17
22. Stainton, S. M. *et al.* Assessment of the In Vivo Activity of SPR741 in Combination with Azithromycin against Multidrug-Resistant Enterobacteriaceae Isolates in the Neutropenic Murine Thigh Infection Model. *Antimicrob. Agents Chemother.* **62,** (2018).
23. Martin-Loeches, I., Dale, G. E. & Torres, A. Murepavadin: a new antibiotic class in the pipeline. *Expert Rev. Anti. Infect. Ther.* (2018). doi:10.1080/14787210.2018.1441024
24. Rello, J., Valenzuela-Sánchez, F., Ruiz-Rodriguez, M. & Moyano, S. Sepsis: A Review of Advances in Management. *Adv. Ther.* **34,** 2393–2411 (2017).
25. Kaconis, Y. *et al.* Biophysical mechanisms of endotoxin neutralization by cationic amphiphilic peptides. *Biophys. J.* (2011). doi:10.1016/j.bpj.2011.04.041

26. Sil, D. *et al.* Biophysical Mechanisms of the Neutralization of Endotoxins by Lipopolyamines. *Open Biochem. J.* (2013). doi:10.2174/1874091X01307010082

27. Morrill, H. J., Pogue, J. M., Kaye, K. S. & LaPlante, K. L. Treatment Options for Carbapenem-Resistant Enterobacteriaceae Infections. *Open Forum Infect. Dis.* **2,** ofv050-ofv050 (2015).

28. Zavascki, A. P., Goldani, L. Z., Li, J. & Nation, R. L. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. *J. Antimicrob. Chemother.* **60,** 1206–15 (2007).

29. Kwa, A., Kasiakou, S. K., Tam, V. H. & Falagas, M. E. Polymyxin B: similarities to and differences from colistin (polymyxin E). *Expert Rev. Anti. Infect. Ther.* **5,** 811–21 (2007).

30. O'Driscoll, N. H., Cushnie, T. P. T., Matthews, K. H. & Lamb, A. J. Colistin causes profound morphological alteration but minimal cytoplasmic membrane perforation in populations of *Escherichia coli* and *Pseudomonas aeruginosa*. *Arch. Microbiol.* (2018). doi:10.1007/s00203-018-1485-3

31. Schindler, M. & Osborn, M. J. Interaction of divalent cations and polymyxin B with lipopolysaccharide. *Biochemistry* **18,** 4425–30 (1979).

32. Deris, Z. Z. *et al.* Probing the penetration of antimicrobial polymyxin lipopeptides into gram-negative bacteria. *Bioconjug. Chem.* (2014). doi:10.1021/bc500094d

33. Zhang, L., Dhillon, P., Yan, H., Farmer, S. & Hancock, R. E. W. Interactions of bacterial cationic peptide antibiotics with outer and cytoplasmic membranes of Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.* **44,** 3317–21 (2000).

34. Yu, Z., Qin, W., Lin, J., Fang, S. & Qiu, J. Antibacterial mechanisms of polymyxin

and bacterial resistance. *Biomed Res. Int.* **2015,** 679109 (2015).

35. Needham, B. D. & Trent, M. S. Fortifying the barrier: the impact of lipid A remodelling on bacterial pathogenesis. *Nat. Rev. Microbiol.* **11,** 467–81 (2013).
36. Liu, Y.-Y. *et al.* Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. *Lancet Infect. Dis.* **16,** 161–168 (2016).
37. Caselli, E., D'Accolti, M., Soffritti, I., Piffanelli, M. & Mazzacane, S. Spread of mcr-1 –Driven Colistin Resistance on Hospital Surfaces, Italy. *Emerg. Infect. Dis.* **24,** 1752–1753 (2018).
38. Saavedra, S. Y. *et al.* Genomic and Molecular Characterization of Clinical Isolates of *Enterobacteriaceae* Harboring mcr-1 in Colombia, 2002 to 2016. *Antimicrob. Agents Chemother.* **61,** (2017).
39. Mendes, A. C. *et al.* mcr-1 in Carbapenemase-Producing *Klebsiella pneumoniae* with Hospitalized Patients, Portugal, 2016–2017. *Emerg. Infect. Dis.* **24,** 762–766 (2018).
40. Watkins, R. R., Smith, T. C. & Bonomo, R. A. On the path to untreatable infections: colistin use in agriculture and the end of 'last resort' antibiotics. *Expert Rev. Anti. Infect. Ther.* **14,** 785–8 (2016).
41. Vaara, M. Agents that increase the permeability of the outer membrane. *Microbiol. Rev.* **56,** 395–411 (1992).
42. Balakrishna, R. *et al.* Structural correlates of antibacterial and membrane-permeabilizing activities in acylpolyamines. *Antimicrob. Agents Chemother.* **50,** 852–61 (2006).

43. Delcour, A. H. Outer membrane permeability and antibiotic resistance. *Biochim. Biophys. Acta* **1794,** 808–16 (2009).

44. Mohamed, Y. F., Abou-Shleib, H. M., Khalil, A. M., El-Guink, N. M. & El-Nakeeb, M. A. Membrane permeabilization of colistin toward pan-drug resistant Gram-negative isolates. *Braz. J. Microbiol.* **47,** 381–8 (2016).

45. MacNair, C. R. *et al.* Overcoming mcr-1 mediated colistin resistance with colistin in combination with other antibiotics. *Nat. Commun.* **9,** 458 (2018).

46. Stokes, J. M. *et al.* Pentamidine sensitizes Gram-negative pathogens to antibiotics and overcomes acquired colistin resistance. *Nat. Microbiol.* **2,** 17028 (2017).

47. Díaz, M. V. *et al.* Pentamidine exerts in vitro and in vivo anti Trypanosoma cruzi activity and inhibits the polyamine transport in Trypanosoma cruzi. *Acta Trop.* **134,** 1–9 (2014).

48. Petiz, L. L. *et al.* Effects of a new antiprotozoal drug, N, N′-diphenyl-4-methoxy-benzamidine, on energy-linked functions of rat liver mitochondria. *Chem. Biol. Interact.* **279,** 34–42 (2018).

49. Sands, M., Kron, M. A. & Brown, R. B. Pentamidine: A Review. *Clin. Infect. Dis.* **7,** 625–6344 (1985).

50. David, S. A., Mathan, V. I. & Balaram, P. Interactions of linear dicationic molecules with lipid A: structural requisites for optimal binding affinity. *J. Endotoxin Res.* **2,** 325–336 (1995).

51. David, S. A., Bechtel, B., Annaiah, C., Mathan, V. I. & Balaram, P. Interaction of cationic amphiphilic drugs with lipid A: Implications for development of endotoxin antagonists. *Biochim. Biophys. Acta - Lipids Lipid Metab.* **1212,** 167–175 (1994).

52. Guo, J.-X., Wood, S. J., David, S. A. & Lushington, G. H. Molecular modeling analysis of the interaction of novel bis-cationic ligands with the lipid A moiety of lipopolysaccharide. *Bioorg. Med. Chem. Lett.* **16,** 714–717 (2006).

53. Wood, S. J., Miller, K. A. & David, S. A. Anti-endotoxin agents. 1. Development of a fluorescent probe displacement method optimized for the rapid identification of lipopolysaccharide-binding agents. *Comb. Chem. High Throughput Screen.* **7,** 239–49 (2004).

54. Brown, D. Antibiotic resistance breakers: Can repurposed drugs fill the antibiotic discovery void? *Nature Reviews Drug Discovery* (2015). doi:10.1038/nrd4675

55. Connolly, S. J. Evidence-based analysis of amiodarone efficacy and safety. *Circulation* **100,** 2025–34 (1999).

56. Peterson, A. A., Hancock, R. E. & McGroarty, E. J. Binding of polycationic antibiotics and polyamines to lipopolysaccharides of *Pseudomonas aeruginosa*. *J. Bacteriol.* **164,** 1256–61 (1985).

57. CLSI. M07-A10: Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Tenth Edition. *CLSI (Clinical Lab. Stand. Institute)* (2015). doi:10.1007/s00259-009-1334-3

58. Gao, R. *et al.* Dissemination and Mechanism for the MCR-1 Colistin Resistance. *PLOS Pathog.* **12,** e1005957 (2016).

59. Yi, L. *et al.* mcr-1− Harboring *Salmonella* enterica Serovar Typhimurium Sequence Type 34 in Pigs, China. *Emerg. Infect. Dis.* **23,** 291–295 (2017).

60. Liu, Y.-Y. *et al.* Structural Modification of Lipopolysaccharide Conferred by mcr-1 in Gram-Negative ESKAPE Pathogens. *Antimicrob. Agents Chemother.* **61,**

(2017).

61. Yang, Q. *et al.* Balancing mcr-1 expression and bacterial survival is a delicate equilibrium between essential cellular defence mechanisms. *Nat. Commun.* **8,** 2054 (2017).

62. Li, H. *et al.* Comprehensive proteomic and metabolomic profiling of mcr-1 mediated colistin resistance in *Escherichia coli. bioRxiv* 434548 (2018). doi:10.1101/434548

63. Novem, V. *et al.* Structural and Biological Diversity of Lipopolysaccharides from *Burkholderia pseudomallei* and *Burkholderia thailandensis*. *Clin. Vaccine Immunol.* **16,** 1420–1428 (2009).

64. Tirsoaga, A. *et al.* Simple Method for Repurification of Endotoxins for Biological Use. *Appl. Environ. Microbiol.* **73,** 1803–1808 (2007).

65. Lee, C.-H. & Tsai, C.-M. Quantification of Bacterial Lipopolysaccharides by the Purpald Assay: Measuring Formaldehyde Generated from 2-keto-3-deoxyoctonate and Heptose at the Inner Core by Periodate Oxidation. *Anal. Biochem.* **267,** 161–168 (1999).

66. Wang, J. *et al.* High specific selectivity and Membrane-Active Mechanism of the synthetic centrosymmetric α-helical peptides with Gly-Gly pairs. *Sci. Rep.* (2015). doi:10.1038/srep15963

67. King, A. M. *et al.* Aspergillomarasmine A overcomes metallo-β-lactamase antibiotic resistance. *Nature* **510,** 503–506 (2014).

Appendix

Appendix 1 – currently approved drugs with an LPS-binding ability

FI	Compound	FI	Compound
1726	BENZETHONIUM CHLORIDE	1546	CLENBUTEROL HYDROCHLORIDE
1514.5	CEFADROXIL	1547	ACENEURAMIC ACID
2991.5	CETYLPYRIDINIUM CHLORIDE	3495	CETRIMONIUM BROMIDE
2746.5	CHLORHEXIDINE HYDROCHLORIDE	1706	CHLORAZANIL HYDROCHLORIDE
1450	METOLAZONE	1528	EFAROXAN HYDROCHLORIDE
1365.5	METHYLERGONOVINE MALEATE	1551.5	CARZENIDE
2322	POLYMYXIN B SULFATE	1786	CLEMIZOLE HYDROCHLORIDE
2033	BEKANAMYCIN SULFATE	1622	TIMONACIC
1575.5	AMIODARONE HYDROCHLORIDE	1729	LOMERIZINE HYDROCHLORIDE
1516.5	BETAINE HYDROCHLORIDE	1699	PAZUFLOXACIN MESYLATE
1783.5	METHYLBENZETHONIUM CHLORIDE	1440	RUFLOXACIN HYDROCHLORIDE
1883.5	MITOXANTRONE HYDROCHLORIDE	1480.5	DIMINAZENE ACETURATE
1664	BENZALKONIUM CHLORIDE	1679	IRSOGLADINE MALEATE
1939.5	THONZONIUM BROMIDE	1482.5	OXINIACIC ACID
2663	COLISTIN SULFATE	2160.5	TRICHLORMETHINE HYDROCHLORIDE
1958.5	SUCRALOSE	1623	TRIMEBUTINE MALEATE
1877	PENTAMIDINE ISETHIONATE	1544	SULFAPHENAZOLE
1602	PIMETHIXENE MALEATE	1972	ALEXIDINE HYDROCHLORIDE
1683.5	ETHACRIDINE LACTATE	1457.5	HEXETIDINE

FI = (fluorescence intensity)