Treatment of multidrug-resistant *Acinetobacter baumannii* with novel combinations of polymyxin B and other non-antibiotic drugs

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

by

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This thesis is dedicated to my best friend and wonderful wife Dr Nga Ngoc Lam.

CONTENTS

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ABSTRACT

The opportunistic pathogen *Acinetobacter baumannii* is becoming a major burden to the healthcare system globally. Infections caused by this pathogen are difficult to treat owing to their naturally high resistance profile. Due to the current limited effective antibiotics, carbapenem-resistant *A. baumannii* (CRAB) has recently been identified by the World Health Organization as a critical organism requiring top priority for research and development of new antibiotics. Consequently, the 'old' polymyxin antibiotics previously abandoned for fear of nephrotoxicity and neurotoxicity are increasingly used for the treatment of *A. baumannii* and other multidrug-resistant (MDR) Gram-negative bacilli. Polymyxin E (also known as colistin) and polymyxin B, the two polymyxins used clinically, are currently considered the 'last-line' of defence against these MDR organisms. Although polymyxins are still effective against these bacteria, polymyxin resistance has been observed following polymyxin monotherapy. As limited antibiotics are available for the treatment of these problematic bacteria, it is important that the efficacy of polymyxins is preserved. *In vitro* and animal studies have demonstrated that combinations of a polymyxin with another antibiotic can enhance bacterial killing and prevent the emergence of polymyxin resistance in MDR GNB. Given this background, this thesis aimed to (1) investigate the possibility of improving the antimicrobial activity of polymyxins and preventing polymyxin resistance in *A. baumannii* through the combinations of a polymyxin with an approved non-antibiotic drug, (2) understand the molecular mechanisms of the synergistic combinations, and (3) identify potential targets for other novel polymyxin combinations. This novel repurposing approach takes advantage of a large number of the currently available non-antibiotic drugs and can expedite the discovery of new antibiotics through the rapid drug repositioning process.

To investigate the potential synergistic activity of a non-antibiotic drug with a polymyxin against GNB, each non-antibiotic drug (10 μM) from a Johns Hopkins Clinical Compound Library of 1504 drugs was tested alone or in combination with polymyxin B (2 mg/L) against polymyxin-resistant (MIC ≥4mg/L) *A. baumannii*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. Identified non-antibiotic drugs with antimicrobial activity in the presence of polymyxin B were further evaluated against additional

MDR GNB strains. Preliminary studies with time-kill assays demonstrated closantel, an anthelmintic drug, was highly effective in combination with polymyxin B for the treatment of various MDR including polymyxin-resistant *A. baumannii*. Subsequent time-kill studies and animal infection models revealed mitotane, a neoplastic drug, to be an even better potential candidate for combination treatment with polymyxins, given its current use in humans. A polymyxin B/mitotane combination was highly effective *in vitro* against MDR including polymyxin-resistant *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*. Promisingly, the synergistic activity was also observed *in vivo* with a mouse burn wound infection model.

In order to understand the molecular mechanisms of polymyxin B/mitotane in combination against *A. baumannii*, liquid chromatography–mass spectrometry (LC-MS) based untargeted metabolomics was conducted with polymyxin-susceptible (ATCC 19606 and ATCC 17978) and polymyxin-resistant (FADDI-AB065 [formally known as ATCC 19606R] and FADDI-AB225 [formally known as ATCC 17978R2]) strains in the presence of polymyxin B, mitotane, and their combination. Significantly perturbed lipids and metabolites caused by either genetic background alteration or in the presence of antimicrobial substances were identified with multivariate and univariate analysis. The results indicated polymyxin B as the major cause of metabolic perturbation in *A. baumannii*. When polymyxin B was used in combination with mitotane, metabolic perturbation was substantially enhanced. Mitotane alone, however, had little impact on metabolite levels. Pathway enrichment analysis showed that glycerophospholipid metabolism, pentose phosphate pathway, citric acid cycle, pyrimidine ribonucleotide biogenesis, guanine ribonucleotide biogenesis, and histidine degradation pathway were affected by polymyxin B/mitotane combination. Collectively, the results suggested that polymyxin B/mitotane combination affects DNA replication, L-glutamate level, the activity of urocanate reductase and imidazolonepropionase, energy production, and membrane remodeling.

Given that *A. baumannii* can cause life-threatening pneumonia in critically-ill patients, it is important to understand how the pathogen and the host respiratory epithelial cell interact to identify new

targets for effective novel polymyxin combinations. To achieve this, the interaction of *A. baumannii* and human respiratory epithelial cells (A549 cells) in the presence of polymyxin B (2 mg/L) was investigated using transcriptomics. Simultaneous transcriptional profiling of A549 cells and *A. baumannii* ATCC 19606 from a host-pathogen-drug interaction was conducted with microarray and RNAseq, respectively. In A549 cells, exposure to *A. baumannii* alone was identified to be the major cause of differential gene expressions (DEGs); predictably, the genes were enriched for inflammatory responses. In *A. baumannii*, exposure to A549 cells alone upregulated DEGs enriched for arginine and tyrosine degradation pathways. In bacteria, arginine degradation is important for acid tolerance and tyrosine degradation for energy production. Interestingly, *A. baumannii* exposed to polymyxin B alone upregulated DEGs highly enriched for the tightly-regulated histidine degradation pathway. Additionally, exposure to polymyxin B led to upregulation of the *rcnB* gene involved in nickel/cobalt homeostasis. Time-kill studies showed that an *rcnB* mutant was more susceptible to polymyxin B, while population analysis profiles (PAPs) revealed the same mutant treated with polymyxin B monotherapy give rise to a highly polymyxin-resistant mutant. The findings indicated that nickel/cobalt homeostasis may play an important role in polymyxin resistance and that this pathway may be a promising target to prevent polymyxin resistance.

In conclusion, this thesis demonstrated that the combination of a polymyxin and non-antibiotic drug has potential therapeutic value for the treatment of MDR *A. baumannii*, and potentially other MDR GNB. The off-label use of non-antibiotic drugs for antibacterial purposes in combination with existing antibiotics is a currently underexplored area with significant potential to expedite the discovery of new treatment. This thesis is the first to identify that the antineoplastic drug mitotane possesses antimicrobial activity in combination with polymyxins and is a promising candidate for repositioning for treatment of MDR GNB. The simultaneous transcriptional profiling approach from this thesis has identified important pathways in *A. baumannii* responsible for its pathogenesis. This thesis also identified an interesting protein (putative RcnB) with regard to polymyxin treatment that deserves further investigation. Importantly, the generated transcriptomic and metabolomic data from this

thesis are valuable for future development of *in silico* model of *A. baumannii* to predict novel targets for other novel combination therapies involving polymyxins.

DECLARATION

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes two original papers and two reviews published or submitted in peer reviewed journals. The core theme of the thesis is Antimicrobial Therapy. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the Faculty of Medicine, Nursing and Health Sciences under the supervision of Professor Jian Li, Doctor Tony Velkov, and Doctor Phillip Bergen.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of thesis chapter 1, 2 and 3 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-author(s), Monash student Y/N
1	Pharmacokinetics/ pharmacodynamics of colistin and polymyxin B: are we there yet?	Published	80%. Reviewing the literature, writing the entire first draft, reviewing and addressing all co-authors comments.	1) Tony Velkov 2) Roger L Nation 3) Alan Forrest 4) Brian T Tsuji 5) Phillip J Bergen 6) Jian Li 20%. Reviewing and providing feedback for the drafts	No No No No No No
1	Agents of Last Resort: Polymyxin Resistance	Published	20%. Reviewing the literature and writing the first draft for the sections on mechanism of action, mechanism of resistance, strategies to minimise polymyxin resistance, optimising dosing regimens, and combination therapy	1) Keith S Kaye 2) Jason M Pogue 3) Roger L Nation 4) Jian Li 80%. Writing the other sections of the first draft, reviewing and improving the final version	No No No No

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-author(s), Monash student Y/N
2	Anthelmintic closantel enhances bacterial killing of polymyxin B against multidrug-resistant *Acinetobacter baumannii*	Published	80%. Performing all laboratory experiments, analysing and interpreting all data, writing the first draft and revising all subsequent versions, and formulating conclusions and hypothesis arising from the results of the study	1) Soon-Ee Cheah 2) Heidi H Yu 3) Phillip J Bergen 4) Roger L Nation 5) Darren J Creek 6) Anthony Purcell 7) Alan Forrest 8) Yohei Doi 9) Jiangning Song 10) Tony Velkov 11) Jian Li 20%. Providing support with laboratory experiments and data analysis, supervising and providing advice regarding the concept and design of studies, reviewing manuscript drafts and revisions, and formulating conclusions and hypothesis arising from the results of the study	No No No No No No No No No No No

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-author(s), Monash student Y/N
3	Novel polymyxin combination with antineoplastic mitotane improved the bacterial killing against polymyxin-resistant multidrug-resistant Gram-negative pathogens	Accepted	80%. Performing all laboratory experiments, analysing and interpreting all data, writing the first draft and revising all subsequent versions, and formulating conclusions and hypothesis arising from the the results of the study	1) Jiping Wang 2) Yohei Doi 3) Tony Velkov 4) Phillip J Bergen 5) Jian Li 20%. Providing support with laboratory experiments and data analysis, supervising and providing advice regarding the concept and design of studies, reviewing manuscript drafts and revisions, and formulating conclusions and hypothesis arising from the the results of the study	No No No No No
4	Synergistic killing of polymyxin B in combination with the antineoplastic drug mitotane against polymyxin-susceptible and resistant Acinetobacter baumannii: A metabolomic study	Accepted	80%. Performing all laboratory experiments, analysing and interpreting all data, writing the first draft and revising all subsequent versions, and formulating conclusions and hypothesis arising from the the results of the study	1) Phillip J Bergen 2) Darren J Creek 3) Tony Velkov 4) Jian Li 20%. Providing support with laboratory experiments and data analysis, supervising and providing advice regarding the concept and design of studies, reviewing manuscript drafts and revisions, and formulating conclusions and hypothesis arising from the the results of the study	No No No No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date:** 4/1/2018

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: [redacted] **Date:** 4/1/2018

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PUBLICATIONS IN SUPPORT OF THIS THESIS

1. **Tran TB**, Velkov T, Nation RL, Forrest A, Tsuji BT, Bergen PJ, Li J. 2016. Pharmacokinetics/pharmacodynamics of colistin and polymyxin B: are we there yet? Int J Antimicrob Agents 48:592-597. Parts of Chapter 1 only

2. Kaye KS, Pogue JM, **Tran TB**, Nation RL, Li J. 2016. Agents of Last Resort: Polymyxin Resistance. Infect Dis Clin North Am 30:391-414. Parts of Chapter 1 only

3. **Tran TB**, Cheah SE, Yu HH, Bergen PJ, Nation RL, Creek DJ, Purcell A, Forrest A, Doi Y, Song J, Velkov T, Li J. 2016. Anthelmintic closantel enhances bacterial killing of polymyxin B against multidrug-resistant Acinetobacter baumannii. J Antibiot (Tokyo) 69:415-421. Parts of Chapter 2 only

GLOSSARY OF ABBREVIATIONS

µg	microgram
µL	microlitre
AAC	acetyltransferase
AME	aminoglycoside-modifying enzyme
ANT	nucleotidyltransferase
APH	phosphotransferase
Ara4N	4-amino-4-deoxy-L-arabinose
ATCC	American Type Culture Collection
AUC	area under the concentration-time curve
CAMHB	cation-adjusted Mueller Hinton broth
CBA	colistin base activity
CCL	C-C motif ligand
CDC	Centers for Disease Control and Prevention
cfu	colony forming units
CL	total body clearance
CLR	renal clearance
CLSI	Clinical and Laboratory Standards Institute
*C*max	maximum concentration
CMS	sodium colistin methanesulphonate
CNS	central nervous system
CrCL	creatinine clearance
CRRT	continuous renal replacement therapy
*C*ss,avg	average steady-state plasma concentration
CXCL	C-X-C motif ligand

Dab	diaminobutyric acid
DEG	differentially expressed gene
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FA	fatty acyl
FC	fold-change
FDA	Food and Drug Administration
FDR	false discovery rate
g	g-force
Gly	glycine
GNB	Gram-negative bacilli
GO	gene ontology
GPL	glycerophospholipid
h	hour
HCl	hydrochloric acid
HILIC	hydrophilic interaction liquid chromatography
HPLC	high-performance liquid chromatography
HRMS	high-resolution mass spectrometry
ICU	intensive care unit
IL	interleukin
IU	international units
i.v.	intravenous
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg	kilogram
L	litre

LC-MS	liquid chromatography–mass spectrometry
LPS	lipopolysaccharide
MATE	multi antimicrobial extrusion protein
MBL	metallo-β-lactamase
MCP	monocyte chemoattractant protein
MDC	macrophage-derived chemokine
MDR	multidrug-resistant
mg	milligram
MIC	minimum inhibitory concentration
min	minute
MIP	macrophage inflammatory protein
MIU	million international unit
mL	millilitre
mRNA	messenger ribonucleic acid
NADH	nicotinamide adenine dinucleotide
OMP	outer membrane protein
PA	phosphatidic acid
PAE	post–antibiotic effect
PAPs	population analysis profiles
PBS	phosphate-buffered saline
PC	phosphatidylcholine
PD	pharmacodynamics
PDR	pandrug-resistant
PE	phosphatidylethanolamine
PEtN	phosphoethanolamine
PG	phosphatidylglycerol

PI	phosphatidylinositol
PMB	polymyxin B
PK	pharmacokinetic
PR	polymyxin-resistant
PS	phosphatidylserine
RANTES	regulated on activation normal T cell expressed and secreted
RNA	ribonucleic acid
RND	resistance-nodulation-cell division
rRNA	ribosomal ribonucleic acid
SD	standard deviation
SEM	scanning electron microscopy
*t*1/2	half-life
TCA	tricarboxylic acid cycle
TEA	tetraethylammonium
TEM	transmission electron microscopy
TCS	two-component system
TLR	toll-like receptor
TNF	tumor necrosis factor
XDR	extensively drug-resistant

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Emergence and growing challenge of multidrug-resistant bacteria

The discovery of antibiotics is one of the most significant achievements in modern medicine. Since their first introduction into clinical practice in the 1930s, antibiotics have been contributing significantly to the treatment and control of bacterial infectious diseases, which had been the leading cause of human morbidity and mortality for most of human existence (1-4). In the United States, the mortality rate from bacterial infections reduced considerably from 1938 to 1952, a period that coincided with the introduction of several major classes of antibiotics (3, 5). However, in recent times the success of antibiotics has come under threat. Inappropriate usage of antibiotics in humans, and in animals for growth promotion and infection prophylaxis, has led to a dramatic increase in antibiotic resistance (2, 6-11). The large amounts of antibiotics used globally has caused a profound impact on the life on Earth (12), so much so that for new antibiotics introduced resistance to a given class can subsequently emerged quickly (**Figure 1.1**) (2).

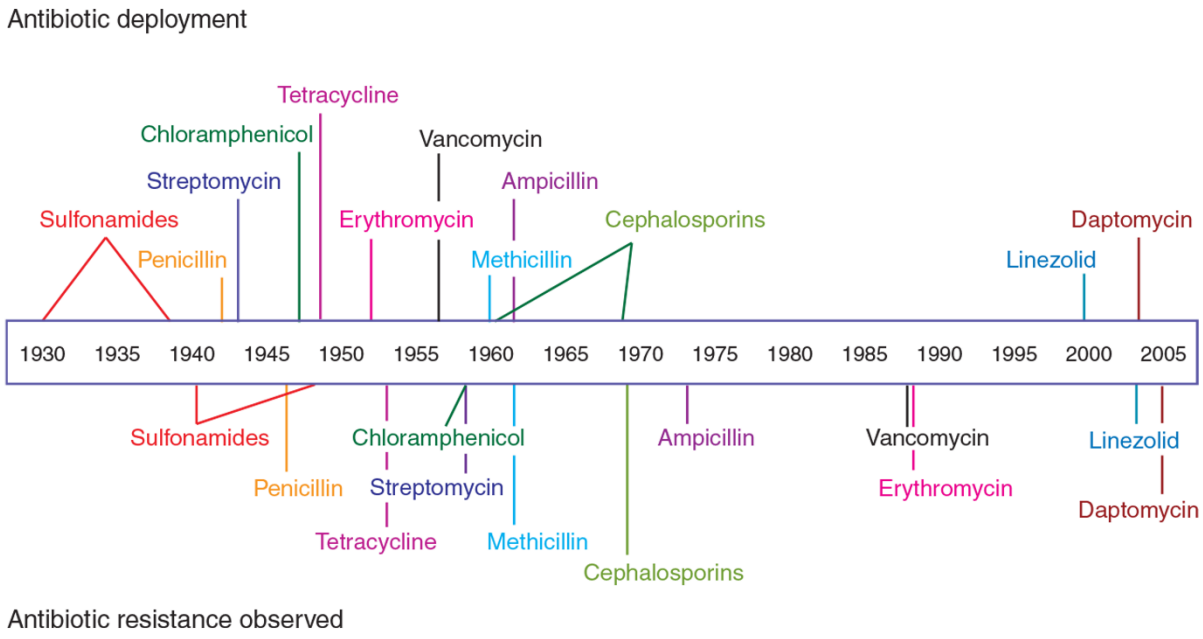

Figure 1.1 Timeline of antibiotic deployment (top) and the emergence of antibiotic resistance (bottom). Figure adapted from Clatworthy *et al.* (2), with permission.

Previously, antibiotics were described as chemical substances produced by micro-organisms only (13, 14). However, current antibiotics are described as natural, synthetic, and semi-synthetic compounds that can destroy or inhibit the growth of bacteria or fungi in human and animal hosts by interacting with their microbial targets (1, 15). Most antibiotics are relatively non-toxic drugs as the targets that they interact with are different or distinct to those in eukaryotic cells (4, 16, 17). Of the different antibiotic classes introduced into clinical practice since the 1930s, five major microbial targets have been discovered, namely i) the cell wall, ii) the cell membrane, iii) protein synthesis, iv) DNA and RNA synthesis, and v) folic acid (vitamin B9) metabolism (**Figure 1.2A**) (2, 16-18).

In order to survive, bacteria have developed multiple antibiotic resistance mechanisms (12, 16, 19, 20). These mechanisms include reducing the antibiotic concentration through efflux pumps, using an alternative metabolic pathway to bypass the affected target, preventing the interaction of the target with its respective antibiotic through target modification, and/or inactivating the antibiotic with enzymes (**Figure 1.2B**) (12, 16). Furthermore, bacteria are highly efficient at exchanging genetic information with one another through the process of horizontal gene transfer, facilitating the sharing of antibiotic resistance genes (19, 21, 22). Collectively, these processes have resulted in the emergence of many multidrug-resistant (MDR), including extensively drug-resistant (XDR) and pandrug-resistant (PDR) bacteria, which are defined as non-susceptible to ≥1 agent in ≥3 antimicrobial categories, non-susceptible to ≥1 agent in all but ≤2 categories, and non-susceptible to all antimicrobial agents, respectively (23).

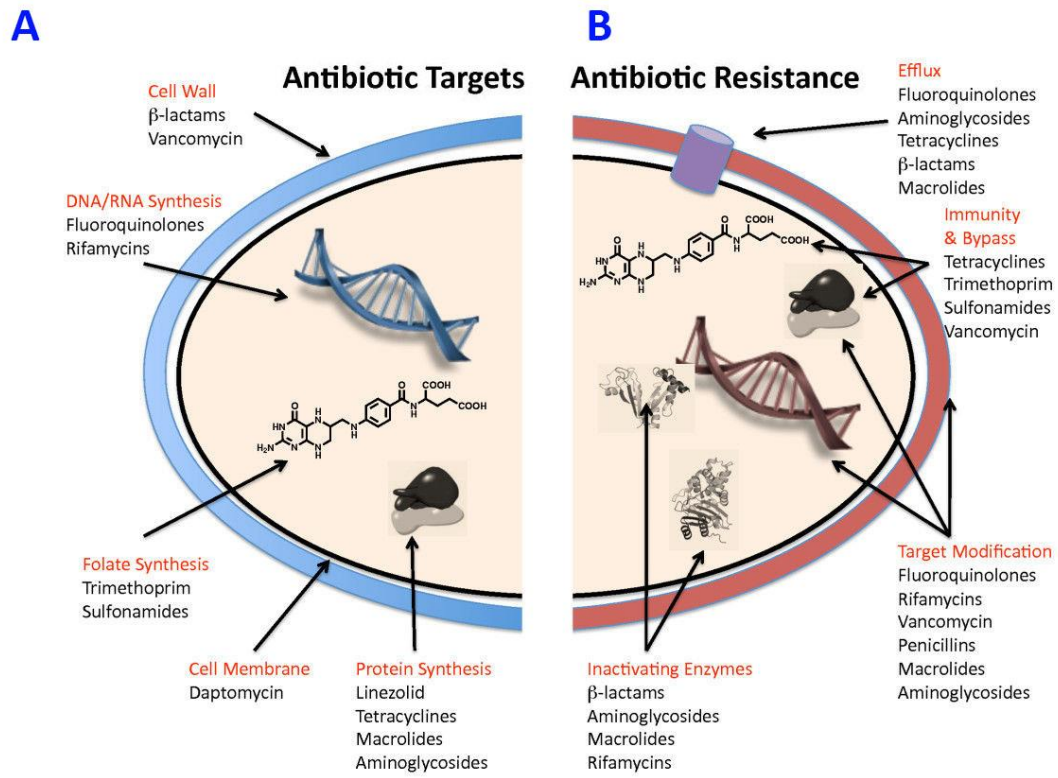

Figure 1.2 Bacterial targets of different classes of antibiotics (A) and mechanisms of antibiotic resistance in bacteria (B). Figure adapted from Wright (16), with permission.

Unfortunately, while bacteria have developed new ways to evade the existing antibiotics, very few new antibiotics with novel mechanisms of action have been developed over the last decade. Complicated regulations involved in the in developmental process, coupled with low economic returns, have resulted in only a handful of new drugs being introduced to the clinic in recent years (24). Notwithstanding, a few new agents have recently been introduced to target Gram-positive organisms; the situation is more dire for Gram-negative organisms where no novel agents have been approved in the last decade. Thus, treatment options for Gram-negative bacteria are becoming more limited as resistance increases to currently available agents.

Among the Gram-negative bacteria, MDR Gram-negative bacilli (GNB) are currently posing a major challenge to the healthcare system globally (10, 25, 26). Over the last few decades, the number of

MDR-GNB has risen significantly while the number of systemic antibiotics approved for the treatment of infections caused by GNB has dramatically reduced (10, 25). Data from the surveillance of antibiotic use and bacterial resistance in intensive care units from Germany showed a six-fold increase in the number of MDR-GNB from 2001 to 2009 (11). Meanwhile, from 2008 to 2012, only two new systemic antibiotics against GNB were approved by the United States Food and Drug Administration (FDA) (25); this number is eight-fold lower compared to the number of systemic antibiotics approved for the period of 1983 to 1987 (25, 27). The decline in the number of new antibiotics is due to regulatory uncertainty, which caused many major pharmaceutical companies to withdraw from new antibiotic development (10). Consequently, infections caused by MDR-GNB are becoming more difficult to treat, leading to increased health care costs, morbidity, and mortality (10, 11, 28). Among the MDR-GNB, carbapenem-resistant *Acinetobacter baumannii* have been classified as critical organisms that require top priority for research and development of new antibiotics (29). Carbapenem-resistant *A. baumannii* are usually MDR as carbapenem antibiotics are often used for the treatment of the MDR *A. baumannii*. In recent years*, A. baumannii* has emerged to be a significant pathogen in critically-ill patients (30, 31).

1.2 *Acinetobacter baumannii*

1.2.1 Infection and transmission

A. baumannii are non-fermentative, oxidase-negative, non-motile, GNB commonly found in humans, soil, meat and vegetables (32, 33). They belong to the genus *Acinetobacter*, which comprises of over 23 named and 12 unnamed (genomic) species (34, 35). Based on phenotypic properties, they are very closely related to *Acinetobacter calcoaceticus*, *Acinetobacter pittii* (formally genomic species 3), and *Acinetobacter nosocomialis* (formally genomic species 13TU) (35). Thus, collectively these species are designated *A. calcoaceticus-baumannii* complex (36). This classification, however, can be misleading as *A. calcoaceticus* has not been known to cause disease in humans (36).

Acinetobacter spp. are typically opportunistic pathogens that can cause serious infections in the critically-ill patients (37). Among these species, *A. baumannii* is most often associated with human infections (34), accounting for ~80% of reported infections caused by *Acinetobacter* spp.; of these, ~63% are considered MDR (28). Initial sites of infection are generally moist tissues such as mucous membranes or exposed areas of the skin, the latter either through accident or injury (38). Once this organism has gained entry to the body, it can cause pneumonia, urinary tract and wound infections, which and can progress to bacteraemia or meningitis (31). Although *A. baumannii* is predominantly associated with nosocomial infections, it has been implicated in serious infections of severely war-wounded soldiers (39). From 2003 to 2005, twenty-three soldiers wounded in Iraq admitted to the Walter Reed Army Medical Center developed an infection with bacteria identified as *Acinetobacter calcoaceticus-baumannii* complex. Among the wounded soldiers there were eighteen cases of osteomyelitis, two infections associated with burns, and three cases of deep wound infection (39). Infrequently, *A. baumannii* also causes community-acquired infections, usually community-acquired pneumonia. The number of these infections have been increasing in regions of Southeast Asia and tropical Australia (40, 41). A retrospective case-control study performed at United Christian Hospital in Hong Kong between July 2000 and December 2003 identified nineteen cases of community-acquired pneumonia by *A. baumannii* (CAP-AB) (40). The study showed that CAP-AB caused a higher incidence of bacteraemia, acute respiratory distress syndrome, and disseminated intravascular coagulation and death when compared to hospital-acquired pneumonia by *A. baumannii* (40).

The ability of *A. baumannii* to survive on environmental surfaces for long periods is suspected to be an important element for their transmission within the health care setting (36). In such settings, *A. baumannii* is commonly transmitted through contaminated medical devices such as ventilators and central venous catheters (34). Colonization or infection by *A. baumannii* is often associated with prolonged hospital stays, admission to the intensive care unit (ICU), recent surgical procedures, mechanical ventilation, central venous catheter use, exposure to antimicrobial agents, invasive procedures, and underlying severity of illness (31, 42).

1.2.2 Antimicrobial resistance

A. baumannii are normally resistant to a broad spectrum of antibiotics. This high-resistance profile is believed to be due to their protective outer membrane and exposure to a large number of resistance genes in the environment (43). Antimicrobial resistance among *A. baumannii* in patients in the intensive care unit has recently increased dramatically (11, 44). Strains resistant to all available antibiotics have been reported, making some infections virtually impossible to treat (31, 45, 46). Mechanism employed by *A. baumannii* to develope resistance include changes to outer membrane proteins (OMPs), reduced access to the target through efflux pumps, modification or protection of the target binding site, and antibiotic inactivation (36, 47).

A wide range of enzymes, classified as β-lactamases, exists among *A. baumannii* that inactivates β-lactam antibiotics, such as penicillins, cephalosporins, and carbapenems (43). Many of these enzymes are inducible, chromosomally encoded AmpC-type β-lactamases, which confer resistance to broad-spectrum cephalosporins (43, 48). *A. baumannii* also produces class D OXA-type enzymes, which can have activity against carbapenems (49). Of all the enzymes, the most clinically significant enzymes produced by *A. baumannii* are IMP- and VIM-type carbapenemases, which are classified as class B metallo-β-lactamases (MBLs) (43, 48). These enzymes inactivate a broad array of antimicrobial agents, including carbapenems, and are often found on highly mobile genetic elements (43, 48). Additionally, *A. baumannii* also use multiple other strategies to become resistant to carbapenems. In some carbapenem-resistant *A. baumannii* strains, reduction or loss of porin channels has been observed (50). The loss of porin channels from the outer membrane of *A. baumannii* is suspected to contribute to antibiotic resistance as they are important for the transportation of antibiotics to their targets (50). It has been suggested that the combined effect of both β-lactamases and loss of porin channels is essential for resistance to β-lactam antibiotics (43). *A. baumannii* can also reduce the entry of carbapenems into the cell and limit target interaction via efflux pumps that actively remove a broad range of antibiotics that have gained entry into the cell (43). Finally, resistance to carbapenems in *A.*

baumannii can also be mediated by alterations to penicillin binding proteins (PBPs), the major target of the β-lactams (51, 52). In carbapenem-resistant *A. baumannii,* down regulation of most PBPs found in the susceptible strains and emergence of new PBPs has been observed (51, 52).

Resistance to aminoglycoside antibiotics in *A. baumannii* is mainly caused by aminoglycoside-modifying enzymes (AMEs) (36, 53-55), which are encoded by genes normally found on transmissible plasmids or transposons (36, 53, 54). There are three classes of AMEs: acetyltransferases (AACs), nucleotidyltransferases (ANTs) or phosphotransferases (APHs) (55). Accordingly, these AMEs acetylate, adenylate or phosphorylate aminoglycoside antibiotics, preventing their interaction with their ribosomal RNA (rRNA) targets (53, 55). *A. baumannii* can also prevent the interaction of aminoglycosides and their target through methylation of the 16S rRNA subunit (36, 56, 57). The enzyme required for this process, 16S rRNA methylase, is encoded by *armA* in *A. baumannii* (56, 57). Efflux pumps also play an important role in aminoglycoside resistance. Resistance nodulation cell division (RND) type pump AdeABC affects the transportation of amikacin and kanamycin (58), while multidrug and toxic compound extrusion (MATE) pump AdeM affects gentamicin and kanamycin transportation (59).

For quinolone antibiotics, modifications to DNA gyrase or topoisomerase IV through mutations in the *gyrA* and *parC* genes that interfere with target site binding may result in resistance in *A. baumannii* (36, 60, 61). Additionally, resistance can be mediated by multidrug efflux pumps for which most quinolones are substrates. RND type pump AdeABC and MATE pump AdeM are the efflux pumps responsible for quinolones resistance (58, 59).

Resistance to tetracyclines is mediated by tetracycline-specific efflux, ribosomal protection, and multidrug efflux (36). In *A. baumannii*, two tetracycline-specific efflux pumps encoded by the *tet*(A) and *tet*(B) determinants have so far been identified (62-64). *Tet*(A) however only confers resistance to tetracycline and not the more active minocycline. Tetracyclines resistance by ribosomal protection is

mediated by *tet*(M) and *tet*(O) determinants, with *tet*(M) occurring less regularly in *A. baumannii* (64). Tetracyclines are also susceptible to efflux by the AdeABC pump (58).

A. baumannii can acquire antibiotic resistance from other organisms, mutation, or sub-populations with pre-existing resistance. It is believed that *A. baumannii* often uses multiple of these strategies to gain resistance. A recent comparative genomic study identified 45 resistance genes from multiple bacterial genera accumulated in an epidemic, MDR *A. baumannii* strain AYE (65).

1.2.3 Treatment of *A. baumannii* infections

Antimicrobial therapy is not always required for *A. baumannii* as their presence may represent colonisation (34). In the event of *A. baumannii* infection, antibiotics commonly used are ceftazidime, carbapenems, sulbactam, piperacillin/tazobactam, aminoglycosides, quinolones (e.g., levofloxacin), cefepime, polymyxin E (i.e. colistin) and polymyxin B (PMB), minocycline, doxycycline, and tigecycline (34, 66, 67). The selection of these antimicrobial agents is guided by the susceptibilities of the pathogen. In Asian and European countries, where resistance incidence to aminoglycosides and piperacillin/tazobactam is high, these antibiotics would not normally be selected (68). The resistance pattern in *A. baumannii* is another good guideline for the selection of appropriate antimicrobial agent as strains that developed resistance to fluoroquinolones have also been found to be resistant to other drug classes through active drug efflux (34, 69). For MDR strains, sulbactam (a β-lactamase inhibitor) may be used. The bacterial killing demonstrated by sulbactam against MDR *A. baumannii* is suspected to relate to its affinity for penicillin-binding proteins in these bacteria (70). This activity is not enhanced by the presence of a β-lactam agent. Nevertheless, the combination of ampicillin and sulbactam has been effective against MDR *A. baumannii* (34). Recently, the bacterial killing of ampicillin plus sulbactam has declined, especially in carbapenem-resistant *A. baumannii* (71).

Currently, the most important drugs for the treatment of *A. baumannii* are the carbapenems, which include imipenem, meropenem, and doripenem. This, however, is about to change due to the rapid

worldwide emergence of carbapenem-resistant *A. baumannii* strains (10, 28). A relatively new glycylcycline antibiotic, tigecycline, can be used for the treatment of MDR, including carbapenem-resistant, *A. baumannii* (72). Tigecycline is only approved by the FDA for treatment of complicated intra-abdominal, skin, and skin-structure infections (34), and is normally used as part of combination therapy (73). Despite high rates of susceptibility of *A. baumannii* to tigecycline indicated by large surveillance trials (with susceptibility provisionally defined by a minimum inhibitory concentration (MIC) ≤2 mg/L and resistance by a MIC ≥8 mg/L) (74-76), conflicting data have also been reported (77-79). A study of 82 clinical isolates of MDR *A. baumannii* collected from the Tel Aviv Medical Center in 2003 showed 66% of the isolates were resistant to tigecyline (78). Similarly, an evaluation of 41 MDR *A. baumannii* from the Third Affiliated Hospital of Sun Yat-sen University from April 2009 to March 2010 revealed tigecycline resistance in 80.4% of the isolates (79). For MDR *A. baumannii*, polymyxins are currently considered the 'last-resort' antibiotics (80, 81). These antibiotics remain effective against the majority of *A. baumannii* as well as other problematic GNB (45). The use of polymyxins to treat *A. baumannii* will form the basis of this thesis.

1.3 Polymyxins - the 'last resort' antibiotics

1.3.1 History of polymyxins

Polymyxins are antibiotics of the lipopeptide class and are active against many GNB (81, 82). They are non-ribosomal products that were first identified from *Paenibacillus polymyxa* (previously known as *Bacillus polymyxa*) in 1947 (83, 84). There are five classes of polymyxins: A, B, C, D and E (85). However, only colistin and PMB are used clinically (80-82). The polymyxins were approved for clinical use in the late 1950s but fell out of favour during the mid-1970s due to concern over their potential to cause nephrotoxicity and neurotoxicity (86). Over the last two decades, clinical interest in polymyxins has dramatically increased following the emergence of MDR- including XDR-GNB, coupled with the dry antibiotic development pipeline (81). Colistin and PMB are currently considered a last-line defence against problematic Gram-negative 'superbugs', notably carbapenem-resistant Enterobacteriaceae, *P.*

aeruginosa and *A. baumannii*, which are classified under the urgent or serious threat level by the Centers for Disease Control and Prevention (CDC) (28).

1.3.2 Chemical structure

Structurally, colistin and PMB are very similar. Their decapeptide chains consist of an intra-molecular cyclic hepta-peptide loop that is formed by the amino group of the side chain of the non-proteogenic diaminobutyric acid (Dab) residue at position 4 and the carboxyl group of the C-terminal threonine residue at position 10 (87, 88). They also possess five diaminobutyric acid residues that are polycationic at pH 7.4, hydrophobic residues at position 6 and 7 and an N-terminal fatty acyl group. Nine of the ten amino acids are of the L-configuration except for the one at position 6 being the D-configuration. The only difference between the structures of the two polymyxins is the amino acid at position 6, with D-leucine for colistin and D-phenylalanine for polymyxin B (**Figure 1.3**) (87, 88). The combination of hydrophilic and lipophilic groups in polymyxins results in their amphipathic character, which is an essential property for their antibacterial activity. Due to the similarities in their structures, the two antibiotics possess comparable activities against a range of GNB (89) and cross-resistance exists (82).

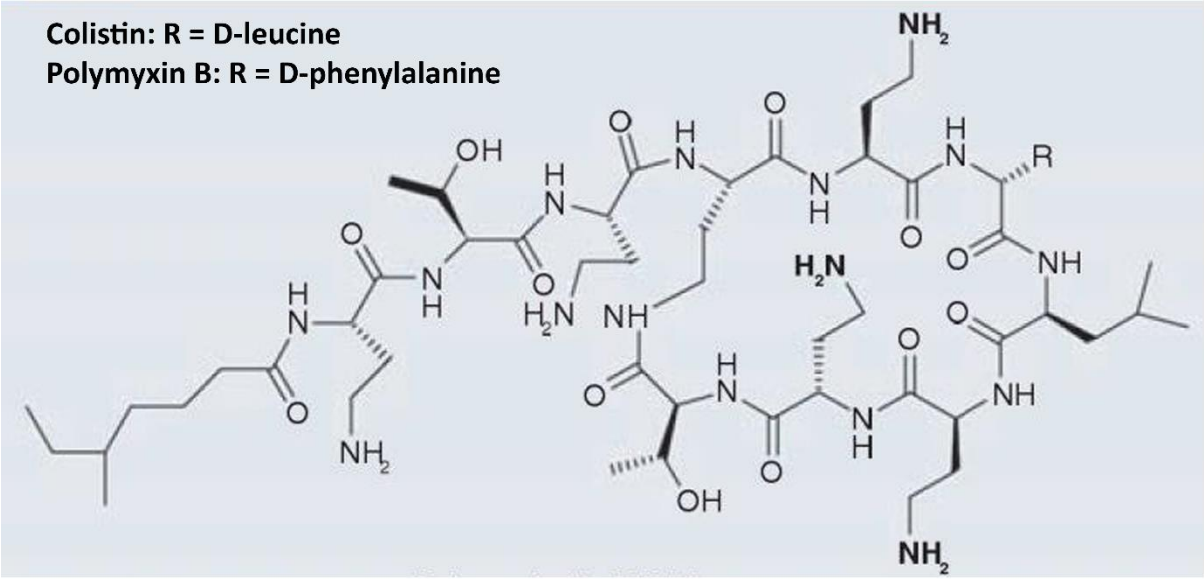

Figure 1.3 Structure of colistin (also known as polymyxin E) and PMB. The molecules are similar in structure with the exception of the amino acid at position 6. Figure adapted from Biswas *et al.* (88), with permission.

1.3.3 Mode of action

The precise mechanism of antibacterial activity of polymyxins is not completely understood. However, the view most commonly held is that polymyxins kill bacteria by disrupting the bacterial outer membrane through the 'self-promoted uptake' pathway and interacting with the inner membrane (**Figure 1.4**) (90, 91). The initial binding target of polymyxins are the lipid A component of lipopolysaccharide (LPS) in the outer membrane of GNB, with both electrostatic and hydrophobic interactions being important (92). Electrostatic interaction via the positively charged Dab residues of the polymyxin (**Figure 1.5**) and the negatively charged phosphate groups on the lipid A moiety of LPS leads to displacement of divalent cations (Mg^{2+} and Ca^{2+}) that bridge the lipid A phosphoesters, thereby destabilising the outer membrane (93). This event allows the polymyxin to insert its hydrophobic regions (fatty acyl tail and amino acids at positions 6 and 7) into the bacterial outer membrane to interact with the fatty acyl tails of lipid A, and cause further outer membrane disruption that promotes the uptake of the polymyxin (90, 94). It has been proposed that after transiting the

outer membrane, polymyxins mediate the fusion of the inner leaflet of the outer membrane with the outer leaflet of the cytoplasmic membrane, which induces phospholipid exchange and causes an osmotic imbalance that leads to cell death (95). The amphipathic property of polymyxins (i.e. presence of both cationic and hydrophobic regions) is necessary for the killing of GNB. PMB nonapeptide (i.e. PMB lacking the fatty acyl tail and the Dab residue at position 1) and colistimethate (in which the Dab residues are masked by negatively charged methanesulfonate moieties but the fatty acyl tail remains) do not possess antibacterial activity (96, 97). In addition to their membrane disrupting effect in GNB, binding of polymyxins to lipid A also neutralises the toxicity of endotoxins (98, 99).

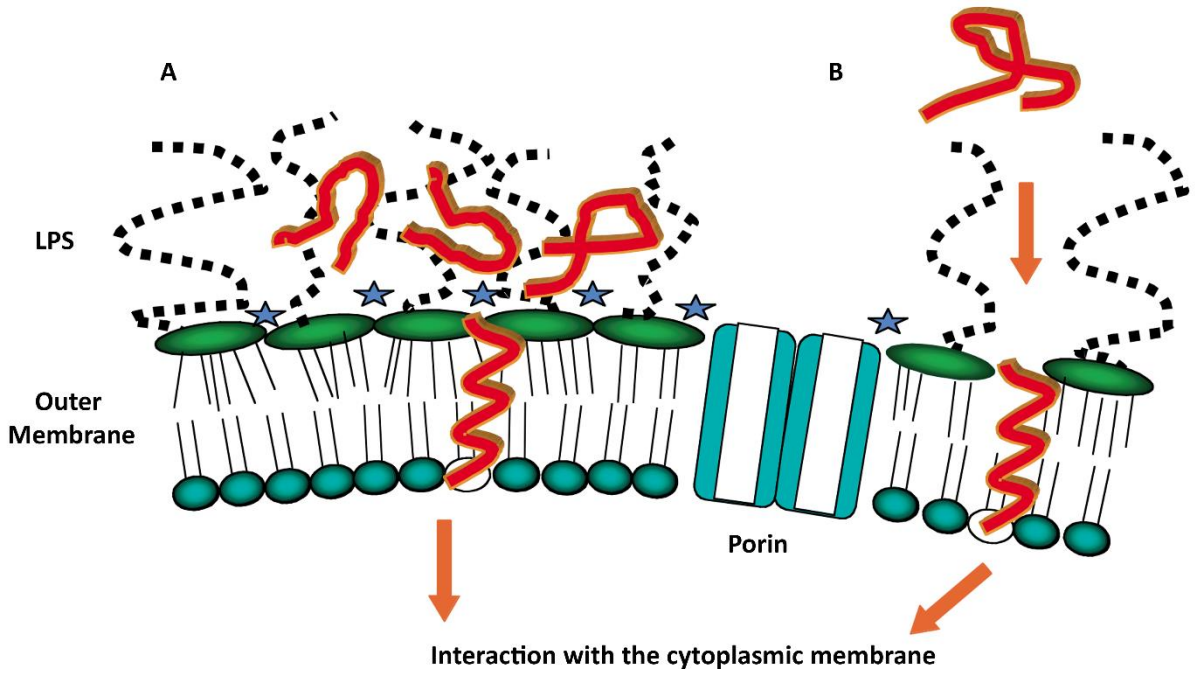

Figure 1.4 Proposed mechanism of self-promoted uptake of cationic antimicrobial peptides (including polymyxins) across the outer membrane of Gram-negative bacteria. The cationic peptides interact with the negatively charged surface (the anionic lipopolysaccharide [LPS]) in the outer membrane. The interaction creates cracks allowing the peptides to cross the outer membrane (A) or displaces the divalent cations that bind to the LPS and disrupts the outer membrane (B). Figure adapted from Hancock (91), with permission.

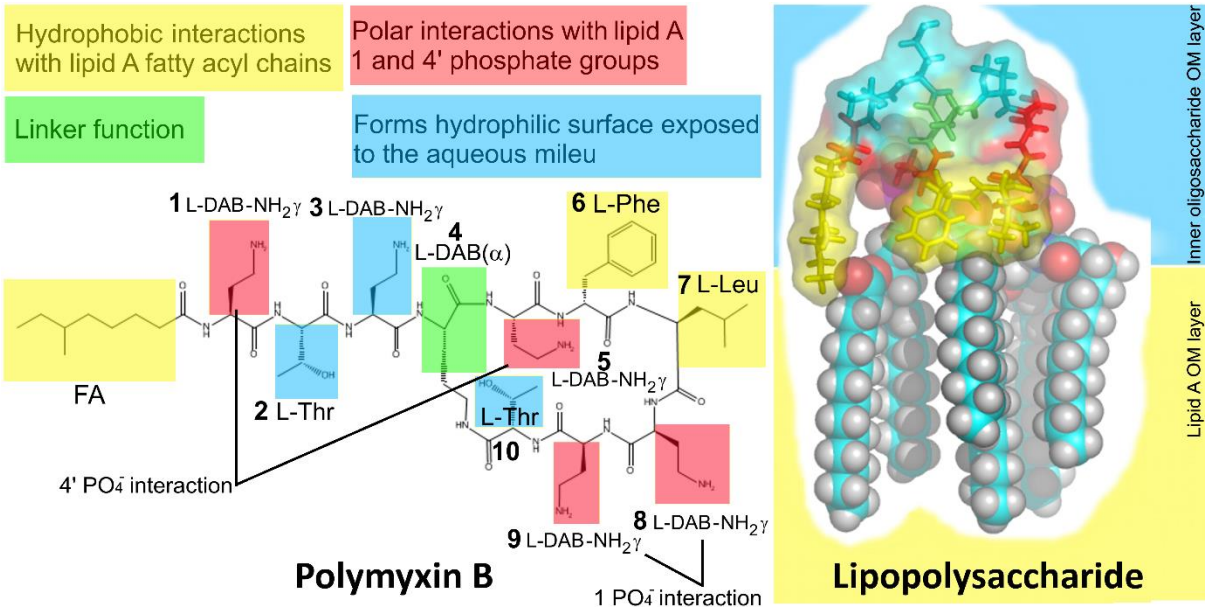

Figure 1.5 A schematic diagram showing the key contacts involved in the complex formation between polymyxin B and the lipid A component of lipopolysaccharide (LPS). FA = *N*-terminal fatty acyl chain. Figure adapted from Velkov *et al.* (87), with permission.

Other secondary mechanisms have also been implicated in the mode of action of polymyxins (100, 101). Recent studies have suggested that the mechanism of polymyxin activity may be associated with oxidative stress via the formation of hydroxyl radicals and inhibition of the NADH oxidase enzyme family of GNB (100, 101). A 2012 study showed that *A. baumannii*, *E. coli* and *Francisella novicida* treated with either PMB or colistin produced an ~2-fold increase in hydroxyl radicals compared to the untreated groups (100). The rapid killing of *A. baumannii*, *E. coli* and *F. novicida* by polymyxins was found to be mediated by hydroxyl radicals as bacterial killing was lower in the presence of the hydroxyl radical scavenging compound, thiourea (100). Subsequently, PMB and colistin were found to inhibit type II NADH-quinone oxidoreductases (NDH-2) activity (a critical enzyme in the bacterial respiratory chain) in a study involving inner membrane preparations of *A. baumannii, K. pneumoniae* and *E. coli* (101). Inhibition of NDH-2 activity by PMB was also observed in acid-fast bacteria, *Mycobacterium smegmatis* (102).

Although the association of bacterial killing of PMB and colistin with free radicals has been challenged (103, 104), it is supported by recent transcriptomics and metabolomics studies (105, 106). Transcriptomics data from *A. baumannii* treated with colistin showed significant upregulation of two superoxide dismutase (SOD) enzymes, HMPREF0010_02336 (*sodB* encoding a predicted FeSOD) and HMPREF0010_02564 (encoding a predicted Cu-ZnSOD) (105). The untargeted metabolomics analysis of *A. baumannii* showed significant depletion of total glutathione content following colistin treatment (106), a finding suggesting utilisation of glutathione pools to compensate for antibiotic-induced oxidative damage. The metabolomics study also supported the role of polymyxins in the inhibition of respiratory chain enzyme NDH-2 as tricarboxylic acid cycle (TCA) metabolites (i.e. fumarate and cis-aconitate) were significantly perturbed following colistin exposure (106).

1.3.4 Mechanisms of polymyxin resistance

The interaction of polymyxins with lipid A is essential for their bacterial killing activity. Consequently, polymyxins are inactive against Gram-positive bacteria where lipid A is absent. In GNB intrinsically resistant to polymyxins, the lipid A molecules have reduced binding affinity for polymyxins causing a diminished interaction. In these lipid A molecules, lipid A usually contains modified phosphate groups, thereby decreasing their overall net negative charge (107-109). Likewise, in bacteria that are initially susceptible to polymyxins, resistance is usually acquired through lipid A modifications (**Figure 1.6**) (110, 111).

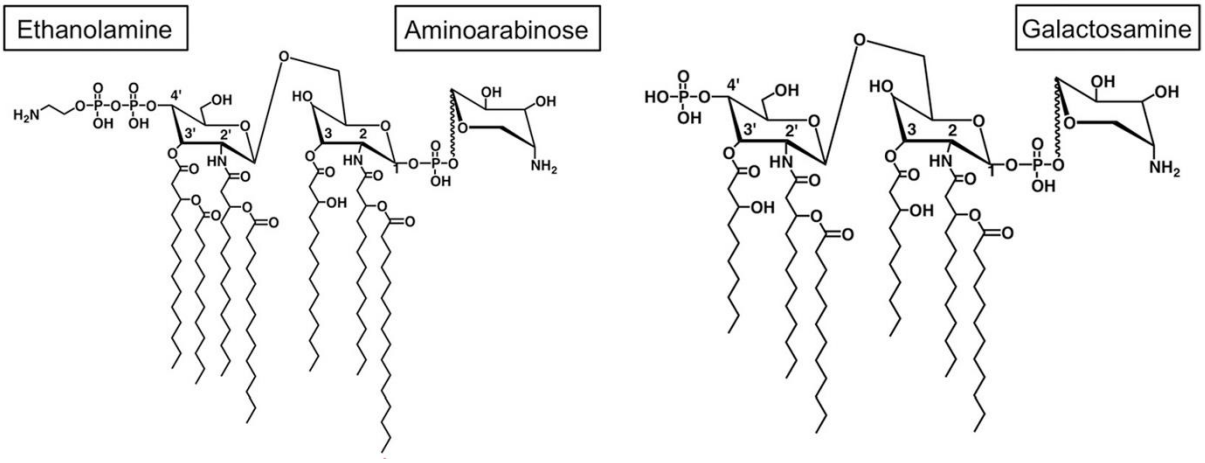

Figure 1.6 Potential modification of the lipid A component of lipopolysaccharide by positively charged residues, including ethanolamine, aminoarabinose, and glucosamine that leads to the resistance of polymyxins. Figure adapted from Pellier *et al.* (111), with permission.

The modification of LPS that most commonly leads to polymyxin resistance in *P. aeruginosa* involves the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the phosphate group in lipid A (110). This modification is usually controlled by the *arn* (*pmr*) operon, which is regulated by the PmrA/PmrB, PhoP/PhoQ, and ParR/ParS two-component systems (TCSs) (112, 113). These systems can also be activated by changes in the environment (e.g. high Fe^{3+} concentration, low Mg^{2+} or Ca^{2+} concentrations, and low pH) and lead to decreased bridging of adjacent lipid A molecules via divalent cations and an unstable outer membrane (114-116). PmrB and PhoQ are sensor cytoplasmic membrane-bound kinases that phosphorylate their respective regulator proteins PmrA and PhoP upon activation. Once phosphorylated, PmrA and PhoP promote the upregulation of the *arn* operon leading to the addition of L-Ara4N to the phosphate groups of lipid A (117). Resistance to polymyxins can develop when mutations occur in the PmrA/PmrB and PhoP/PhoQ systems (118). The addition of phosphoethanolamine (PEtN) to lipid A has also been identified in the modification of LPS of polymyxin-resistant *P. aeruginosa*. This modification is controlled by the ColR/ColS TCS, which is also upregulated in the presence of excess extracellular Zn^{2+} (119).

In *K. pneumoniae*, resistance to polymyxins may involve several strategies. One involves the modification of lipid A by the addition of either L-Ara4N or PEtN (118). These modifications are caused by mutations in pmrA, prmB or phoQ genes that upregulate the PhoP/PhoQ and PmrA/PmrB systems (120-122). It has also been reported that the upregulation of the PhoP/PhoQ and PmrA/PmrB systems can be caused by a deletion in the mgrB locus (123). Another resistance mechanism in *K. pneumoniae* involves over-production of surface capsular polysaccharides (CPS). It is believed that CPS acts as a barrier to limit the interaction of polymyxins with lipid A by 'trapping' or binding polymyxins (124, 125). The AcrAB-TolC efflux pump may play a role in polymyxin resistance in *K. pneumoniae* (126).

In *A. baumannii* where L-Ara4N biosynthesis and attachment genes are generally lacking, one way in which polymyxin resistance is achieved is from modification of LPS by the addition of PEtN to lipid A (127). This modification can be caused by mutations in the pmrA and/or pmrB that induce the autoregulation of the promoter region of the pmrCAB operon (118). Recent findings from polymyxin-resistant clinical isolates indicate that modification of LPS with galactosamine (GalN) also contributes to polymyxin resistance, even though the precise regulatory pathway is not yet understood (111). In addition to LPS modification, *A. baumannii* also possesses a unique polymyxin resistance mechanism that involves loss of LPS (128). This phenotype can be caused by mutations or insertional inactivation of lipid A biosynthesis genes. In these polymyxin-resistant *A. baumannii* isolates, genes responsible for transport of phospholipids and lipoproteins and production of poly-β-1,6-*N*-acetylglucosamine are upregulated to compensate for the missing LPS in the outer leaflet of the outer membrane (129). An untargeted metabolomics study comparing the metabolite levels of polymyxin-susceptible *A. baumannii* ATCC 19606 and its polymyxin-resistant derivative through LPS loss (*A. baumannii* ATCC 19606R) showed several putative sugar phosphate metabolites, including metabolites associated with the pentose phosphate pathway (PPP), were significantly perturbed in the 19606R strain (130). Compared to the parent polymyxin-susceptible ATCC 19606 strain, the LPS deficient strain also displayed different levels of metabolites involved in phenylalanine, tyrosine, tryptophan, and histidine

metabolic pathways and a shift in its glycerophospholipid profile towards an increased abundance of short-chain lipids (130).

Resistance to polymyxins can also arise from polymyxin-heteroresistant bacteria. Heteroresistance is defined as a polymyxin-susceptible isolate with sub-populations able to grow in the presence of PMB or colistin concentrations higher than the minimal inhibitory concentration (MIC) (131). When polymyxins are administered as monotherapy to such isolates, the polymyxin-resistant subpopulations survive and multiply and replace the susceptible population (131-135). Consequently, polymyxin resistance deriving from the polymyxin-heteroresistance is different to the resistance that resulted from adaptive responses. Heteroresistance implies that the susceptibility of the resistant subpopulation is not affected by drug exposure. Recent studies indicate that polymyxin heteroresistance in *P. aeruginosa* is uncommon (136); however, it is very common in MDR *K. pneumoniae* (137, 138) and carbapenem-resistant *A. baumannii* (139, 140).

Fortunately, resistance to polymyxins may come at a fitness cost. *A. baumannii* isolates with polymyxin resistance usually grow at a much slower rate and are less capable of causing infection compared to their non-resistant counterparts (141, 142). A study comparing the fitness cost of lipid A modification and LPS loss in *A. baumannii* showed that both mechanisms lead to reduced fitness and virulence; however, reduction in biological fitness associated with LPS loss was greater than with PEtN addition (141). Impaired virulence in *A. baumannii* is also linked to reduced expression of metabolic proteins and of the OmpA porin (143). Significant biological fitness cost due to polymyxin resistance has yet to be observed in *P. aeruginosa* and *K. pneumoniae*.

1.3.5 Pharmacokinetics of polymyxins

1.3.5.1 Colistin methanesulphonate/colistin

Colistin is more widely used for the treatment of infections with GNB than is PMB. Colistin is administered parenterally in the form of an inactive prodrug, the sodium salt of (CMS) (82). Currently,

there are two different labeling systems in use for parenteral CMS (82). In Europe, international unit (IU) is used for CMS, while colistin base activity (CBA) is employed in North America, South America, and Southeast Asia. One million IU is equivalent to ~30 mg of CBA. Awareness of the labeling differences is important for proper conversion and interpretation of different dosage regimens reported in the literature (144, 145).

Following intravenous (i.v.) administration of CMS, this inactive prodrug is hydrolysed to the active form, colistin, which exhibits a different pharmacokinetic (PK) profile. CMS is eliminated mainly by the kidneys while colistin is eliminated mainly by the non-renal mechanisms (82). In an early study in rats with glomerular filtration of ~2.3 mL/(min kg), following an i.v. bolus dose of 1mg/kg colistin sulfate, the total body clearance (CL) of colistin was 5.2 ± 0.4 mL/(min kg) while the renal clearance (CL_R) was only 0.010 ± 0.008 mL/(min kg) (146). Over 24 h, only 0.18 ± 0.14% of the total colistin dose was recovered in urine. In a separate study with isolated perfused rat kidney, the clearance of colistin when perfused alone was significantly lower compared to when colistin was perfused with tetraethylammonium [TEA], glycine-glycine [Gly-Gly], or hydrochloric acid [HCl] (147). The combined findings indicated extensive renal reabsorption of colistin, the process that is sensitive to the pH of urine and may involve organic cation transporters (inhibited by TEA) and peptide transporters (inhibited by Gly-Gly). When CMS was administered at 15 mg/kg to rats with glomerular filtration rate of ~5.2 mL/(min kg), the CL of CMS was 11.7 mL/(min kg) and the CL_R was 7.2 ± 2.2 mL/(min kg) (148). Compared to the glomerular filtration rate, the higher CL_R of CMS indicated tubular secretion of CMS into the urine. PK analysis showed that approximately 7% of the administered dose of CMS was converted to colistin systemically. After 24 h, 61.1 ± 14.4% of the total dose of CMS was recovered in urine, around 50% of which was colistin. Compared to the previous animal study with colistin sulfate (146), the higher concentration of colistin recovered in the urine is likely due to increased conversion of CMS in the renal tubular cells, the bladder, and the collection vessel. The rat study with CMS also showed the elimination of colistin is not rate limited by its formation from CMS as the terminal $t_{1/2}$ of

formed colistin was approximately twice that of the administered CMS (55.7 ± 19.3 versus 23.6 ± 3.9 min).

In several recent PK studies in critically-ill patients (149-151), total CMS clearance was found to be higher than the apparent clearance of formed colistin. The higher CMS clearance leads to low plasma concentration of formed colistin and delays attainment of effective colistin concentrations following i.v. therapy with CMS. After i.v. administration of CMS at 3 MIU, it can take >36 h to reach a colistin plasma concentration of 2 mg/L (150). This dilemma can be partially counteracted with the use of a loading dose. In studies where a loading dose of CMS has been used (6-9 MU) (151, 152), colistin plasma concentrations peaked after 8 h leading to the faster elimination of pathogens. In critically-ill patients (149, 153), it was noted that the kidney function and renal replacement therapy (RRT) have a dramatic impact on the PK of CMS and formed colistin. The largest population PK study undertaken in critically-ill patients involved 105 patient with a wide range of renal function (3-169 mL/min), 12 of whom were receiving intermittent haemodialysis and 4 continuous RRT (CRRT) (149). This study showed substantial inter-patient variation in the average steady-state plasma colistin concentration ($C_{ss,avg}$) ranging from 0.48-9.38 mg/L. Significant inter-patient variation was observed even among patients with similar creatinine clearance and those given the same daily dose of CMS (**Figure 1.7**). Studies in patients with RRT showed that both CMS and formed colistin are cleared by continuous venovenous hemodiafiltration (149, 153). Clearly, given that the plasma concentration of formed colistin is highly influenced by the renal function of patients, it is essential that the dosage regimen of CMS is adjusted to ensure appropriate colistin exposure is obtained. In patients with a creatinine clearance (CL_{Cr}) of >80 mL/min, only 65-75% of patients receiving the approved updated dose recommended by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) achieved a $C_{ss,avg}$ of formed colistin ≥1 mg/L (154). As the minimum inhibitory concentration required to inhibit the growth of 90% of organisms (MIC_{90}) for colistin is ≤0.5-1 mg/L against *A. baumannii, P. aeruginosa*, and *K. pneumoniae* (155) (see Section 1.3.6), it would be clinically useful to administer the maximal CMS dose in patients with CL_{Cr} > 80 mL/min, ideally in combination with

another antibiotic that may provide synergistic bacterial killing (149, 156). Since colistin is ~50% unbound in human plasma (156) (and unpublished data), a colistin $C_{ss,avg}$ of ~2 mg/L is necessary for effective treatment of bacteria with an MIC of 1 mg/L. For patients on RRT, in order to achieve a colistin $C_{ss,avg}$ of 1 mg/L, the current scientifically-based recommendation suggests a CMS loading dose of 9 MIU follow by followed at 24 h by 1 MIU every 12 h (q12h) for patients on intermittent haemodialysis, and 4.3 MIU q8h or 6.3 MIU q12h for patients on CRRT (149). Furthermore, haemodialysis patients should aim to have their dialysis performed towards the end of the CMS dosing interval to avoid excessive removal of CMS from the body. After dialysis, a CMS dose of 1.7 MIU is required to replenish the removed CMS.

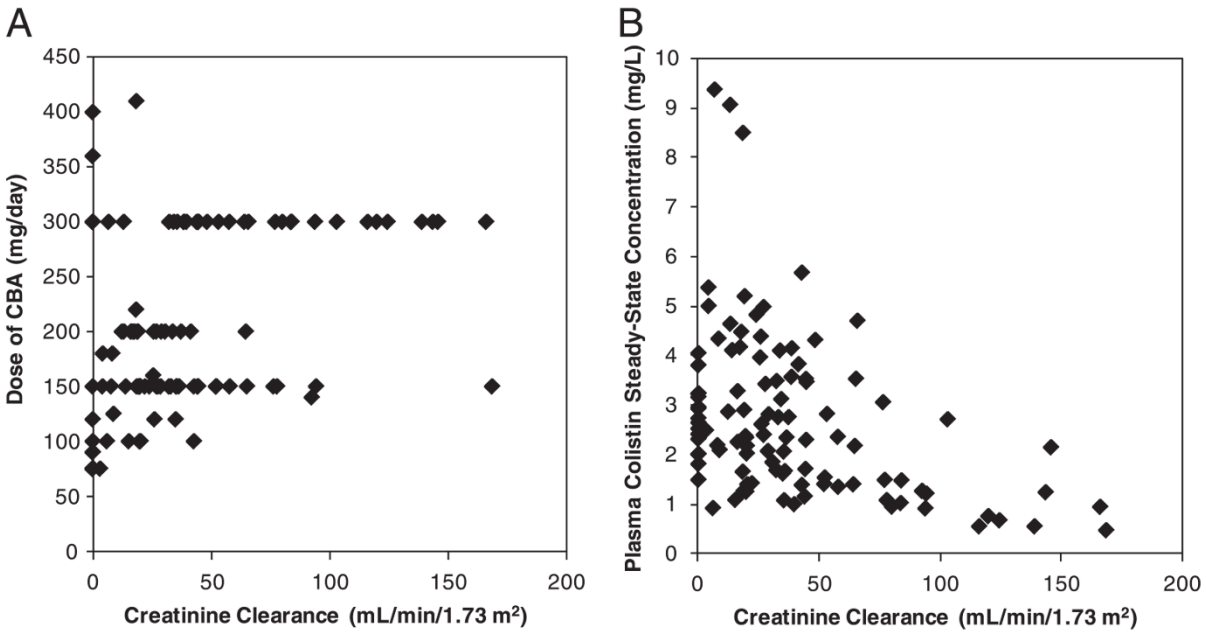

Figure 1.7 Relationship of physician-selected daily dose of colistin base activity (CBA) (A) and the resultant average steady-state plasma colistin concentration (B) versus creatinine clearance in 105 critically-ill patients. Figure adapted from Garonzik *et al.* (149), with permission.

Currently, little is known about the PK of CMS/colistin in the extravascular fluid. In critically-ill patients with and without central nervous system (CNS) infection, the distribution of colistin into the cerebral

spinal fluid is very low following i.v. administration of CMS (157). In critically-ill patients without CNS infection, the mean AUC_{CSF}/AUC_{SERUM} ratio from samples collected at 1, 4, and 8 h after CMS administration showed that the CSF concentration of formed colistin was only around 7% of the unbound serum colistin concentration after an i.v. dose of 3 MIU CMS every 8 h (157). When a combination of i.v. (3 MIU CMS every 8h) and intraventricular (0.125 MIU CMS once daily) CMS was administered, concentrations of formed colistin in CSF after 1, 4, and 8 h were >40% of the unbound colistin serum concentration (157). This study showed that the combination of i.v. and intraventricular administration of CMS may be useful for the treatment of CNS infection with Gram-negative bacteria, although further studies are necessary.

A recent study in patients with cystic fibrosis showed that the concentration of formed colistin in sputum is very low after i.v. administration (158). Less than 1 mg/L of unbound formed colistin was detected in the sputum at 12 h after a single i.v. administration of 5 MIU of CMS. The study showed that higher concentrations (>10 mg/L) of unbound formed colistin in the sputum can be achieved through inhalation of CMS. After a single inhalation dose of 4 MIU CMS, an average maximum concentration of ~12.8 mg/L was achieved in the sputum at ~4.6 h (158). Unbound plasma concentration of formed colistin following CMS inhalation is very low. Following a single nebulisation dose of CMS at 2 or 4 MU, the maximum plasma colistin concentrations were 0.22±0.055 mg/L and 0.33±0.092 mg/L, respectively, and <3% of the nebulised CMS dose was recovered in the urine in 24 h. In a study that compared intrapulmonary and systemic PK of CMS and formed colistin after inhalation and i.v. administration of CMS in critically-ill patients (159), unbound colistin concentrations were higher within the epithelial lining fluid (ELF, 9.53-1,137 mg/L) and lower in plasma (0.15-0.73 mg/L) after aerosol delivery compared with i.v. administration (1.48-28.9 mg/L in ELF and 0.15-4.7 mg/L in plasma). These findings show the potential of CMS inhalation for the treatment of pneumonia caused by GNB. Clearly, systematic PK/PD studies are warranted for optimising the use of inhaled CMS.

Although recent studies indicated that low extracellular concentrations of colistin was achieved with i.v. administration of CMS, there is possibility that the CSF, sputum and ELF concentrations were under predicted. Colistin has been reported to adsorb to plastic ware (160), consequently, a high proportion of colistin in low-protein media can be lost through the use of plastic pipette tips.

1.3.5.2 Polymyxin B

For PMB, a parenteral formulation (as its sulfate salt) is available in a number of countries, including the USA, but is not available in Europe (80, 94). Consequently, PMB is administered directly in its active antibacterial form while CMS requires conversion *in vivo* to generate the active entity, colistin. This difference in the form administered to patients has a major effect on the clinical pharmacological profile of the two polymyxins, an understanding of which is critical to their optimal clinical use (161).

Unlike CMS, to date limited PK studies have been conducted for PMB with no study examining the distribution into the extracellular fluid. A study involving eight critically-ill patients showed that, similar to colistin in rats, PMB is mainly eliminated by non-renal pathway(s) (<1% recovered in the unchanged form in urine) (80). After i.v. administration (0.5-1.5 mg/kg over 60 min every 12-48 h), the total body clearance ranged from 1.2-3.4 L/h and C_{max} from 2.38-13.9 mg/L. The largest population PK study to date involved 24 critically-ill patients with a wide range of kidney function (creatinine clearance of 10-143 mL/min), including two patients on CRRT (162). With i.v. doses between 0.45-3.38 mg/kg/day, the mean total body clearance was 1.7 L/h with little inter-individual variability and a median urinary recovery of 4.04%. This study showed the PMB $C_{ss,avg}$ ranging from 0.68-4.88 mg/L, with minimal inter-patient variability in the majority of patients (**Figure 1.8**), a finding in stark contrast with that discussed above for the influence of renal function on the $C_{ss,avg}$ of plasma colistin after administration of CMS. These PK data demonstrated that renal function does not affect PMB plasma concentration and should not be used for dosage regimen adjustment. However, in the two patients on CRRT, 12.2% and 5.62% of the dose was recovered as unchanged PMB in the dialysates during the 12 h dosing interval (162). These data suggest that, not unlike colistin, PMB can be cleared during

dialysis and higher doses may be required. Results from a study undertaken by Sandri *et al.* in 2013 suggest that PMB doses should be adjusted using total body weight. The current recommendation for the loading dose of PMB is 25,000 IU/kg and the maintenance dose is 15,000 IU/kg every 12 hours (162).

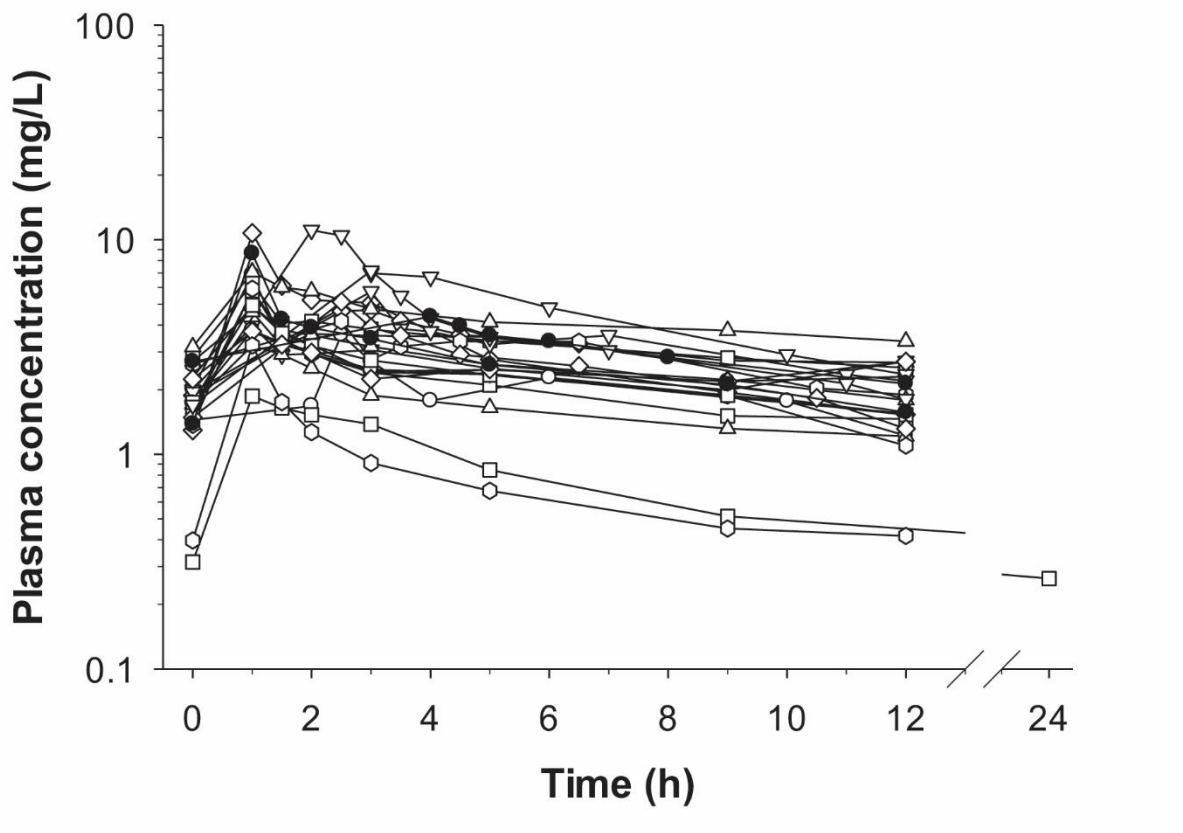

Figure 1.8 Plasma concentration-time profiles of PMB in 24 critically-ill patients. Concentrations from the patients undergoing continuous venovenous hemodialysis are shown by filled symbols. Figure adapted from Sandri *et al.* (162), with permission.

In summary, it is evident that the PK of CMS/colistin is influenced by renal function; hence, it is important to adjust the dosage regimens of CMS accordingly for optimal efficacy. For PMB, such adjustment is not required as it is mainly cleared by non-renal pathway(s). Currently, PMB appears to have superior PK characteristics for treatment of infections that rely on rapid attainment of efficacious plasma concentrations, as high plasma exposure is more rapidly achieved and maintained in patients

including those with varying renal function (161, 162). Since CMS is mainly eliminated by the kidneys, high urinary colistin concentrations can be produced through hydrolysis of CMS. Thus, CMS may be a better option than PMB for treatment of urinary tract infections with Gram-negative bacteria (161).

1.3.6 Pharmacodynamics of polymyxins

Colistin and PMB exhibit comparable potency *in vitro* due to their similar chemical structures (87, 155). The antimicrobial activity spectrum of polymyxins consists of many GNB including *Acinetobacter* spp. (45, 73, 80, 132, 155, 163-166)*, P. aeruginosa* (45, 73, 80, 155, 164-167)*, Klebsiella* spp. (45, 73, 80, 155, 165, 166, 168, 169), *Enterobacter* spp. (45, 165, 166), *Escherichia coli* (45, 155, 163, 165, 166, 170), *Salmonella* spp. (166, 170), *Shigella* spp. (166, 170), *Citrobacter* spp. (45, 166), *Haemophilus* spp. (171), *Bordetella pertussis* (172), *Legionella* spp. (173) and *Aeromonas* spp. (174). Apart from GNB, polymyxins are also active against numerous mycobacterial species (175) including *Mycobacterium xenopi*, *M. intracellulare*, *M. tuberculosis*, *M. fortuitum*, *M. phlei* and *M. smegmatis*. Additionally, polymyxins possess variable activity against *Campylobacter* spp. (176, 177) and *Stenotrophomonas maltophilia* (163, 164, 178, 179) and borderline activity against *Bartonella* spp. (180, 181).

Polymyxins are generally inactive against *Vibrio* spp. (182), *Providentia* spp. (166, 183), *Serratia* spp. (166, 170, 183, 184), *Proteus* spp. (170, 183)*, Morganella morganii* (185), *Helicobacter pylori* (186, 187), *Neisseria* spp. (170), *Brucella* spp. (170), *Edwardsiella tarda* (188), *Burkholderia cepacia* complex (164, 189), *P. pseudomallei* (190) and *Moraxella catarrhalis* (191). Polymyxins have no significant activity against the majority oft Gram-positive species (85, 192, 193).

The potency of an antimicrobial agent is generally indicated by its MIC, defined as the lowest concentration that inhibits the visible growth of a microorganism after overnight incubation (194, 195). This concentration varies depending on the agent and the organism (194, 195). As CMS is an inactive prodrug of colistin, MIC measurement of colistin is normally performed with colistin sulphate (81, 97). To date, SENTRY Antimicrobial Surveillance Program (2006–2009) is the largest surveillance

programme examining the MICs of colistin and PMB. The compiled data from this programme showed that colistin and PMB possess similar *in vitro* activities (MIC_{90}, ≤0.5-1 mg/L) against *A. baumannii, P. aeruginosa* and *K. pneumoniae,* with very low resistance rates globally (<0.1-1.5%) (155). However, questions have been raised regarding the susceptibility testing methods used for polymyxins, including their potential adsorption onto plasticwares used in the MIC measurement and poor diffusion of polymyxins in agar (160). In this regard, polysorbate 80 (P-80) was initially proposed to improve the broth microdilution MIC results for colistin and PMB as it can prevent their binding to plastic. However, its use was contraindicated by the Clinical and Laboratory Standards Institute (CLSI) owing to potential synergism between P-80 and the polymyxins (160, 194, 196). In the recent CLSI protocol, P-80 is not recommended in the measurement of colistin and PMB MICs (194). Presently, broth microdilution is regarded as the best method for polymyxin susceptibility testing. According to the recent CLSI guidelines, a colistin or PMB MIC ≤2 mg/L is susceptible and ≥4 mg/L is resistant for *Acinetobacter* spp. while ≤2 mg/L is susceptible and ≥8 mg/L is resistant for *P. aeruginosa* and other non-Enterobacteriaceae (194). The susceptibility breakpoints of colistin by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recently updated to MIC ≤2 mg/L is susceptible and >2 mg/L is resistant for *Acinetobacter* spp., *Pseudomonas* spp. and Enterobacteriaceae (197). However, data from recent pharmacokinetic/pharmacodynamic (PK/PD) studies (described in detail below) suggested the breakpoints for the above Gram-negative pathogens could be even lower. Consequently, a joint CLSI and EUCAST Working Group is re-evaluating the existing breakpoints (81, 160).

Based on the patterns of killing, antibiotics can be divided into two groups: concentration-dependent and time-dependent antibiotics (198, 199). Concentration-dependent antibiotics exhibit activity over a wide range of concentrations with moderate to prolonged persistent effects while time-dependent antibiotics exhibit activity over a narrow range of concentrations with minimal to moderate persistent effects (198, 199). Since the MIC is determined using only one time point and static drug concentrations, it does not provide details on the antimicrobial agent's activity over time or its post-

antibiotic effect (PAE) (200-202); such information is critical for the designing of effective dosage regimens. Consequently, PD studies are normally conducted using time-kill assays, *in vitro* PK/PD models and small animals.

Most studies on the PD of the polymyxins have been conducted using colistin. Time-kill studies with colistin (sulphate) indicated that polymyxins display concentration-dependent activity with potent activity against *A. baumannii* (**Figure 1.9**), *P. aeruginosa* and *K. pneumoniae*, including MDR strains (137, 140, 203-207). Bacterial killing is extremely rapid. At concentrations close to MIC, colistin significant reduced the bacterial count of *A .baumannii* as early as 30 minutes (131, 207).

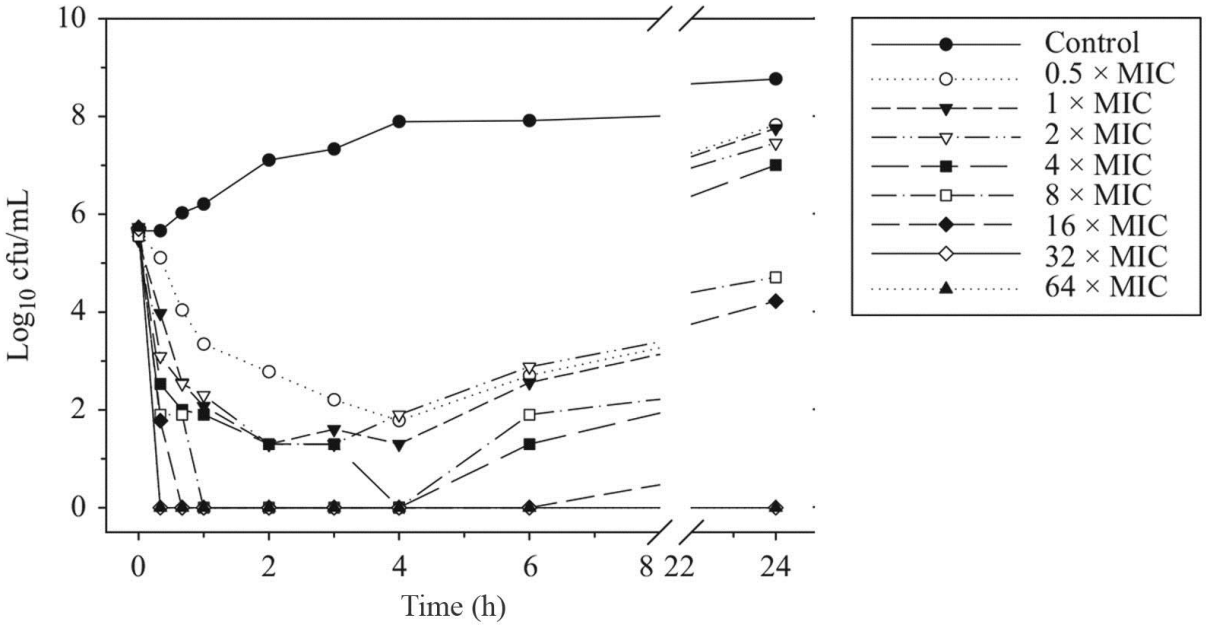

Figure 1.9 Time-kill studies showing the concentration-dependent activity of colistin against a clinical isolate of *A. baumannii*. Figure adapted from Owen *et al.* (207), with permission.

Although a concentration-dependent antibiotic, colistin exhibited minimal to moderate PAE against *P. aeruginosa* and *K. pneumoniae* (137, 203). Greater than one hour of PAE was observed against *P. aeruginosa* only when colistin was used at a high multiplicity of the MIC (8 and 16) (203). Against *K. pneumoniae*, a very modest 1.6 h PAE was observed only at concentrations ≥64 times MIC (137). In

multiple studies with *A. baumannii*, colistin exhibited both a negative and significant PAE (207-209). However, the study utilising the highest number (19) of MDR clinical isolates of *A. baumannii* indicated mean PAEs of 3.90 and 4.48 h for 1 x MIC and 4 x MIC concentrations of colistin, respectively (209). Currently, no studies have been conducted to investigate the PAE of PMB.

Time-kill studies with *P. aeruginosa* indicated that the bacterial killing of both colistin and PMB is influenced by the size of the bacterial inoculum (210, 211). At high bacterial inocula (10^8 and 10^9 colony-forming units per mL [cfu/mL]), both the rate and extent of killing by colistin was shown to be significantly reduced compared to a lower inoculum (10^6 cfu/mL); the rate of killing was 6- and 23-fold slower for inoculum size of 10^8 and 10^9 cfu/mL, respectively, compared to the inoculum size of 10^6 cfu/mL (211). At the 10^9 cfu/mL inoculum, to achieve equivalent bactericidal activity at the 10^6 cfu/mL inoculum (i.e. ≥3-$\log_{10}$ cf/mL reduction) up to 32-fold higher concentrations were required. Similar findings were observed for PMB where the rate and extent of the killing was significantly reduced at an inoculum of 10^7 cfu/mL compared with 10^5 cfu/mL for four strains for *P. aeruginosa* (210). Through MIC measurements, the activity of polymyxins against *A. baumannii* also appeared to be affected by different inoculum sizes. Against 19 *A. baumannii* isolates, the MIC_{50} and MIC_{90} were both 0.50 μg/mL for the inoculum size of 10^5 cfu/mL, 0.12 μg/mL and 4 μg/mL, respectively, for 10^6 cfu/mL inoculum, and both ≥512 μg/mL for 10^7 cfu/mL inoculum (209). Most studies with colistin showed rapid concentration-dependent killing against *A. baumannii*, *K. pneumoniae,* and *P. aeruginosa* with minimal post-antibiotic effects at clinically achievable concentrations (137, 207, 212). Although the antimicrobial killing of colistin was rapid, regrowth was observed for many isolates as early as within 2 h. In *A. baumannii* and *K. pneumoniae*, heteroresistance to polymyxins plays an important role in the rapid emergence of resistance (see Section 1.3.4). PMB displays very similar PD to that of colistin with rapid killing against *P. aeruginosa in vitro* and regrowth around 4 h (210).

The time course of antimicrobial activity for different antibiotics can vary significantly based on their PD characteristics (202). Thus, the interrelationship between the PK and the PD characteristics is an

important determinant for effective dosage regimens (202, 213, 214). For example, antibiotics that exhibit minimal concentration-dependent killing and prolonged PAE would be most effective in regimens that prioritise duration of antibiotic exposure over high antibiotic concentrations since higher concentrations do not provide better bacterial killing. For concentration-dependent antibiotics with prolonged PAE, effective regimens would involve infrequent dosing of large doses to achieve high plasma drug concentrations. Based on the pattern of bactericidal activity and the presence or absence of PAE in antibiotics, the antimicrobial activity for a particular antimicrobial agent can be best described by one of three PK/PD indices (215, 216) (**Figure 1.10**); (1) the maximal concentration to MIC ratio (C_{max}/MIC), (2) the area under the concentration-time curve over 24 h to MIC ratio (AUC/MIC), and (3) the cumulative percentage of a 24-h period that concentrations exceed the MIC (T>MIC).

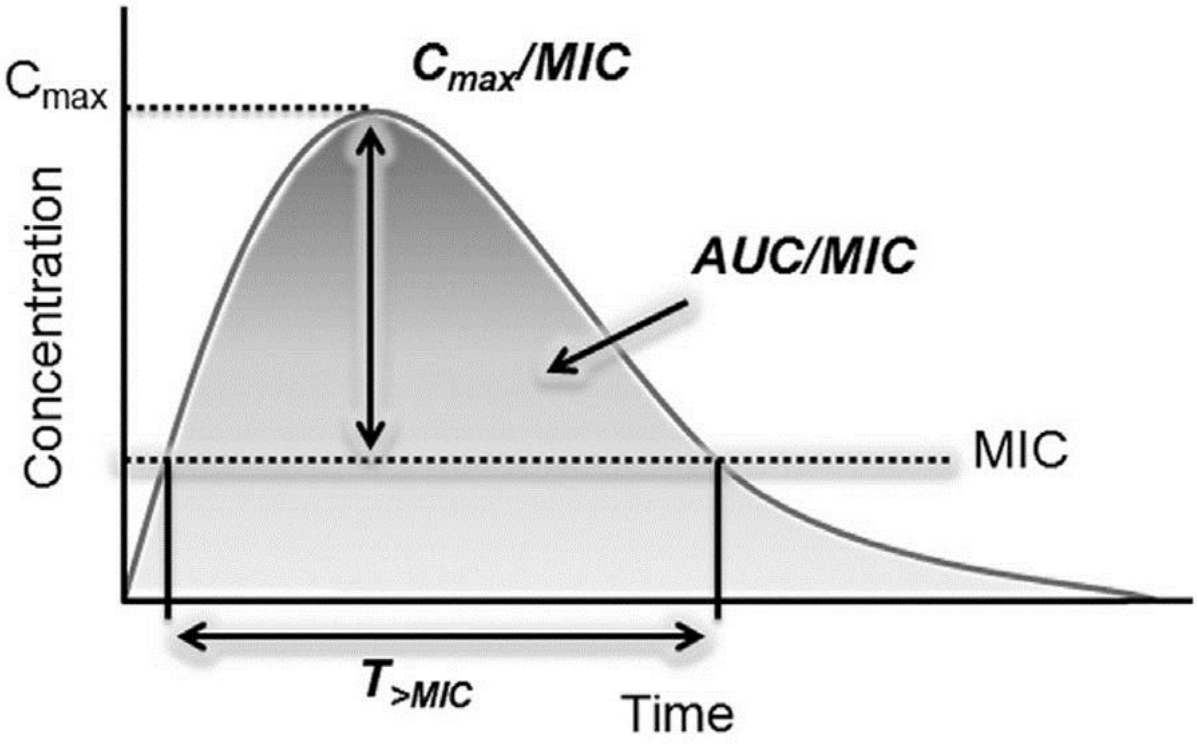

Figure 1.10 PK/PD indices associated with the efficacy of the antibiotics. AUC/MIC and T>MIC are calculated over a 24 h period. Figure adapted from Asín-Prieto *et al.* (216), with permission.

There is no true PK/PD index for an antibiotic as the index depends on both the input and elimination rate of the antibiotic in the performed study, the chosen design, and typically not consistent across a wider range of MICs. Nevertheless, using *A. baumannii* and *P. aeruginosa* in neutropenic mouse thigh and lung infection models, the PK/PD index that best describes the antimicrobial activity of colistin is the ratio of the area under the unbound (free) concentration–time curve to the MIC (fAUC/MIC) (**Figure 1.11**) (156); for *P. aeruginosa*, this has also been demonstrated *in vitro* (212). Owing to the potential binding of polymyxins to the plasticware or ultrafiltration membranes, our research group identified that ultrafiltration can be problematic (146), and rapid equilibrium dialysis methods are superior for measuring plasma binding of polymyxins (156). Our research group recent PK/PD study using ultracentrifugation and rapid equilibrium dialysis methods in neutropenic mice showed that the unbound fraction of colistin of 0.084 is ca. 6-fold lower than in humans (ca. 0.5) (156) (unpublished data). For three strains *of P. aeruginosa* [ATCC 27853, PAO1 and a multi-drug resistant (MDR) clinical isolate] and three strains of *A. baumannii* (ATCC 19606 and two MDR clinical isolates), an fAUC/MIC value of 7.4–13.7 and 7.4–17.6, respectively, was required for a 2 $\log_{10}$ reduction in bacterial load in the thigh of neutropenic mice. In the neutropenic mouse lung infection model, subcutaneous colistin was substantially less effective at killing *A. baumannii* and *P. aeruginosa* compared with in the thigh infection model (156).With the highest tolerable dose (40 mg/kg administered 6- or 8-hourly with cumulative daily doses of 120-160 mg/kg), 2 $\log_{10}$ killing in the lungs was not achievable for all six of the tested strains. The lower antibacterial activity in the lungs relative to the thigh is most likely due to limited drug exposure in the lungs following parenteral administration.

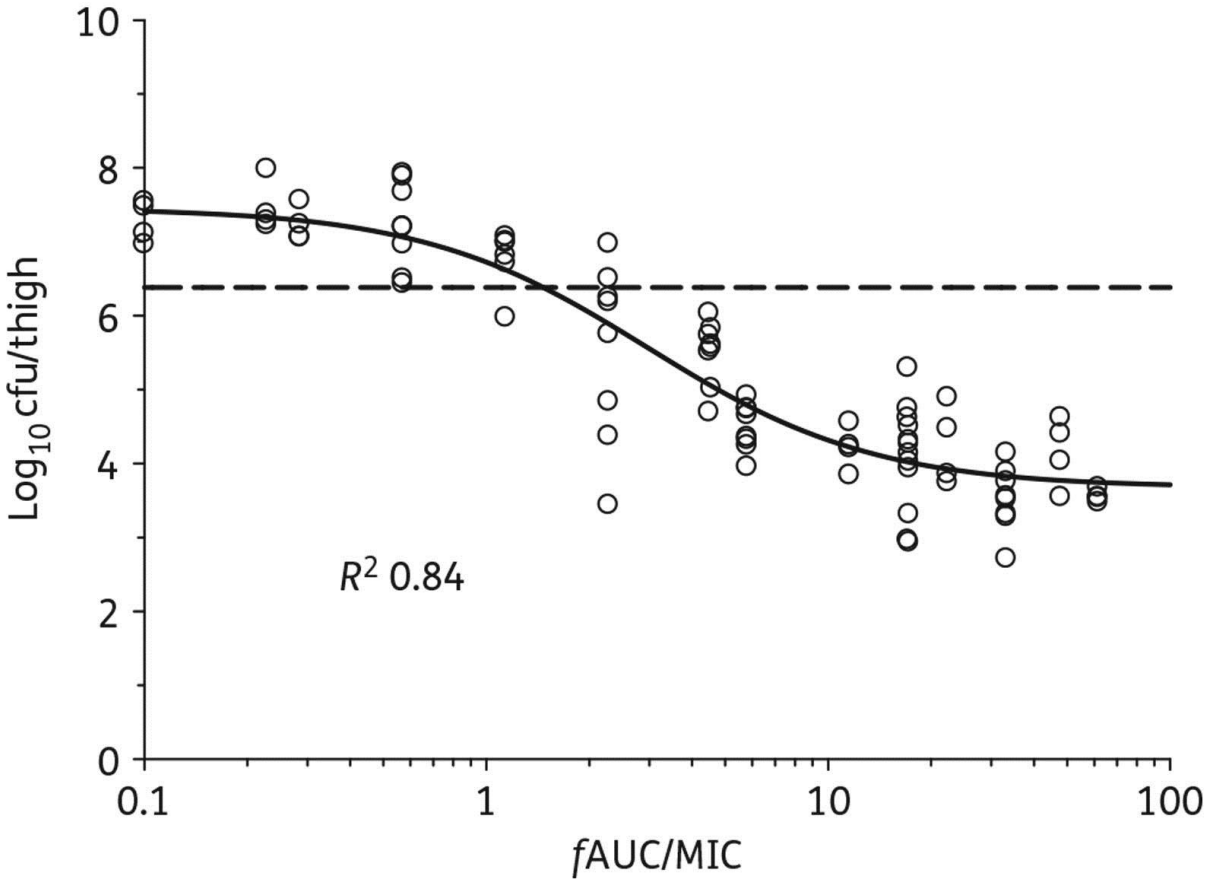

Figure 1.11 Relationship between $\log_{10}$ colony forming unit per thigh at 24 h and fAUC/MIC for *A. baumannii* ATCC 19606. Figure adapted from Cheah *et al.* (156), with permission.

Currently available data from animal and clinical studies suggest that colistin (and CMS) may have limited efficacy against respiratory tract infections (156, 158). Limited studies to date have examined the PK/PD index driving the activity of PMB. Given the similarity in the structure, it is very likely that fAUC/MIC is the most predictive PK/PD parameter for parenteral PMB (210). In patients with good renal function, however, i.v. administration of PMB is very likely to generate higher fAUC/MIC values than CMS at similar dose because: (i) CMS distribution is influenced by kidney function while PMB is not; and (ii) CMS conversion to colistin in vivo is slow and incomplete. To optimise the clinical use of PMB, more PD studies are needed.

Although polymyxins largely remain effective against many GNB, recent PK and PD data on polymyxins suggest that caution is required with monotherapy due to emergence of resistance. Studies with *in*

vitro PK/PD models that simulate the clinically achievable unbound plasma concentration-time profiles of colistin or PMB in critically-ill patients showed early regrowth of heteroresistant strains of *A. baumannii* (217, 218)*, P. aeruginosa* (210, 219, 220), and *K. pneumoniae* (221, 222) following monotherapy; population analysis profiles (PAPs) revealed that the regrowth of bacteria exposed to colistin for 72 h or PMB for 96 h possessed substantially higher proportion of polymyxin-resistant subpopulations compared to the bacteria culture prior to polymyxin therapy and the growth controls. Polymyxin monotherapy has also been demonstrated to produce similar increases in the proportion of polymyxin-resistant subpopulations (**Figure 1.12**) in other *in vitro* studies including static and dynamic time-kill infection models, even in the presence of polymyxin concentrations much higher than the MIC of the organism (137, 140, 207, 211, 223-227). For *A. baumannii*, similar finding has also been observed in a murine thigh and lung infection models (228). It is suspected that, in bacteria with polymyxin heteroresistance, the emergence of the polymyxin resistance from polymyxin monotherapy is due to the selective killing of the polymyxin susceptible sub-populations and subsequent unchallenged growth of the subpopulations with mutations that confer polymyxin resistance (e.g. LPS-deficient *A. baumannii* (128); [see Section 1.3.4]). In addition to the selection of resistant subpopulations, it is also suspected polymyxin resistance may develop from genetic mutations that are induced through polymyxin exposure. In a recent study investigating the mutant prevention concentrations (MPC; the drug concentration required to prevent the emergence of all single-step mutations from a susceptible population of more than 10^{10} cells (229)) of colistin in 40 *A. baumannii*, 40 *P. aeruginosa* and 40 *K. pneumoniae* clinical isolates, including imipenem resistant isolates, amino acid alterations of PmrA/PmrB, PhoP/PhoQ and ParR/ParS (two component systems involved in polymyxin resistance through lipid A modification [see Section 1.3.4]) occurred *in vitro* within the period of selection of single-step mutants (230). Additionally, the investigation revealed high MPCs (≥64 mg/L) of colistin for all isolates, which cannot be achieved in patients with the current polymyxin dosing recommendations (see Section 1.3.5.1). Collectively, the findings indicated that polymyxin combinations may be necessary to minimise the emergence of polymyxin resistance.

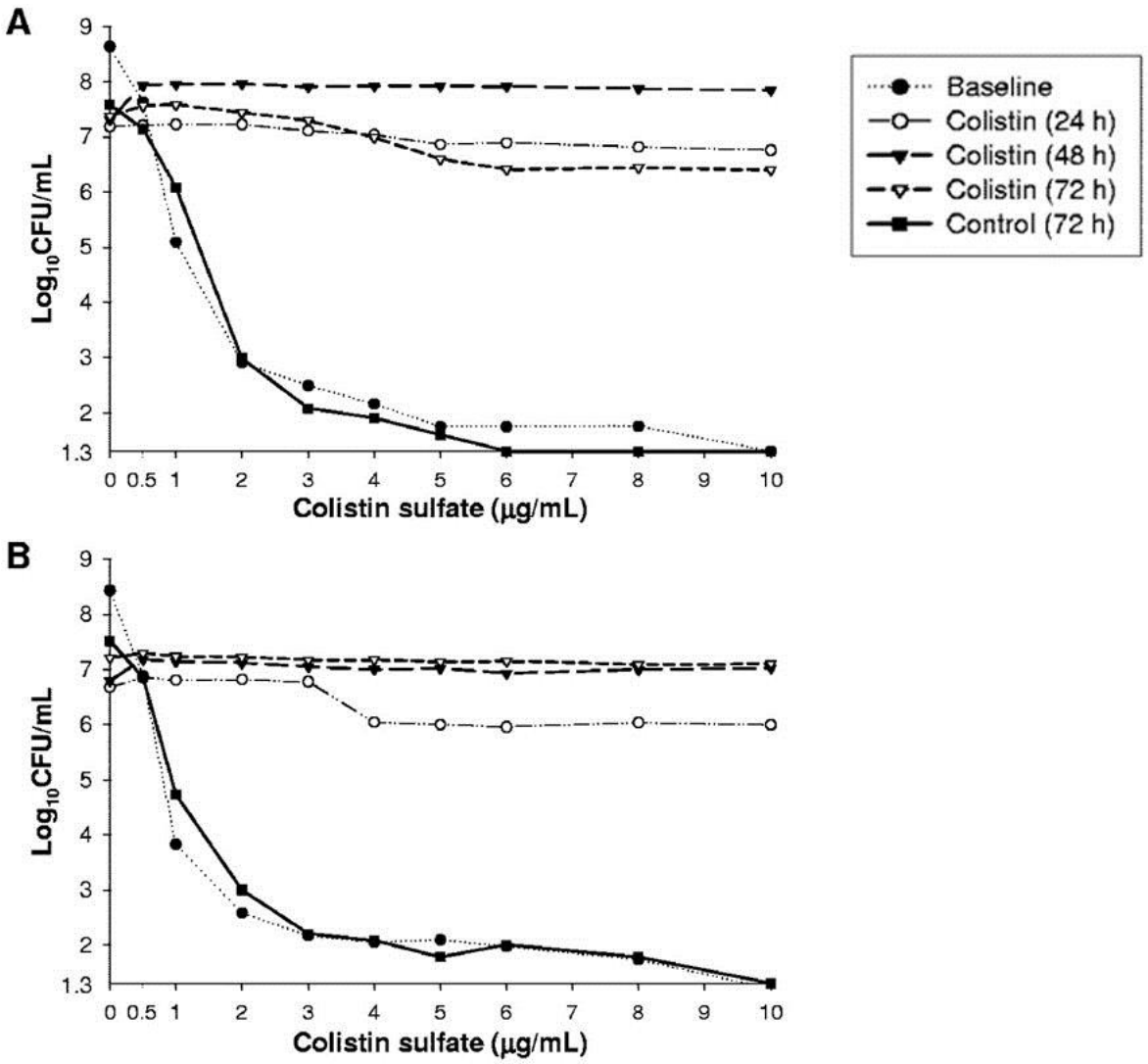

Figure 1.12 Population analysis profiles of an *A. baumannii* clinical strain (A) and ATCC 19606 (B) from an *in vitro* PK/PD model. Figure adapted from Tan *et al.* (140), with permission.

1.3.7 Toxicodynamics of polymyxins

In the early years of their use, polymyxin-associated neurotoxicity occurred in patients with an incidence as high as 27% following parenteral administration (86, 231). However, recent retrospective clinical studies have not shown neurotoxicity to be a major concern (232, 233). Nephrotoxicity is by

far the most common and dose-limiting side effect associated with parenteral polymyxins, with incidence rates in patients as high as 60% (234, 235). However, the rate of nephrotoxicity in patients receiving i.v. polymyxins is somewhat variable and depends on the definition of nephrotoxicity employed [e.g. RIFLE (risk, injury, failure, loss, and end-stage kidney disease) and AKIN (Acute Kidney Injury Network) scoring systems] (236).

Nephrotoxicity has also been observed both with colistin and PMB following parenteral administration (236-239). Recent toxicodynamic (TD) analyses of colistin showed that patients with colistin $C_{ss,avg}$ > 2.5 mg/L and patients with CL_{Cr} > 80 mL/min are more likely to develop nephrotoxicity (237, 238). The minimum colistin plasma concentration was also identified as an independent risk factor for nephrotoxicity, which occurred in the majority of patients when the minimum colistin plasma concentration was ≥2.2 mg/L (odds ratio = 4.6 on Day 7) (237).

For PMB, a daily dose of ≥150 mg (hazard ratio = 1.92) has been identified as the risk factor of nephrotoxicity (239). A retrospective study showed the earliest onset of nephrotoxicity reported for i.v. CMS or PMB occurred 2 days after initiation of therapy, with the majority of cases occurring after 15 days of therapy (236). Fortunately, polymyxin-associated nephrotoxicity is reversible in the majority of patients (237, 240).

In regard to the mechanisms of polymyxin-induced nephrotoxicity, cell culture and animal studies have demonstrated that colistin and PMB accumulate in renal tubular cells possibly through active uptake mechanisms mediated by megalin and PEPT2 transporters (241, 242). Immunostaining of kidney sections from a mouse with PMB-induced nephrotoxicity showed PMB distributed mainly within the proximal tubular cells of the renal cortex (243). A study of PMB accumulation with synchrotron X-ray fluorescence microscopy (XFM) showed the concentrations were approximately 1930- to 4760-fold higher in single rat (NRK-52E) and human (HK-2) kidney tubular cells than extracellular concentrations (244). The XFM results also showed a significant increase in the intracellular calcium concentration, a potential stimulus for triggering apoptosis (244, 245). The

extremely high intracellular concentrations of polymyxin caused dramatic changes in mitochondrial morphology, disrupted the mitochondrial membrane potential, induced apoptosis and cell cycle arrest in renal tubular cells (246, 247). A study with rat kidney proximal tubular cells (NRK-52E) showed that PMB induces activation of caspase-3, -8, and -9, expression of FasL, mitochondrial fragmentation, loss of mitochondrial membrane potential, and formation of reactive oxygen species (ROS) (248). Clearly, developing a solid knowledge-base of the precise mechanisms of the uptake of polymyxins by renal tubular cells and subsequent cell death are crucial for the development of novel approaches to attenuate polymyxin-induced nephrotoxicity, optimising their use in patients and the discovery of safer new-generation polymyxins.

1.3.8 Polymyxin combination therapies

Due to the lack of new antibiotics and potential for development of resistance with polymyxin monotherapy (see Sections 1.1 and 1.3.6), it is important that both polymyxins are used optimally to maximise bacterial killing and minimise resistance and nephrotoxicity. There are a number of approaches to minimise resistance to polymyxins, including optimising their dosage regimens in patients using PK/PD/TD and employment of rational combinations. Optimising polymyxin dosages may minimise resistance as suboptimal plasma concentration can promote adaptive responses in bacteria.

Based upon recent animal PK/PD and clinical PK data (see Sections 1.3.5 and 1.3.6), colistin combination therapy is likely to be beneficial in patients infected by a causative pathogen with a MIC >1 mg/L, or in patients with moderate-to-good renal function receiving i.v. CMS as many of these patients may not achieve an unbound colistin plasma concentration of >1 mg/L (156, 228, 249). Given the high incidences of polymyxin heteroresistance in *A. baumannii* and *K. pneumonia* and the potential for the rapid emergence of resistance with polymyxin monotherapy (see Sections 1.3.4 and 1.3.6) (137-140, 217, 218, 221, 222, 228, 229), polymyxin combinations have been explored as a potential way to increase the bacterial killing and prevent polymyxin resistance development in these bacteria

(229, 249-251). Sub-population and mechanistic synergy have been proposed as two possible mechanisms for the enhanced bacterial killing that exists for the combination of different antibiotics (251, 252). Sub-population synergy occurs when the presence of a second antibiotic helps eliminate the sub-population that is resistant to the other antibiotic, whereas mechanistic synergy occurs when the second antibiotics targets different cellular pathways to the other antibiotic and leads to the overall enhanced bacterial killing (251, 252) (**Figure 1.13**). It has also been proposed that, as polymyxins disrupt the outer membrane of GNB, their synergystic activity with other antibiotics may evolve through their ability to promote the entry of the other antibiotic across the OM which may be normally impermeable to the secondary antibiotic (253). Unfortunately, many polymyxin combinations used in the clinic have been chosen empirically. A more rational approaches towards what secondary antibiotic to use in the combination with polymyxins should take into account the effect of the secondary antibiotic on pre-existing polymyxin-resistant sub-populations, the site of its target, as well as the changes in bacterial responses to the new combination treatment using omic 'technologies'.

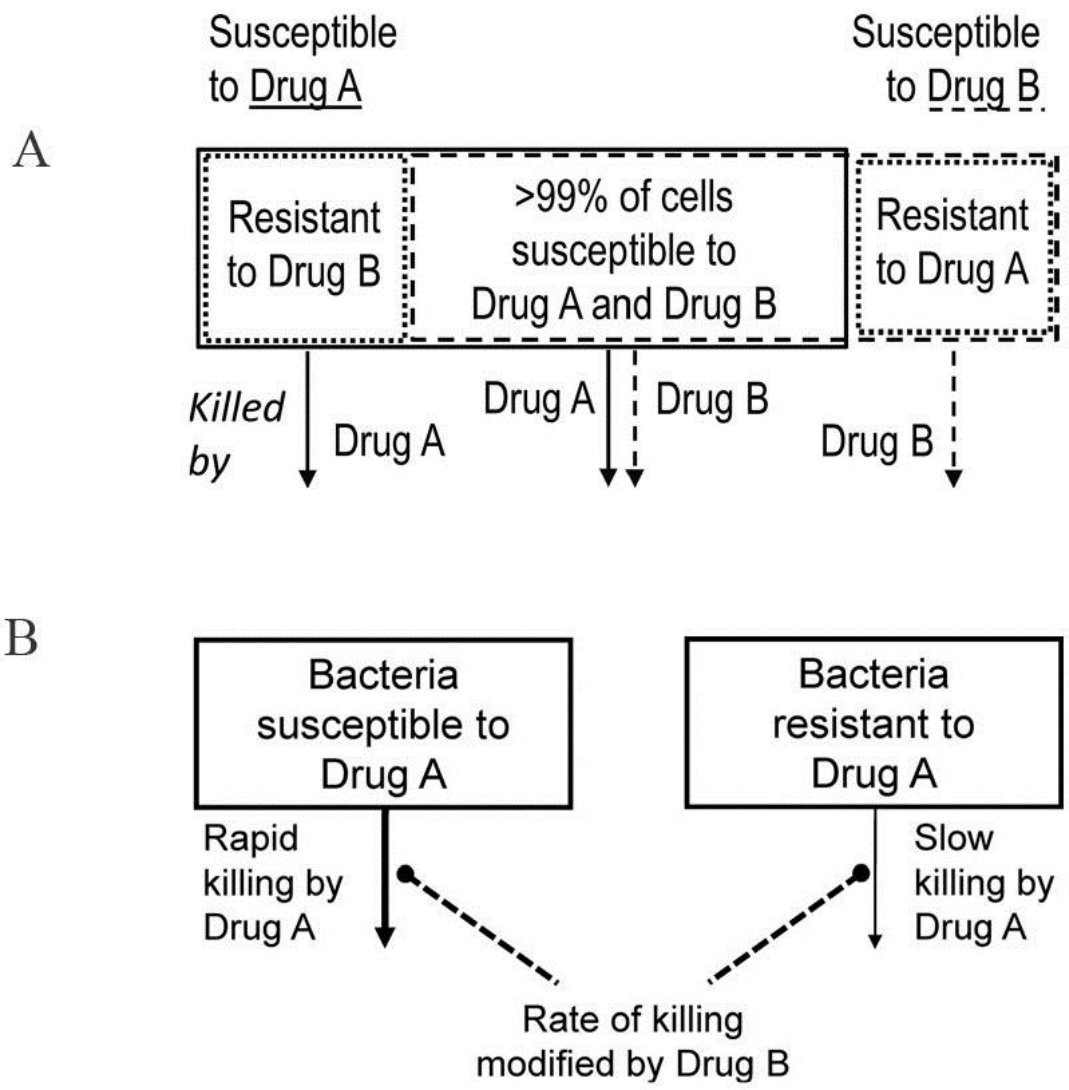

Figure 1.13 Schematic representations for subpopulation synergy (A) and mechanistic synergy (B). In subpopulation synergy, the resistant subpopulations of one drug is susceptible to the second drug. In mechanistic synergy, the second antibiotic enhances the bacterial killing by targeting different cellular pathways to the other antibiotic. Figure adapted from Landersdorfer *et al.* (252), with permission.

The combination of drugs can have various effects on the bacterial killing (254). The effects are caused by different PD interactions that are commonly classified by i*n vitro* studies as additivity, synergy, indifference or antagonism (254). The type of interaction may depend on the concentrations of the drugs being used and an interaction is only clinically relevant when the combination is superior than

the most effective drug when administered alone. The criteria used to classify the PD interactions depend on the experimental methods employed. In studies using the checkerboard microbroth dilution method, the fractional inhibitor concentration (FIC) index is used to classify these PD interactions. The FIC is calculated as follow (254):

$$\text{FIC index} = \frac{\text{MIC of drug A in combination}}{\text{MIC of drug A alone}} + \frac{\text{MIC of drug B in combination}}{\text{MIC of drug B alone}}$$

With the checkerboard method, traditionally the PD interaction is defined as synergistic, additive or antagonistic when the FIC index is ≤0.5, 1.0 or 2.0, respectively (255, 256). Recently, however, it has been suggested to use FIC index >4 to define antagonism and FIC of 2 to define indifference due to the inherent imprecision of the technique when two-fold dilutions are used (257). Alternatively, static or PK/PD time-kill methods are often used for the assessment of the PD interaction in combination therapy. These methods are considered superior than the checkerboard method as they measure the bactericidal activity of the combination over time while the checkerboard method only reveals the inhibitory information for a single time point (254). For time-kill methods, synergism and antagonism are traditionally defined as a 100-fold increase and a 100-fold decrease in bacterial killing at 24 h, respectively, with the combination therapy compared most active drug used in monotherapy (254).

To date a number of different antibiotics have been investigated in combination with polymyxins. The most common combinations reported are with carbapenems, rifampicin, and tigecycline (258-263). A systematic review and meta-analysis of polymyxin combinations with carbapenems showed that *in vitro* time-kill studies synergism occurred for all three species *A. baumannii, P. aeruginosa* and *K. pneumoniae*; however, it occurred more frequently against *A. baumannii* (77%) than *P. aeruginosa* (50%) and *K. pneumoniae* (44%) (258). In two static time-kill studies, colistin (0.06 - 8 mg/L) and meropenem (0.03 - 64 mg/L) combinations were synergistic against 94.2% (49/52) of *A. baumannii* (264) (including colistin-resistant and meropenem-resistant isolates), while colistin (0.12 - 16 mg/L) and doripenem (0.06 - 32 mg/L) combinations were synergistic against 100% (25/25) of *A. baumannii*

(265) (including colistin-resistant and doripenem-resistant isolates). The same studies showed colistin (0.12 - 1 mg/L) and meropenem (0.06 - 8 mg/L) combinations were synergistic against 25.5% (13/51) of *P. aeruginosa* (264) (including meropenem resistant isolates), while colistin (0.12 - 16 mg/L) and doripenem (0.03 - 128 mg/L) combinations were synergistic against 76.0% (19/25) of *P. aeruginosa* (265) (including colistin-resistant and doripenem-resistant isolates). For *K. pneumoniae*, a static time-kill study showed the combinations of colitin (5 mg/L) and imipenem (10 mg/L) were synergistic against 33.3% (14/42) of *K. pneumoniae* isolates (266); those that were susceptible either to both agents or to colistin. For polymyxin and rifampicin combinations, time-kill studies showed synergy rate of >50% against *A. baumannii* (259) and 100% against carbapenemase-producing *K. pneumoniae* isolates (260). Although less frequently investigated, *in vitro* studies indicated that synergism also exists for polymyxin/ tigecycline combinations (261-263). The synergistic activity between polymyxins and tigecycline was reported for carbapenem-resistant *A. baumannii* (263), and various KPC-producing *Enterobacteriaceae* including *E. coli* (262), *K. pneumoniae* (261, 262)*, Serratia marcescens* (261, 262) and *Enterobacter cloacae* (261, 262).

In addition to the enhanced bacterial killing, *in vitro* studies have also demonstrated that the combinations of polymyxin and carbapenem or rifampicin also suppressed the emergence of polymyxin-resistance (217, 220, 221, 258, 267). Colistin/rifampicin combinations were shown to suppress the development of polymyxin resistance in *A. baumannii* (217), while colistin/doripenem combinations were shown to suppress the development of polymyxin resistance in *P. aeruginosa* (220, 267) and *K. pneumoniae* (221). The combination of PMB and tigecycline, however, it did not suppress the emergence of polymyxin resistance in *A. baumannii* according to one *in vitro* pharmacodynamic model (263).

Limited animal data are available examining polymyxin combinations. In a study with a model of sepsis in mice, all animals infected with either *P. aeruginosa* ATCC 27853 or MDR clinical strain died by 72 h. When the animals were given a single i.v. dose of colistin (1 mg/kg), imipenem (20 mg/kg), or both

immediately after the bacterial inoculation, the death rates at 72 h reduced to 30%, 40%, and 10%, respectively, for animals infected with *P. aeruginosa* ATCC 27853, and 30%, 80%, and 15% for those infected with the MDR clinical strain (268). In a similar study involving sepsis model in rats where the animals were given single i.v. dose colistin (1 mg/kg), rifampicin (10 mg/kg), or both as therapy instead, for animals infected with *P. aeruginosa* ATCC 27853, the death rates at 72 h were 26.7%, 93.3%, and 6.6%, respectively; for the animals infected with the MDR clinical strain, the death rates were 53.3%, 100%, and 26.6% (269). In a mouse model of pneumonia, a combination of colistin (administered as CMS intranasally at 5 mg/kg/12h) with either imipenem (30 mg/kg/12 h administered subcutaneously) or rifampicin (25 mg/kg/24 h administered orally) increased the survival of the animals infected with a MDR *P. aeruginosa* strain to 62.5% and 75% from 0% after 72 h, respectively ($P < 0.05$), compared to the control and monotherapy groups (270). Similarly, against MDR *A. baumannii* in a thigh infection rat model, the combination treatment with colistin (3 mg/kg administered intramuscularly) and rifampicin (5 mg/kg administered intravenously) also increased the survival (30% on day 6) of the animal compared to either drug alone (0% on day 6) (271). Unlike *in vitro* studies, colistin (administered as CMS intraperitoneally at 1.25 mg/kg/6h) plus tigecycline (administered intraperitoneally at 10 mg/kg/12h) did not show statistical significant differences in activity compared to either drug alone against XDR *A. baumannii* in a rat pneumonia model (272).

Although some results from animal studies are promising, the potential benefit of polymyxin combinations in patients remains unclear due to several shortcomings. One major challenge is animal studies often do not clearly indicate whether the 'colistin' administered was colistin (sulphate) or CMS (sodium) (268, 269), which makes results difficult to interpret as the PK of the two forms of colistin are highly different. Another important shortcoming is the administered doses used in animals often selected based on the doses used in human, which does not take into consideration the importance of animal scaling to compensate for the PK dissimilarities across species (273). Currently, few clinical studies have been conducted to assess the benefit of combining polymyxins with another antibiotics. Most clinical studies that were conducted are retrospective in nature. For the few prospective studies

reported, the number of patients is generally small and thus the power calculation is low. In a recent retrospective study, the bacterial killing of colistin alone or combined with sulbactam or carbapenem against *A. baumanniii* were evaluated in 70 patients with ventilator-associated pneumonia (274). Colistin was administered intraveneosly in the form of CMS at 75 mg/8h or 150 mg/12h (the dose was adjusted for patients with renal impairment). Carbapenem was administered intravenously either as imipenem at 500 mg/6h or meropenem at 1 g/8 h (meropenem was applied as prolonged infusion to achieve optimal efficacy). Sulbactam was administered intravenously at 1 g/8 h. Of the 70 patients, 17 patients were administered CMS alone, 20 patients were administered CMS and sulbactam, and 33 patients were administered CMS and carbapenem. The clinical and microbiological responses from the investigation showed no significant difference statistically ($p>0.05$), although both responses were higher in the CMS plus carbapenem group (274). In a recent analysis conducted on all clinical studies that compared colistin monotherapy with colistin-based combination therapy for the treatment of carbapenem-resistant GNB, the findings indicated that both colistin *per se* and the colistin/carbapenem combination produced similar outcomes (275). The investigators noted that there are potential sources of bias in the original studies, including selection bias (different criteria required for the selection of polymyxin monotherapy or combination therapy), small study size (does not permit adjustment for other risk factors), potentially suboptimal dosing strategies, and the appropriateness of the initial empirical antibiotic treatment.

The potential benefit of polymyxin and rifampicin combinations was recently investigated in a multicenter randomised controlled trial (RCT) involving 210 patients with serious infections due to extensively drug-resistant *A baumannii* (276). In this study, the patients were randomly allocated (1:1) to either CMS alone (i.v. 2 MIU/8h), or CMS (at the same dose specified) plus rifampicin (i.v. 600 mg/12h). The primary end point of the study was overall 30-day mortality and the secondary end points were infection related death, microbiologic eradication, and length of hospitalization. The results showed a significant increase in microbiologic eradication rate for the colistin and rifampicin combination group; however, no difference was observed for infection-related death and length of

hospitalization (276). The multicenter RCT conducted by Durante-Mangoni and colleagues (276) also included polymyxin resistance emergence as one of its secondary outcome measures, but no difference between the combination and monotherapy groups was found. In a single-center RCT, the benefit of using colistin combined with rifampicin over colistin alone was evaluated in 43 patients with ventilator-associated pneumonia (277). In this study, 22 patients were administered only colistin intravenously (300 mg CBA [~10 MIU] per day), and the other 21 patients were administered colistin intravenously (at the same dose) combined with rifampicin (600 mg/day) nasogastrically. Similar to the multicenter RCT (276), the time to microbiological clearance was significantly shorter in the combination group. The findings also showed that clinical, laboratory, radiological, and microbiological response rates were better in the combination group; however, they were not statistically significant ($P>0.05$). At present, the available clinical data do not support the combination of colistin and rifampicin because of the lack of improved clinical outcomes with the combination therapy.

To understand the real benefit of polymyxin combination treatments, future RCTs should include a loading dose and/or use higher daily maintenance doses to achieve optimal plasma polymyxin concentrations throughout the treatment course (see Section 1.3.5.1). Furthermore, several clinical studies were underpowered and/or suffered from the ethical constraints involved in conducting RCTs in critically-ill patients. With regard to the latter, in most studies, patients in both the polymyxin monotherapy and combination groups received multiple other antibiotics in addition to the index second antibiotic under consideration (276). Because of the last-line status of the polymyxins, in many studies it is likely (but usually not reported) that the time from diagnosis of infection to the initiation of polymyxin in the polymyxin monotherapy and combination groups differed, very likely favouring the monotherapy group. Most studies have had multiple endpoints, but many have neglected to identify the potential benefit of polymyxin combinations to prevent the development of polymyxin resistance. Future studies should also be appropriately designed to evaluate the emergence of polymyxin resistance following combination and monotherapy. Although the clinical benefit of

polymyxin combinations remains unproven, it may be beneficial to use polymyxin combination therapy considering the polymyxin-associated nephrotoxicity and PK/PD profiles (see Sections 1.3.6 and 1.3.7). Well-designed and appropriately powered RCTs are required to examine the potential advantage of polymyxin combination therapy versus polymyxin monotherapy.

1.4 Treatment of MDR GNB with novel polymyxin/non-antibiotic drug combinations

Although much effort has been invested in the discovery of new antibiotics for the treatment of MDR GNB, the process of *de novo* drug discovery is lengthy and the success rate is very low (278-280). It has been shown that the process from discovery to market can be around 10 - 17 years with a success rate <10% (278). The repositioning of non-antibiotic drugs for antimicrobial purposes represents a promising alternative approach to combat MDR GNB (281). Through drug repositioning, where safety and PK uncertainties are reduced, new antimicrobial agents can be available as early as three years (278). Various studies have already identified multiple classes of non-antibiotic drugs that possess bacterial killing (281-284). Recently, bacterial killing against *A. baumannii* was identified in gallium nitrate, a drug normally used for the treatment of hypercalcemia of malignancy (282). The antihistamine terfenadine has also been shown to possess bacterial killing against *Staphylococcus aureus* and other Gram-positive bacteria (283), and the anti-cancer drug 5-Florouracil was recently shown to possess bacterial killing against both Gram-positive and Gram-negative bacteria (284). Consequently, the use of synergistic combinations of non-antibiotic drugs with antibiotics is emerging as a potentially valuable and cost-effective approach to improve the clinical efficacy of currently available antibiotics against problematic MDR bacterial pathogens (285). Given polymyxins ability to permeabilise the protective outer membrane of GNB, it raises the possibility that polymyxin may help to reveal the antimicrobial activity of non-antibiotic drugs by enabling them to gain entry into the bacterial cells and interact with their intracellular targets. In fact, the bacterial killing of the earlier mentioned gallium nitrate was enhanced in the presence of colistin (282). The potential use of a non-antibiotic drug in combination with polymyxins to treat MDR-GNB represents a temporary solution for

the currently limited number of effective antibiotics. However, Care should be taken when it comes to using to using non-antibiotic such as anticancer drugs. The use of anticancer drugs may have adverse side effect on the patients and lead to the development of anticancer drug. Similar problem with regards to the emergence of resistance may also develop following the use of other non-antibiotic drugs. To address this problem it is critical to understand the activity of the combination at the systemic level so optimal usage of such combination may be achieved.

Increasingly, systems-oriented approach is becoming widely employed in research and has been successfully applied in drug discovery, biotechnology and in the clinic (286-290). Systems biology is an area of study that aims at understanding biology at the system level, i.e. the networks that form the whole of living organisms rather than the characteristics of isolated parts of a cell or organism (291, 292). This area of research incorporates multiple scientific disciplines including biology, computer science, engineering, bioinformatics, and physics (291-293). Major progress in measurement technologies that allowed better acquisition of comprehensive data sets on system performance and information on the underlying molecules, and computational methods that allowed better analysis and interpretation of large data sets, has been the main driving factor for the shift from functional genomics to systems biology (293, 294). With systems biology, it is possible to predict changes in biological systems over time and under varying conditions, which are useful for the development of the solutions to these changes.

When it comes to bacterial killing, antibiotic-mediated cell death is a complex process and the drug-target interaction only signals its initiation (295). A better understanding of the specific sequences of events following the binding of an antibiotic to its target and the ultimate mechanism underpinning bacterial cell death is useful for the development of new antibiotics and the improvement of currently available antibiotics. Knowledge of how antibiotics work at the systemic level in bacteria is also essential for better understanding of how antibiotic resistance develops and to prevent it from emerging. Recently, systems-oriented research with transcriptomics and metabolomics has been used

for generating novel insights on the activity of polymyxins and the emergence of its resistance (105, 106, 130, 296-298). In one transcriptomic study, transcriptomes of the stable and non-stable polymyxin-resistant *A. baumannii* following exposure to PMB showed altered expression of genes associated with outer membrane structure and biogenesis (296). In a separate study, six genes encoding PmrAB two-component regulatory enzymes, PmrC (a lipid A phosphoethanolamine transferase), a glycosyltransferase, a poly-beta-1,6-N-acetylglucosamine deacetylase, and a putative membrane protein were found to be associated with polymyxin resistance in *A. baumannii* (297). Transcriptomic studies with *K. pneumoniae* revealed polymyxin B induced expression of several two-regulatory components, such as ArcA-ArcB, and this is suspected to be involved in the re-routing of the *K. pneumoniae* metabolism, and genes that correlated to growth in acid stress (298). Recent metabolomics studies identified a range of metabolites associated with treatment with polymyxins (discussed in Sections 1.3.3) and polymyxin resistance due to loss of LPS (discussed in Sections 1.3.4) in *A. baumannii*. Collectively, these results indicated that the activity of polymyxins is beyond that of outer membrane damage. These studies suggest that transcriptomic and metabolomic studies are potentially valuable tools for the investigation of the bacterial killing of novel combinations of polymyxins and non-antibiotic drugs. The data generated from such investigations can also be useful for computational models that predict effective new antimicrobial agents in combination with polymyxins.

In addition to the understanding of the bacterial killing of antibiotics at the systemic level, systemic understanding of bacterial pathogenesis is equally important for effective treatment of infectious diseases; this is possible through the studies of host-pathogen interaction. In the past, bacterial pathogenesis studies only focused either on the pathogen or the host (299). With the advance transcriptomic techniques, it is now possible to monitor the gene expression of the host and the pathogen simultaneously (300-302). Simultaneous transcriptional profiling of the interaction of HEp-2 cell monolayers and *Chlamydia trachomatis* serovar E EBs discovered a possible chlamydial strategy for early iron acquisition, putative immune dampening effects, and a potential *Chlamydia*-induced

fibrotic scarring (302). Since simultaneous transcriptional profiling can interogate the gene expression of the host and pathogen at the same time, it is a valuable tool to better understand the pathogenesis of bacterial diseases at the moleclar level. As pneumonia is a significant pathological form of infection caused by *A. baumannii*, simultaneous transcriptional profiling on the interaction of this pathogen and human respiratory epithelial cells may be beneficial as it could allow identification of novel bacterial targets for future drug development and repurposing.

1.5 Summary

Treatment options for infections caused by MDR GNB, especially MDR *A. baumannii*, are rapidly diminishing. *A. baumannii* is rapidly gaining global attention due to serious infections caused in critically-ill patients, including war-wounded soldiers. Consequently, the 'old' polymyxin antibiotics are being used as the 'last-resort' agents for these problematic pathogens. Although still effective, reports of polymyxin resistance are emerging in GNB pathogens following polymyxin monotherapy.

As new antibiotics for MDR GNB are not yet available, it is important to investigate novel approaches to enhance the bacterial killing of polymyxins and prevent the emergence of polymyxin resistance. Synergistic combinations of approved non-antibiotic drugs with existing antibiotics is emerging as a potentially valuable and cost-effective means to improve the clinical efficacy of currently available antibiotics against problematic MDR bacterial pathogens (285). Currently, the use of a combination of a polymyxin with another antibiotic has been shown to produce improved antimicrobial activity and reduced polymyxin resistance by *in vitro* and animal studies (see Section 1.3.7) (217, 220, 258, 259, 270). The use of a combination of a polymyxin and a non-antibiotic drug for the same purpose, however, has yet to be investigated.

This thesis investigates the potential repositioning of non-antibiotics to be used in combination with colistin or polymyxin B for the treatment of MDR GNB, in particular, *A. baumannii*. The objectives of this thesis were:

1. To identify non-antibiotic drugs that have synergistic activity with polymyxin B against MDR GNB (Chapter 2).

2. To evaluate the *in vitro* antimicrobial activity of polymyxin B combined with the most active non-antibiotic drug identified in Objective 1 above (Chapter 3).

3. To evaluate the *in vivo* antimicrobial activity of polymyxin B combined with the most active non-antibiotic drug identified in Objective 1 above (Chapter 3).

4. To undertake metabolomics studies to investigate the molecular mechanisms underpinning enhanced bacterial killing of polymyxin B combined with the most active non-antibiotic drug (Chapter 4).

5. To understand the molecular interaction of *A. baumannii* and host cells in the presence of polymyxins using transcriptomic studies, and to identify molecular targets for novel polymyxin B and non-antibiotic drugs combinations (Chapter 5).

1.6 Thesis structure

In this thesis, the materials and methods are described in detail separately under each result chapter instead of a separate chapter. Parts of Chapter 1 (Introduction and Literature review) formed the basis of two recent reviews on polymyxins, which have been published. Chapters 2 to 4 (research findings) contains materials from manuscripts which have been published or submitted for publication.

CHAPTER TWO: THE ANTIMICROBIAL ACTIVITY OF POLYMYXIN B AGAINST GNB IS SYNERGYSTIC IN COMBINATION WITH CERTAIN NON-ANTIBIOTIC DRUGS

2.1 Abstract

Polymyxins, an old class of antibiotics, are currently used as the last resort for the treatment of MDR GNB. However, recent PK and PD data indicate that polymyxin monotherapy can lead to the development of polymyxin resistance (PR) (see Section 1.3.6). Novel approaches are urgently needed to preserve and improve the efficacy of this last-line class of antibiotics. This chapter examines (1) the potential benefit of combining polymyxin B with other non-antibiotic drugs for the treatment of various PR and MDR GNB and (2) the antimicrobial activity of novel combination of polymyxin B with anthelmintic closantel against problematic MDR and PR *A. baumannii*. In the presence of 2 mg/L of polymyxin B, 66 non-antibiotic drugs (10 μM) from the Johns Hopkins Clinical Compound Library (JHCCL [version 1.3]) showed antimicrobial activity against PR- *A. baumannii*, *P. aeruginosa* or *K. pneumoniae*. From the identified non-antibiotics, closantel possessed activity against *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* species and was selected as a potential candidate for combination with polymyxin B to treat MDR and PR *A. baumannii*. Time-kill studies showed closantel monotherapy (16 mg/L) was ineffective against most *A. baumannii* isolates tested. However, closantel at 4-16 mg/L with a clinically achievable concentration of polymyxin B (2 mg/L) (see Section 2.4.3) successfully inhibited the development of polymyxin resistance in polymyxin-susceptible isolates, and provided synergistic killing against PR isolates (MIC ≥4mg/L). The findings in this chapter suggested that the combination of polymyxin B with non-antibiotic drugs is a possible alternative to combat MDR GNB and the combination of closantel and polymyxin B could be potentially useful for the treatment of MDR, including PR, *A. baumannii* infections. The repositioning of non-antibiotic drugs to treat bacterial infections may significantly expedite discovery of new treatment options for bacterial 'superbugs'.

2.2 Introduction

The past two decades has seen a substantial increase in Gram-negative 'superbugs' resistant to almost all clinically available antibiotics (25). This dire situation is exacerbated by a lack of novel antibiotics in the drug discovery pipeline, leaving the world in a vulnerable state against these life-threatening bacteria (25). 'Old' polymyxin class of antibiotics, polymyxin B and E (the latter also known as colistin), are now used as a last line of defense against Gram-negative 'superbugs' (94). Of these pathogens, *A. baumannii* is one of the most problematic, causing a range of infections in the nosocomial setting and in injured military personnel (see Section 1.2.1) (36). Although polymyxins largely remain effective against problematic Gram-negative bacteria such as *A. baumannii*, recent PK and PD data on polymyxins suggest that caution is required with monotherapy due to emergence of resistance (149, 228) (see Section 1.3.6). Worryingly, there have been increasing reports of infections caused by *A. baumannii* which are resistant to all available antibiotics, including polymyxins (303, 304). The emergence of polymyxin-resistant *A. baumannii* highlights the urgent need to investigate novel approaches for maintaining and improving the clinical efficacy of polymyxins. The use of synergistic combinations of non-antibiotic drugs with antibiotics is emerging as a potentially valuable and cost-effective approach to improve the clinical efficacy of currently available antibiotics against problematic MDR bacterial pathogens (285). In the present study, the potential benefit of combining polymyxin B with other non-antibiotic drugs for the treatment of various PR and MDR GNB was investigated using the JHCCL. Additionally, the bacterial killing and the rapid emergence of polymyxin resistance in *A. baumannii* was investigated using clinically relevant concentrations of polymyxin B in combination with a promising non-antibiotic closantel.

2.3 Materials and methods

2.3.1 Construction of non-antibiotic drug library

A total of 1504 drugs (consisted of 1248 non-antibiotic drugs; i.e. excluding drugs with antibiotic, antibacterial or antiseptic indications) that were approved by the FDA, approved for use abroad or undergoing phase 2 clinical trials were assembled according to the JHCCL in 96-well mother plates (305, 306). Approximately 90% of the drugs were initially prepared in 50 µL of dimethyl sulfoxide (DMSO) and the rest in 50 µL of water at a concentration of ~10 mM. The drugs were arrayed in a total of 27 96-well mother plates, leaving the first and last columns in each plate for controls. Subsequently, prior to storage at -80 ° C, each drug was diluted to ~5 mM stock solution with 50 µL of dimethyl sulfoxide.

2.3.2 GNB isolates used in the screening of the non-antibiotic drugs

To identify non-antibiotic drugs from the JHCCL possessing synergistic activity with polymyxin B, the library was screened against six GNB isolates consisted of polymyxin-susceptible (MIC ≤2 mg/L) *A. baumannii* ATCC 19606, *P. aeruginosa* ATCC 27853, and *K. pneumoniae* ATCC 13883, and polymyxin-resistant (MIC ≥4 mg/L) *A. baumannii* FADDI-AB173*, P. aeruginosa* FADDI-PA070 and *K. pneumoniae* FADDI-KP027 (**Table 2.1**). The polymyxin-susceptible isolates were purchased from the American Type Culture Collection (Rockville, MD, USA). Polymyxin-resistant *A. baumannii* FADDI-AB173 was kindly provided by JMI laboratory (North Liberty, Iowa, USA), *P. aeruginosa* FADDI-PA070 was a clinical isolate from a patient with cystic fibrosis from the Women's and Children's Hospital (South Autralia, Australia), and *K. pneumoniae* FADDI-KP027 was a clinical isolate kindly provided by the University of Queensland (QLD, Australia); MICs to polymyxin B were determined as described below in Section 2.3.3. Each drug was screened alone (at 10 µM) against all six GNB isolates, and in the presence of 2 mg/L polymyxin B (Sigma-Aldrich, Castle Hill, NSW, Australia; Batch number BCBD1065V) against the three polymyxin-resistant GNB isolates (**Table 2.2**); stock solutions of polymyxin B were prepared as

described below in Section 2.3.3. Each JHCCL mother plate was used to create nine replicate daughter plates (96-well microtiter plates [Techno Plas, St Marys, SA, Australia]) with ~0.6 µL of a drug solution per well. For the screening of the drugs alone, 50 µL of 10% DMSO:water and 150 uL of a corresponding bacterial suspension (polymyxin-susceptible and polymyxin-resistant isolates) was added to each well (**Table 2.2**). The bacterial suspensions were prepared by emulsifying overnight bacterial culture on nutrient agar plates (Media Preparation Unit, VIC, Astralia) in 0.9% saline to ~0.5 McFarland and subsequently diluting 100-fold in 4/3 cation-adjusted Mueller Hinton broth (CAMHB, lot number: 1238500; Ca^{2+} at 23.0 mg/L and Mg^{2+} at 12.2 mg/L; Oxoid, Hampshire, UK; 4/3 indicates CAMHB at ~1.3 times the standard concentration). For the screening of drugs in the presence of 2 mg/L of polymyxin B, 50 µL of 10% DMSO:water, 10 µL of 40 mg/L polymyxin B in milli-Q water (Millipore, North Ryde, Australia), and 140 µL of a corresponding bacterial suspension (polymyxin-resistant isolates only) was added to each well (**Table 2.2**). For the negative controls, bacterial suspensions were replaced with bacteria-free 4/3 CAMHB. For the positive controls, the JHCCL drugs were not included and polymyxin B solution was replaced with drug-free Milli-Q water. All plates were incubated at 35-37^{o} C. The antimicrobial activity of the JHCCL drugs alone or in combination with polymyxin B against polymyxin-susceptible and -resistant *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* was evaluated at ~20 and ~40 h visually through the detection of turbidity. Drugs with antimicrobial activity were identified as those that successfully inhibited bacterial growth at 20 and/or 40 h; that is, no visible growth was observed at these times. Drugs with antimicrobial activity with polymyxin B against at least two GNB isolates were repeated two additional times for confirmation. At 40 h, 100 µL the culture from the wells showing antimicrobial activity was subculture onto nutrient agar and incubated at 35-37^{o} C overnight for viable cell counting.

Table 2.1 GNB isolates and their polymyxin resistance profiles used in the screening for non-antibiotic drugs with antimicrobial activity from the Johns Hopkins Clinical Compound Library version 3.

Bacterial isolate	Polymyxin B MIC (mg/L)	Susceptibility to polymyxin B
A. baumannii ATCC 19606	0.5	Susceptible
A. baumannii FADDI-AB173	16	Resistant
P. aeruginosa ATCC 27853	0.5	Susceptible
P. aeruginosa FADDI-PA070	256	Resistant
K. pneumoniae ATCC 13883	0.5	Susceptible
K. pneumoniae FADDI-KP027	32	Resistant

Table 2.2 The screening setup for each drug from the Johns Hopkins Clinical Compound Library version 3.

Drug replicate	Screen type	Bacterial isolate	Setup
1	JHCCL drug alone	*P. aeruginosa* ATCC 27853	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 150 µL bacteria in 4/3xCAMHB
2	JHCCL drug alone	*P. aeruginosa* FADDI-PA070	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 150 µL bacteria in 4/3xCAMHB
3	JHCCL drug alone	*A. baumannii* ATCC 19606	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 150 µL bacteria in 4/3xCAMHB
4	JHCCL drug alone	*A. baumannii* FADDI-AB173	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 150 µL bacteria in 4/3xCAMHB
5	JHCCL drug alone	*K. pneumoniae* ATCC 13883	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 150 µL bacteria in 4/3xCAMHB
6	JHCCL drug alone	*K. pneumoniae* FADDI-KP027	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 150 µL bacteria in 4/3xCAMHB
7	JHCCL drug and polymyxin B	*P. aeruginosa* FADDI-PA070	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 10 µL 0.04 mg/mL PB + 140 µL bacteria in 4/3xCAMHB
8	JHCCL drug and polymyxin B	*A. baumannii* FADDI-AB173	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 10 µL 0.04 mg/mL PB + 140 µL bacteria in 4/3xCAMHB
9	JHCCL drug and polymyxin B	*K. pneumoniae* FADDI-KP027	0.6 µL JHCCL drug + 50 µL 10% DMSO:H20 + 10 µL 0.04 mg/mL PB + 140 µL bacteria in 4/3xCAMHB

2.3.3 *A. baumannii* strains and MIC measurements of polymyxin B and non-antibiotic drug closantel

Eight strains of *A. baumannii* representing a mixture of polymyxin-susceptible (MIC ≤2 mg/L) and polymyxin-resistant (MIC ≥4 mg/L) strains, including MDR strains, were employed for the evaluation

of the synergy with the anthelmintic drug closantel in combination with polymyxin B (**Table 2.3**). Of the four polymyxin-susceptible isolates, FADDI-AB009 and 2949 were polymyxin heteroresistant; polymyxin heteroresistance was defined as a polymyxin-susceptible isolate (that is, MIC ≤2 mg /L) with subpopulations able to grow in the presence of >2 mg/L polymyxin B or colistin (see Section 1.2.4) (131). *A. baumannii* ATCC 19606 was purchased from the American Type Culture Collection (Rockville, MD, USA) and the polymyxin-resistant variant FADDI-AB065 (also referred to as ATCC 19606R) was from a previous study (128); polymyxin resistance of FADDI-AB065 is conferred by complete loss of lipopolysaccharide (LPS) from the outer membrane (see Section 1.3.4) (128). FADDI-AB009 was provided by The Alfred Hospital (Melbourne, Australia) and its polymyxin-resistant variant FADDI-AB085 was produced by plating onto Mueller-Hinton agar (Oxoid, Adelaide, Australia) containing 10 mg/L of colistin sulfate (Sigma-Aldrich, Castle Hill, Australia). In addition, two pairs of polymyxin-susceptible and -resistant isolates were obtained from two patients at the University of Pittsburgh Medical Center before (susceptible) and following (resistant) colistin treatment: 2382 vs 2384 and 2949 vs 2949A (111). Polymyxin resistance in isolates 2384 and 2949A is conferred by the modifications of lipid A (111). All four isolates from the University of Pittsburgh Medical Center are MDR (defined as non-susceptible to ≥1 treating agent in ≥3 antimicrobial categories) (23). MICs to polymyxin B (Sigma-Aldrich, Castle Hill, NSW, Australia; Batch number BCBD1065V) and closantel (Sigma-Aldrich; Batch number SZBC320XV) were determined for all isolates in three replicates on separate days using broth microdilution in CAMHB (194). Stock solutions of polymyxin B and closantel were prepared immediately before each experiment. Polymyxin B was dissolved in Milli-Q water and sterilised by passage through a 0.20-µm cellulose acetate syringe filter (Millipore, Bedford, MA, USA). Closantel was first dissolved in dimethyl sulfoxide (Sigma-Aldrich) and then in Milli-Q water to make a 10% (v/v) solution. The solution was further serially diluted in filter-sterilised Milli-Q water to the desired final concentration; preliminary studies demonstrated the final concentration of dimethyl sulfoxide (2.5%, v/v) to which the bacteria were exposed had no effect on their growth. All assays were performed in 96-well microtiter plates in CAMHB with a bacterial inoculum of ~5×10^5 cfu/mL.

Plates were incubated at 37° C for 20 h. MICs were determined as the lowest concentrations that inhibited the visible growth of the bacteria. For polymyxin-resistant isolates, MICs of closantel in the presence of 2 mg/L of polymyxin B were also determined (that is, polymyxin B at the specified concentrations was added to each well of the 96-well plate).

Table 2.3 MICs for polymyxin B and closantel against the *A. baumannii* strains examined in this study.

Strain	MDR[a]	Polymyxin susceptibility[b]	MICs (mg/L)		
			Polymyxin B	Closantel	Closantel in the presence of 2 mg/L polymyxin B[c]
ATCC 19606	No	S	0.5	>128	NP[d]
FADDI-AB009[e]	No	S (HR)	0.5	>128	NP
2382	Yes	S	0.5	>128	NP
2949[e]	Yes	S (HR)	1	>128	NP
FADDI-AB065	No	R	128	0.5	0.5
FADDI-AB085	No	R	32	0.5	0.5
2384	Yes	R	8	>128	1
2949A	Yes	R	64	>128	2

[a] Multidrug resistance (MDR) was defined as non-susceptible to ≥1 treating agent in ≥3 antimicrobial categories (23).

[b] CLSI breakpoints (S, susceptible; R, resistant): Polymyxin B, S ≤2 mg/L, R ≥4 mg/L; breakpoints are not available for closantel.

[c] Closantel MICs in the presence of 2 mg/L of polymyxin B.

[d] Not performed (NP) for polymyxin-susceptible isolates.

[e] Polymyxin B heteroresistant (HR). Heteroresistance to polymyxin B was defined as the existence, in an isolate for which the polymyxin B MIC was ≤2 mg/L, of subpopulations able to grow in the presence of >2 mg/L polymyxin B (131).

2.3.4 Baseline polymyxin population analysis profiles

The existence of polymyxin-resistant subpopulations at baseline (t = 0 h) was determined using population analysis profiles (PAPs) as described previously (223). In brief, bacterial cell suspensions (50 µL) of ~10^8 cfu/mL were appropriately diluted with 0.9% saline and plated onto Mueller-Hinton agar plates (Media Preparation Unit, University of Melbourne, Parkville, Australia) containing polymyxin B (0, 0.5, 1, 2, 4 and 8 mg/L) using an automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted after 24 h incubation at 37°C using a ProtoCOL colony counter (Synbiosis, Cambridge, UK).

2.3.5 Static time-kill studies

Time-kill studies with polymyxin B and closantel alone, and in combination, were conducted. For monotherapy, polymyxin B was used at 2 mg/L and closantel at 16 mg/L. Three polymyxin B/closantel combinations were investigated using polymyxin B at 2 mg/L combined with closantel at 2, 4 or 16 mg/L (dimethyl sulfoxide at 2.5% (v/v) was used for all treatments). Before each experiment, isolates were subcultured onto nutrient agar plates (Media Preparation Unit) and incubated overnight at 35-37°C. One colony was then selected and grown overnight in 20 mL CAMHB at 35-37°C; from this colony an early log-phase culture was obtained. Each drug was added alone or in combination to 20 mL of a log-phase broth culture of ~ 5 × 10^5 cfu/mL to yield the desired concentrations. Each 20 mL culture was placed in a sterile 50 mL polypropylene tube (Greiner Bio-One, Frickenhausen, Germany) containing 20 mL of CAMHB and incubated in a shaking water bath at 37°C (shaking speed, 150 rpm). Serial samples (0.5 mL) were removed aseptically at 0, 0.5, 1, 2, 4, 6 and 24 h for viable-cell counting; the samples were appropriately diluted in 0.9% saline and 50 µL of the resultant bacterial cell suspension was spirally plated onto nutrient agar. In order to examine the rapid emergence of polymyxin-resistant subpopulations, samples at 24 h were additionally plated onto Mueller-Hinton agar containing polymyxin B at 4 mg/L. Enumeration was performed after 24 h of incubation as described above. Microbiological responses of combination therapy relative to monotherapy were

examined descriptively and via the log change method, that is, comparing the change in Log_{10} cfu/mL from 0 h (cfu_0) to time t (4 and 24 h; cfu_t) as shown: log change = $Log_{10}(cfu_t) - Log_{10}(cfu_0)$. Synergy was defined as ≥2 Log_{10} cfu/mL killing for the combination relative to the most active corresponding monotherapy at a specified time (254).

2.3.6 Quantification of antibacterial activity

The antibacterial activity of polymyxin B and closantel, both individually and in combination, was quantified using a recently reported empirical modelling approach (307) that characterises the rate of bacterial killing in addition to the suppression of bacterial regrowth. An empirical model (**Equation 2.1**) was fitted to the time-kill experimental data and estimates were obtained for the parameters A, B, C, K_d and K_r that describe the magnitude of bacterial killing, magnitude of bacterial regrowth, time delay of bacterial regrowth and the rates of bacterial killing and regrowth, respectively.

$$\mathrm{Log}_{10}\left(\frac{CFU}{mL}\right) = A \cdot e^{-K_d \cdot t} + \frac{B}{1+e^{-K_r \cdot (t-C)}} \qquad (1)$$

Estimation was performed by non-linear regression using the solver add-in in Microsoft Excel and the parameter estimates were subsequently used to calculate a model-derived time to 2 Log_{10} killing (T2LK**;** **Equation 2.2**) and time to 3 Log_{10} regrowth (T3LR; **Equation 2.3**). The T2LK was used as a measure of bacterial killing, whereas the T3LR was used as a measure of the suppression of bacterial regrowth. T3LR was constrained to <24 h to account for the duration of the time-kill study.

$$t_{x\text{-}Log10\ kill} = -\frac{1}{K_d} \cdot \ln\left(1 - \frac{x_{\mathrm{Log10\ killing}}}{A}\right) \qquad (2)$$

$$t_{x-Log10\ regrowth} = C + \frac{1}{K_r} \cdot \ln\left(\frac{x_{Log10\ regrowth}}{B - x_{Log10\ regrowth}}\right) \qquad (3)$$

2.4 Results

2.4.1 Non-antibiotic drugs possessing antimicrobial activity

The screening of the drugs at 10 μM revealed a total of 110 non-antibiotics drugs with antimicrobial activity alone or in combination with 2 mg/L polymyxin B against at least one GNB isolates. These drugs were defined as those with indications other than antibiotic, antibacterial or antiseptic, and able to inhibit bacterial growth at 20 h and/or 40 h. 66 drugs were identified to have antimicrobial activity only in the presence of 2 mg/L of polymyxin B, with 12 of these displaying antimicrobial activity against two or more GNB isolates (**Table 2.4**). Closantel (10 μM) plus polymyxin B (2 mg/L) showed the best antimicrobial activity against polymxin-resistant *A. baumannii*, *P. aeruginosa* and *K. pneumoniae* as <10 cfu/mL was detected in each culture after 40 h treatment (**Table 2.4**).

Table 2.4 Non-antibiotic drugs from the JHCCL showing antimicrobial activity (at 10 µM) in combination with polymyxin B (2 mg/L) against polymyxin-resistant GNB isolates of *A. baumannii, P. aeruginosa* or *K. pneumoniae*.

Drug	Indication	No polymyxin B						With polymyxin B (2 mg/L)		
		A. baumannii **ATCC 19606**	***P. aeruginosa*** **ATCC 27853**	***K. pneumoniae*** **ATCC 13883**	***A. baumannii*** **FADDI-AB173**	***P. aeruginosa*** **FADDI-PA070**	***K. pneumoniae*** **FADDI-KP027**	***A. baumannii*** **FADDI-AB173**	***P. aeruginosa*** **FADDI-PA070**	***K. pneumoniae*** **FADDI-KP027**
Closantel	Anthelminithic	×	×	×	×	×	×	✓ (<10)	✓ (<10)	✓ (<10)
Dichlorophen	Anthelminithic	×	×	×	×	×	×	✓ (~10,000)	✓ (<10)	✓ (~50,000)
Chlorosalicylanilide	Anti-inflammatory	×	×	×	×	×	×	✓ (>100,000)	✓ (>100,000)	✓ (>100,000)
Clomiphene	Selective estrogen receptor modulator	×	×	×	×	×	×	✓ (<10)	✓ (>100,000)	×
Prochlorperazine	Antiemetic	×	×	×	×	×	×	✓ (<10)	✓ (>100,000)	×
Chlorpromazine	Antiemetic	×	×	×	×	×	×	✓ (~1,000)	✓ (>100,000)	×
Mitotane	Antineoplastic	×	×	×	×	×	×	✓ (~1,000)	✓ (>100,000)	×
Thiethylperazine	Antiemetic	×	×	×	×	×	×	✓ (~1,000)	✓ (>100,000)	×
Suloctidil	Vasodilator	×	×	×	×	×	×	✓ (~2,000)	✓ (>100,000)	×
Chloroxine	Dermatologic	×	×	×	×	×	×	✓ (~10,000)	✓ (~25,000)	×
Bismuth Subnitrate	Antacid	×	×	×	×	×	×	✓ (>100,000)	✓ (>100,000)	×
Oxiconazole	Antifungal	×	×	×	×	×	×	✓ (>100,000)	✓ (>100,000)	×

The symbol ✓ indicates the presence of antimicrobial activity and the symbol × indicates no antimicrobial activity at 20 and/or 40 h. The numbers in brackets indicate the number of colony forming unit per mL (cfu/mL) after 40 h.

2.4.2 MICs and PAPs

MICs of each drug alone plus MICs to closantel in the presence of polymyxin B (2 mg/L), as well as results for baseline PAPs, are shown in **Table 2.3**. Closantel alone was inactive (MIC >128 mg/L) against the majority of isolates. However, an MIC of 0.5 mg/L for closantel was achieved against two polymyxin-resistant strains (FADDI-AB065 and FADDI-AB085); for these two strains, closantel MICs were unaffected by the addition of polymyxin B (2 mg/L). The addition of polymyxin B substantially reduced closantel MICs in the two remaining polymyxin-resistant isolates (2384 and 2949A; **Table 2.3**). The varying susceptibility to polymyxin B of subpopulations within the polymyxin-susceptible isolates before polymyxin B treatment was evident in the PAPs (**Figure 2.1**). Two isolates (2949 and FADDI-AB009) considered susceptible based upon polymyxin B MIC results were heteroresistant, containing subpopulations able to grow in the presence of 4 mg/L polymyxin B (**Figure 2.1**). For the polymyxin-resistant isolates, virtually the entire bacterial population was highly resistant to polymyxin B and grew in the presence of 8 mg/L polymyxin B.

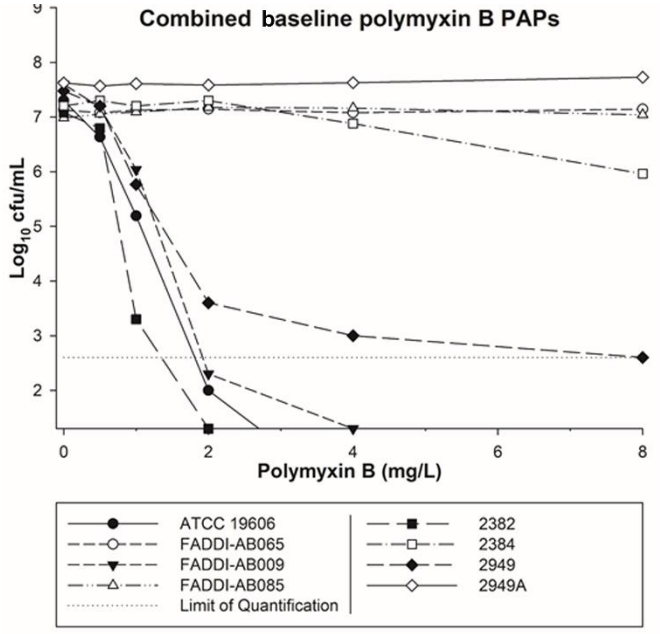

Figure 2.1 Baseline polymyxin B PAPs of the *A. baumannii* isolates employed in the evaluation of the antimicrobial activity of the combination of closantel and polymyxin B. The y-axis starts from the limit of detection and the limit of quantification is indicated by the horizontal dotted line.

2.4.3 Time-kill studies and rapid emergence of polymyxin resistance

Time-kill profiles for polymyxin B and closantel monotherapy and combination therapy against polymyxin-resistant isolates are shown in **Figure 2.2**. The proportions of polymyxin-resistant isolates before and after 24 h of treatment with each regimen are shown in **Table 2.5.** Against the closantel-susceptible isolates FADDI-AB065 and FADDI-AB085, polymyxin B monotherapy (2 mg/L) resulted in no bacterial killing, whereas closantel monotherapy (16 mg/L) resulted in rapid killing (>3 $\log_{10}$ cfu/mL) between 2 and 4 h (T2LK: 178 and 113 min for FADDI-AB065 and FADDI-AB085, respectively). Minimal regrowth was observed at 24 h for FADDI-AB065 (<2 $\log_{10}$ cfu/mL), although substantial regrowth occurred for FADDI-AB085 (>6 $\log_{10}$ cfu/mL, T3LR = 19.3 h; **Figure 2.2**). Despite subsequent regrowth at 24 h, the polymyxin-resistant subpopulations of FADDI-AB085 treated with closantel monotherapy (16 mg/L) were ~2 $\log_{10}$ lower compared with control, treatment with polymyxin B monotherapy (2

mg/L) and treatment with polymyxin B/closantel 2 mg/L combination (**Table 2.5**). Against the remaining polymyxin-resistant isolates 2384 and 2949A, no bacterial killing was observed with either polymyxin B or closantel monotherapy, with growth mirroring that of the controls over 24 h (**Figure 2.2**). Combination therapy of polymyxin B and closantel was highly effective against isolates FADDI-AB065 and FADDI-AB085. For FADDI-AB065, all combinations of polymyxin B and closantel resulted bacterial eradication, with no viable colonies detected at 24 h. For FADDI-AB085, complete inhibition was achieved with combinations of polymyxin B and closantel at concentration 4 and 16 mg/L. Against the isolates 2384 and 2949A, even though regrowth was at or close to control values by 24 h with all polymyxin B/closantel combinations, rapid and extensive bacterial killing was observed soon after the commencement of the combination therapy. Against isolate 2949A, polymyxin B plus closantel at 16 mg/L was synergistic at 4 h (T2LK: 80.7 min), with an additional ~4.5 $\log_{10}$ kill compared with polymyxin B monotherapy observed with the highest closantel concentration (**Figure 2.2**). For isolate 2384, rapid and extensive bacterial killing was observed with all polymyxin B/closantel combinations with a minimum of ~5 $\log_{10}$ greater killing compared with monotherapy at 4 h (T2LK: 46.7, 20.1 and 11.7min for polymyxin B 2 mg/L plus closantel 2, 4 and 16 mg/L, respectively; **Figure 2.2**). Within 2 h of initiation of therapy, no viable bacteria were detected with the polymyxin B/closantel (4 and 16 mg/L) combinations; the killing at 4 h in these cases was ~7.5 Log_{10} more than with equivalent monotherapy.

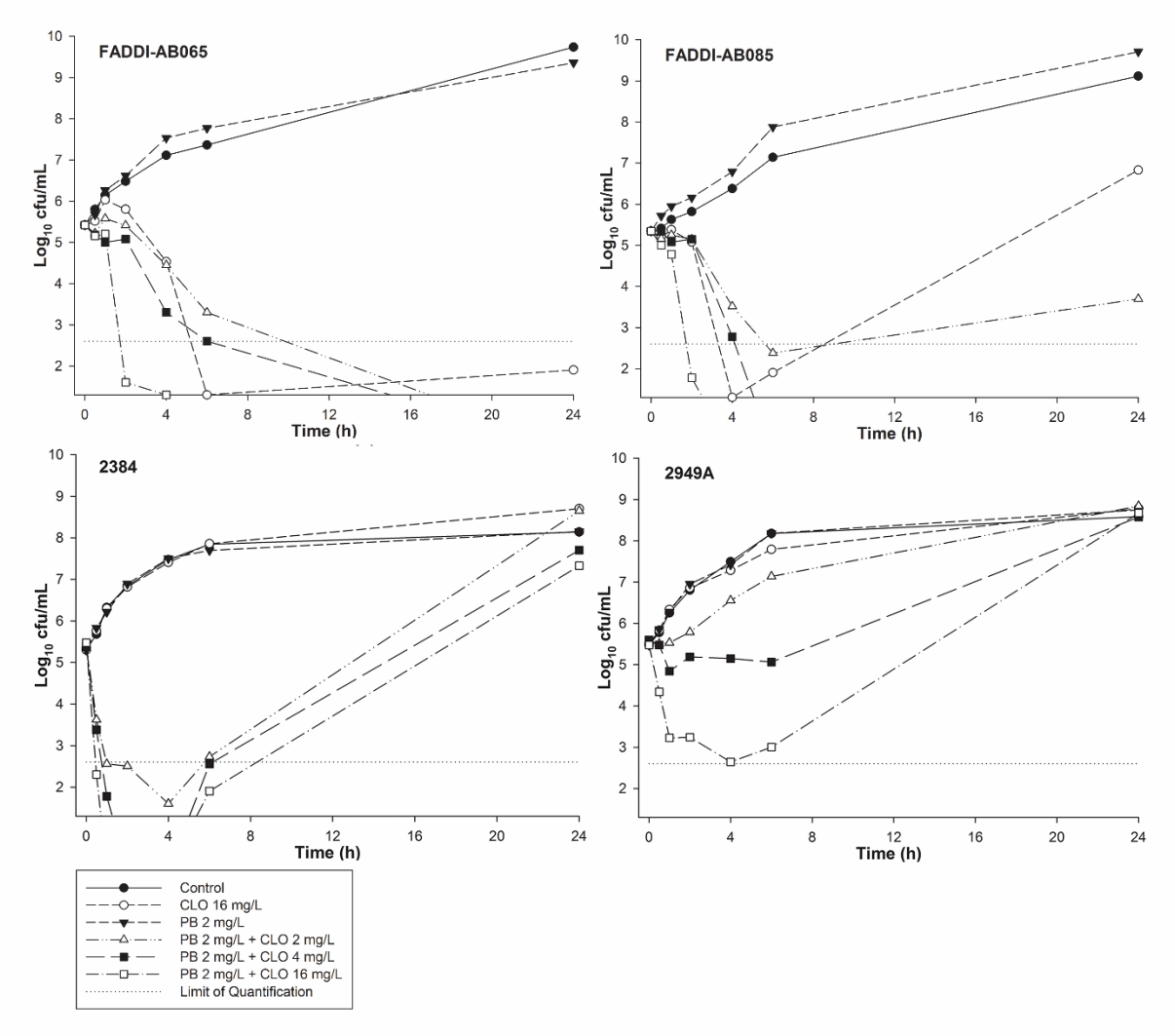

Figure 2.2 Time-kill curves for polymyxin B (PB) and closantel (CLO) monotherapy and combination therapy against polymyxin-resistant *A. baumannii* isolates FADDI-AB065, FADDI-AB085, 2384 and 2949A. The y-axis starts from the limit of detection and the limit of quantification is indicated by the horizontal dotted line.

Table 2.5 Proportion of polymyxin-resistant subpopulations of examined isolates before and after 24 hours treatment with polymyxin B (PB) alone, closantel (CLO) alone, and the combination.

A. baumannii strains	Proportion of polymyxin B-resistant subpopulations able to grow on agar supplemented with 4 mg/L polymyxin B						
	Baseline	After 24 hours treatment					
		Control	PB 2.0 mg/L	CLO 16 mg/L	PB 2.0 mg/L + CLO 2.0 mg/L	PB 2.0 mg/L + CLO 4.0 mg/L	PB 2.0 mg/L + CLO 16 mg/L
Polymyxin-susceptible							
ATCC 19606	ND[a]	3.33×10^{-8}	ND	ND	ND	NG[b]	NG
FADDI-AB009[c]	5.00×10^{-7}	5.00×10^{-6}	NG	1.00×10^{-6}	NG	NG	NG
2382	ND	ND	NG	ND	NG	NG	NG
2949[c]	3.33×10^{-5}	1.67×10^{-5}	9.17×10^{-1}	4.17×10^{-6}	5.91×10^{-3}	NG	NG
Polymyxin-resistant							
FADDI-AB065	8.96×10^{-1}	7.46×10^{-1}	1.86	1.00	NG	NG	NG
FADDI-AB085	1.52	1.29	2.14	1.12×10^{-2}	1.77	NG	NG
2384	4.75×10^{-1}	2.90×10^{-1}	1.97×10^{-1}	5.95×10^{-1}	4.82×10^{-1}	4.89×10^{-1}	2.55×10^{-2}
2949A	1.01	1.74	1.62	1.38	1.13	1.42	1.31

[a]No polymyxin-resistant subpopulations detected (ND)

[b]No growth detected after 24 hours (NG)

[c]Polymyxin B heteroresistant isolates

Time-kill profiles for polymyxin B and closantel monotherapy and combination therapy against polymyxin-susceptible isolates are shown in **Figure 2.3**. Against all polymyxin-susceptible isolates, polymyxin B monotherapy (2 mg/L) resulted in rapid bacterial killing to below the limit of detection within 0.5–1 h, with no viable colonies detected up to 6 h. For FADDI-AB009 and 2382, no regrowth was observed at 24 h. However, regrowth occurred at 24 h with the remaining two isolates (**Figure 2.3**). For heteroresistant isolate 2949, the proportion of polymyxin-resistant subpopulations markedly increased at 24 h following polymyxin B monotherapy, with virtually the entire population able to grow on Mueller-Hinton agar containing 4 mg/L polymyxin B (**Table 2.5**); the substantial bacterial killing observed at this time with all other susceptible isolates precludes meaningful comparison of polymyxin-susceptible and -resistant subpopulations. For isolates ATCC 19606 and 2949 (the isolates where regrowth at 24 h was observed), the addition of closantel at 4 and 16 mg/L to polymyxin B was synergistic at 24 h, preventing regrowth despite closantel having no discernible antibacterial activity as monotherapy against any polymyxin-susceptible isolate (that is, growth with closantel monotherapy was essentially indistinguishable from that of the control). Regrowth similar to that which occurred with polymyxin B monotherapy was observed with the polymyxin B/closantel 2 mg/L combination against isolates ATCC 19606 and 2949. However, with this combination the rapid emergence of polymyxin-resistant subpopulations was 2 $\log_{10}$ lower than polymyxin B monotherapy for isolate 2949 (**Table 2.5**). Antimicrobial activity for the combination of polymyxin B and closantel against polymyxin-susceptible isolates, quantified by the model-derived T2LK, did not differ significantly compared with polymyxin B alone (mean ± s.d.: 11.5 ± 2.60 vs. 10.5 ± 0.73 min, P=0.47). Notably, against isolate 2949, the bacterial regrowth was markedly suppressed following combination therapy with closantel (2, 4 and 16 mg/L) compared with polymyxin B alone (T3LR: 422 h vs 6.08 h).

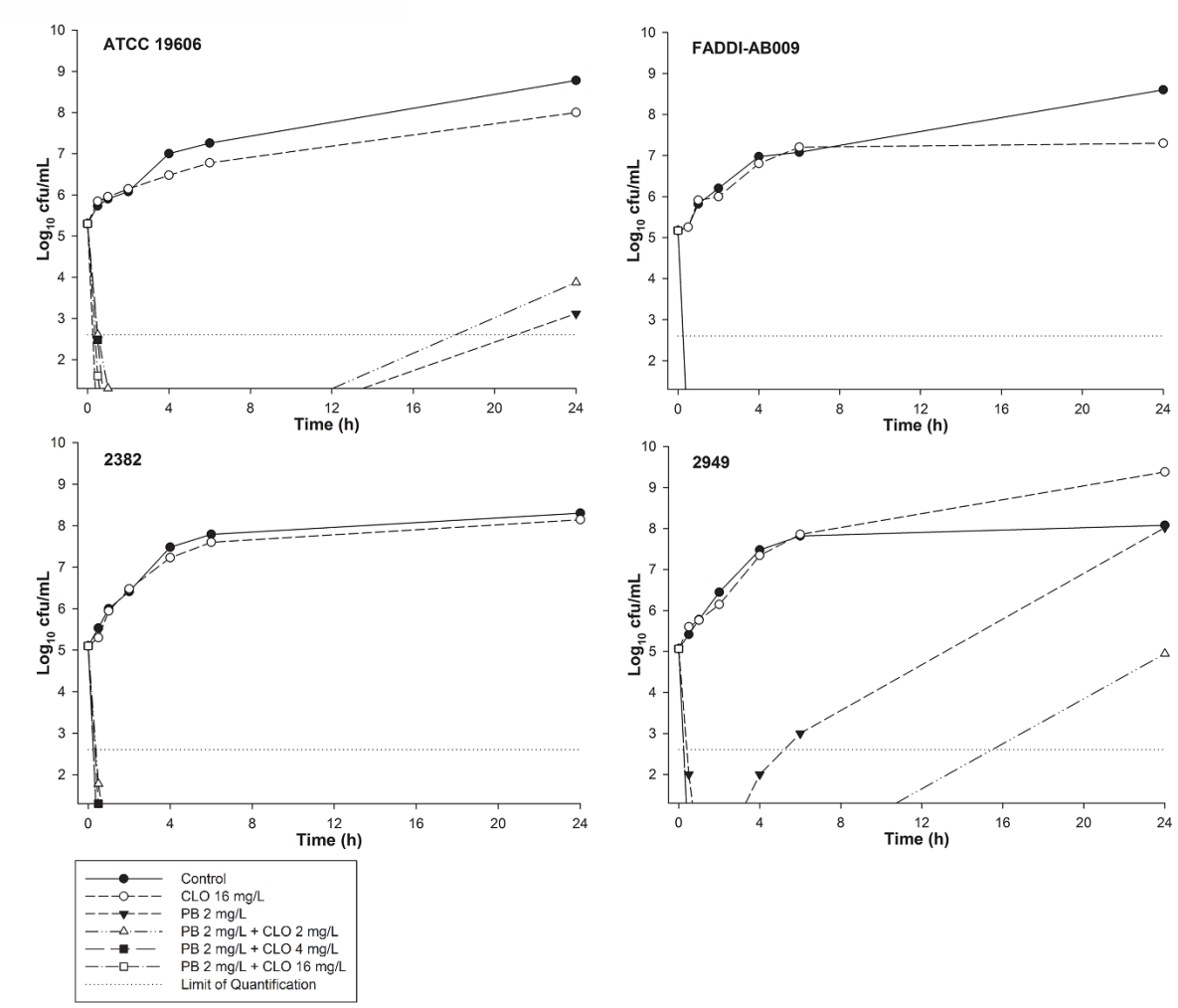

Figure 2.3 Time-kill curves for polymyxin B (PB) and closantel (CLO) monotherapy and combination therapy against polymyxin-susceptible *A. baumannii* isolates ATCC 19606, FADDI-AB009, 2382 and 2949. The y-axis starts from the limit of detection and the limit of quantification is indicated by the horizontal dotted line. For combinations with CLO 2 mg/L (FADDI-AB009 and 2382) and 4 and 16 mg/L (all isolates), regrowth (if present) is below the limit of detection.

2.5 Discussion

Infections caused by MDR *A. baumannii* are increasing globally and are already a major burden on the public health-care system (see Section 1.2.1) (10, 308, 309). Although polymyxins are increasingly used as a last-line therapy against this very problematic Gram-negative pathogen (82, 310), reports of

polymyxin-resistant MDR *A. baumannii* are increasing (311). In addition, emerging pharmacokinetic and pharmacodynamic data for polymyxins suggest caution with polymyxin monotherapy due to the presence of polymyxin heteroresistant isolates (140, 207). Consequently, novel treatment strategies that optimise bacterial killing and minimise the emergence of polymyxin resistance are urgently required (81).

This chapter examined the potential to improve the antimicrobial activity of polymyxin B against *A. baumannii* as well as two other problematic GNB (*P. aeruginosa* and *K. pneumoniae*; **Table 1.1**), using non-antibiotic drugs and evaluated the *in vitro* efficacy of the combination of polymyxin B with the non-antibiotic closantel against a range of clinical isolates (including MDR isolates) of *A. baumannii* with various susceptibilities to polymyxin B (**Table 2.3**). *In vitro* and animal studies have shown that the combination of a polymyxin with another antibiotic can have a synergistic effect as well as prevent the emergence of polymyxin-resistance (see Section 1.3.8). However, this study is the first to examine the effect on bacterial killing and the emergence of resistance with the combination of a polymyxin and a non-antibiotic drug.

To expedite the potential repositioning of non-antibiotic drugs for antibiotic purposes, only drugs approved by the FDA, approved for use abroad or undergoing phase 2 clinical trials were employed in the initial screening. To identify non-antibiotic drugs with antimicrobial activity against a wide range of GNB, the drug library was screened against three species of GNB which included polymyxin-susceptible and -resistant *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*. Of 1248 non-antibiotic drugs, 110 showed antimicrobial activity alone, or in combination with 2 mg/L polymyxin B, against at least one GNB isolates. Comparison of the screenings of the non-antibiotic drugs alone with the non-antibiotic drugs in the presence of polymyxin B, 66 drugs having antimicrobial activity only in the presence of 2 mg/L of polymyxin B. From the 66 non-antibiotic with antimicrobial activity, 12 were active to at least two GNB isolates (**Table 2.4**). For the focus of this project, the 12 non-antibiotic drugs that showed antimicrobial activity against multiple GNB species only in combination with polymyxin B

were considered as potential candidates for combination. Interestingly, in the presence of 2 mg/L polymyxin B, all of these 12 non-antibiotic drugs were effective against the PR *A. baumannii* and *P. aeruginosa* but only 3 were effective against PR *K. pneumoniae*. Currently, the antimicrobial mechanisms of these non-antibiotic drugs are unclear. However, their selective activity against *A. baumannii* and *P. aeruginosa* may be due to the closer phylogenetic relationship of these two bacterial species (43, 312). Evaluation of the chemical structures of the 12 non-antibiotic drugs showed that, with exception of bismuth subnitrate, all possessed at least one benzene ring. Although inconclusive, it is possible that drugs with benzene rings are good candidates for combination therapy with polymyxins. To determine which non-antibiotic drug produced the greatest bacterial killing (i.e. produced the lowest cfu/mL of bacteria) when combined with polymyxin B, viable cell counts were conducted on bacterial cultures from the screening after 40 h incubation. Interestingly, only the two anthelmintic drugs (closantel and dichlorophen) from the 12 identified non-antibiotics demonstrated antimicrobial activity with polymyxin B against all three GNB species. Closantel showed the highest activity with polymyxin B, with bacterial counts of <10 cfu/mL for all three GNB after 40 h. For dichlorophen, the best antimicrobial activity was observed against *P. aeruginosa* (<10 cfu/mL), with moderate activity against *A. baumannii* (~10,000 cfu/mL) and *K. pneumoniae* (~50,000 cfu/mL). Given the superior antimicrobial activity with polymyxin B, closantel was subsequently selected for further investigation against multiple strains of *A. baumannii*, including MDR and PR strains.

Closantel is a veterinary anthelmintic drug with activity against multiple nematode species (313). The anthelmintic activity of closantel involves the uncoupling of oxidative phosphorylation and inhibition of chitinase (314, 315). This study is the first to demonstrate the synergistic antibacterial activity between polymyxins and closantel against MDR *A. baumannii*. The repositioning of veterinary drugs has been successful for drug discoveries for humans. An example is ivermectin (316), a drug currently used to treat onchocerciasis (river blindness) in humans but initially developed for veterinary use. Currently, information on the pharmacokinetics of closantel is unknown in humans; hence, multiple concentrations of closantel (2, 4 and 16mg/L) were employed based on its pharmacokinetics in

animals (317, 318) and to ensure an appropriate concentration range was covered. The concentration of polymyxin B (2 mg/L) employed in this study is clinically achievable as demonstrated by pharmacokinetic studies in critically-ill patients (80, 319).

For *A. baumannii*, regrowth with polymyxin monotherapy (polymyxin B or colistin) is driven in part by the amplification of polymyxin-resistant subpopulations (140, 207). Such regrowth was similarly observed here in two of four polymyxin-susceptible isolates (**Figure 2.3**). This finding again illustrates that caution is required for treatment of *A. baumannii* infections with polymyxin monotherapy. For the polymyxin-resistant isolates, rapid and marked improvements in bacterial killing were observed with all three combinations against isolates 2384, and with the combination of polymyxin B/closantel 16 mg/L against 2949A. These improvements occurred despite the virtual absence of bacterial killing with each monotherapy. For example, against isolate 2384 improvements in bacterial killing of 4-5 $\log_{10}$ cfu/mL compared with each monotherapy were observed within 1 h of the commencement of treatment with the combination containing 4 mg/L closantel. Despite subsequent regrowth, such rapid and extensive initial killing by an antibiotic/non-antibiotic combination against isolates highly resistant to each drug is an important finding. The rapid and extensive reduction in the bacterial load at the commencement of therapy may facilitate clearance of bacteria by the host's immune system. Interestingly, closantel showed antibacterial activity as monotherapy against FADDI-AB065 and FADDI-AB085, but even then the combinations with all concentrations of closantel (2, 4 and 16mg/L) demonstrated superiority through better regrowth suppression after 24 h. The addition of closantel to polymyxin B had no effect on initial bacterial killing of polymyxin-susceptible isolates due to extensive bacterial killing by polymyxin B alone (**Figure 2.3**). However, the addition of closantel at 4 or 16mg/L did suppress the regrowth observed with polymyxin B monotherapy against ATCC 19606 and 2949 (**Figure 2.3**).

These findings merit further research given increasing reports of polymyxin resistance (320-324) and a diminishing arsenal of effective antibiotics (325-327). Similar to previous reports (137, 328, 329), my

current study shows that MIC results do not completely mirror those from time-kill studies (**Table 2.3**; and **Figure 2.2**). For isolates 2384 and 2949A, closantel MICs were 1 and 2 mg/L, respectively, in the presence of 2 mg/L of polymyxin B (**Table 2.3**). However, in the time-kill studies, regrowth was observed for both isolates with 16 mg/L of closantel in the presence of 2 mg/L of polymyxin B (**Figure 2.2**). As MICs are obtained after 20 h incubation via visual observation for turbidity and viable counting using agar plates is not part of the MIC measurement, the MIC results do not necessarily indicate lack of viable cells (for example, in the 24 h time-kill studies).

The antibacterial mechanism of closantel is unclear. However, closantel has been shown to exhibit antimicrobial activity against Gram-positive bacteria in vitro (330, 331) and against *Staphylococcus aureus* in a Caenorhabditis elegans infection model (332). For Gram-negative bacteria, the unique structure of the cell envelope creates a permeability barrier to hydrophobic compounds such as closantel (logP 7.2). LPS, the principal component of the external leaflet of the Gram-negative outer membrane, is the initial binding target of polymyxins via electrostatic interaction of the cationic L-α,γ-diaminobutyric acid (Dab) side chains present on polymyxins with the negatively charged phosphate groups of the lipid A component of LPS (87). Binding displaces the divalent cations (Ca^{2+} and Mg^{2+}) that bridge adjacent LPS molecules, disorganizing the outer membrane and increasing its permeability (93). Although it was originally proposed that bacterial killing by the polymyxins resulted from permeabilisation of the bacterial outer membrane and subsequent leakage of cell contents, the precise mechanism(s) by which polymyxins ultimately kill bacterial cells is/are still unknown and several alternative mechanisms of action have been reported (100, 333-335). A previous study showed polymyxin resistance in isolates 2384 and 2949A is conferred by the modifications of lipid A with cationic galactosamine (111). It is apparent that this outer membrane modification on its own did not lead to enhanced penetration of closantel as the MIC for both isolates was >128mg/L and closantel monotherapy produced no bacterial killing. However, the enhanced bacterial killing observed when combined with polymyxin B suggests sufficient permeabilisation of the outer membrane by the polymyxin to allow closantel to enter into the cell and exert an antibacterial effect. Complete loss of

LPS in *A. baumannii* is also known to confer polymyxin resistance, although such resistance comes at the cost of rendering the outer membrane more permeable to hydrophobic compounds that would otherwise be unable to enter the bacterial cell (128). This may explain the antibacterial activity of closantel in its own right (closantel MICs of 0.5 mg/L) against strains FADDI-AB065 (which is LPS deficient) and FADDI-AB085. This would also be consistent with the previously reported antibacterial activity of closantel against Gram-positive species that do not possess LPS (330, 331). It is possible that the rate of bacterial killing of closantel is slower than that of polymyxin B. Consequently, for FADDI-AB065 and FADDI-AB085 where closantel alone was able to reduce the bacterial count, the number of bacteria declined slowly over time compared to the other strains of bacteria. The slow regrowth rate of FADDI-AB065 and FADDI-AB085 strains suggested that the level of resistance to closantel is low in these isolates.

2.6 Conclusions

In an era of declining antibiotic discovery and rapidly emerging antibiotic resistance, novel treatment strategies for MDR Gram-negative organisms such as *A. baumannii* are urgently needed. The off-label use of non-antibiotic drugs for antibacterial purposes in combination with existing antibiotics is a currently underexplored area with significant potential to expedite discovery of new treatment options for infections caused by MDR pathogens. The findings from the present study demonstrate that the 'unexpected' combination of polymyxin B with an anthelmintic, closantel, may substantially increase the antibacterial activity against MDR, including polymyxin-resistant, *A. baumannii*. Given that closantel is a veterinary drug, care should be taken with its application for human infections. For the meantime, it may be suited to overcome resistance in animals. Further investigations in animal infection models are required for translation into the clinic.

CHAPTER THREE: NOVEL POLYMYXIN COMBINATION WITH ANTINEOPLASTIC MITOTANE IMPROVED THE BACTERIAL KILLING AGAINST POLYMYXIN-RESISTANT MULTIDRUG-RESISTANT GRAM-NEGATIVE PATHOGENS

3.1 Abstract

Due to limited new antibiotics, polymyxins are increasingly used to treat MDR Gram-negative bacteria, in particular carbapenem-resistant *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*. Unfortunately, polymyxin monotherapy has led to the emergence of resistance. Polymyxin combination therapy with other drugs has been demonstrated to improve the efficacy and prevent the emergence of resistance. This study demonstrates that the novel combination of polymyxin B with the FDA-approved antineoplastic drug mitotane enhances *in vitro* antimicrobial activity of polymyxin B against clinical isolates of *A. baumannii, P. aeruginosa* and *K. pneumoniae*. Against five polymyxin-susceptible Gram-negative isolates the combination of polymyxin B (2 mg/L) and mitotane (4 mg/L) provided improved bacterial clearance during the first 6 h of treatment compared to monotherapy and prevented regrowth and emergence of polymyxin resistance. Electron microscopy imaging revealed that the polymyxin B/mitotane combination potentially affected cell division in *A. baumannii*. The enhanced antimicrobial activity of the polymyxin/mitotane combination was also confirmed in a mouse burn infection model against a polymyxin-resistant *A. baumannii* isolate. These results have important implications for repositioning non-antibiotic drugs for antimicrobial purposes, which may expedite the discovery of novel therapies to combat the rapid emergence of antibiotic resistance.

3.2 Introduction

The emergence of Gram-negative bacteria with resistance to multiple classes of antibiotics is causing serious problems for health care centers worldwide (25). Infections caused by MDR Gram-negative bacteria not only have higher mortality rates (336), but also lead to more economic burden than infections caused by susceptible Gram-negative bacteria (337). Among these MDR Gram-negative bacteria, carbapenem-resistant *A. baumannii* has been identified as one of the most difficult-to-treat pathogens and is becoming increasingly problematic for critically-ill patients and war-wounded soldiers (28, 36, 39). More recently, the World Health Organization has classified carbapenem-resistant *A. baumannii*, *P. aeruginosa* and Enterobacteriaceae as the top priority for research and development of new antibiotics (29).

Due to the current lack of effective antibiotics against MDR Gram-negative bacteria, the polymyxins (colistin and polymyxin B) have been revived as antibiotics of last resort (81, 82). However, resistance to polymyxins is on the rise (338-340) and a growing body of evidence suggests resistance to polymyxins can emerge with monotherapy (136, 138, 140, 210, 217-222). Unfortunately, the *de novo* drug discovery and development process is lengthy (10 - 17 years) and has a low success rate (<10%) (278). With limited new antibiotics in the pipeline, an approach to expedite the discovery process is through the repositioning of non-antibiotic FDA-approved drugs. This process can be as short as three years, as these drugs have already passed the FDA safety requirements and have well defined pharmacokinetics (278). In light of the dire resistance problem, in this study I evaluated the *in vitro* antimicrobial activity of the combination of polymyxin B and FDA-approved antineoplastic mitotane against highly resistant clinical isolates of Gram-negative bacteria, including carbapenem-resistant *A. baumannii*, carbapenem-resistant *P. aeruginosa*, and New Delhi metallo-β-lactamase (NDM)-producing *K. pneumoniae*. The current findings highlight the potential of this novel polymyxin/non-antibiotic combination for treatment of these problematic Gram-negative 'superbugs'.

3.3 Materials and methods

3.3.1 Bacterial isolates

Ten bacterial isolates which included multidrug- and polymyxin-resistant isolates were examined in this study (**Table 3.1**). *A. baumannii* ATCC 17978, *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 13883, and *P. aeruginosa* ATCC 27853 were obtained from the American Type Culture Collection (Rockville, MD, USA). *A. baumannii* FADDI-AB225 is a polymyxin-resistant, phosphoethanolamine-modified lipid A, *pmrB* mutant derived from ATCC 17978 (formally designated ATCC 17978-R2) (341). *A. baumannii* FADDI-AB065 is a polymyxin-resistant, LPS-deficient, *lpxA* mutant derived from ATCC 19606 (formally designated ATCC 19606R) (128). Polymyxin-susceptible *A. baumannii* FADDI-AB180 and lipid A modified (with phosphoethanolamine and galactosamine) polymyxin-resistant *A. baumannii* FADDI-AB181 are carbapenem-resistant MDR clinical isolates from the bronchoalveolar lavage fluid of a patient before and after colistin therapy, respectively (formally designated 2949 and 2949A, respectively) (111). *P. aeruginosa* FADDI-PA070 is a non-mucoid, MDR (including carbapenem- and polymyxin-resistant) clinical isolate from the sputum of a patient with cystic fibrosis (formally designated FADDI-PA070) (219). *K. pneumoniae* FADDI-KP027 is a polymyxin-resistant, NDM-producing clinical isolate from the sputum of a patient with respiratory tract infection. Isolates were stored in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) in cryovials at -80°C and subcultured onto nutrient agar plates (Media Preparation Unit, University of Melbourne, Melbourne, VIC, Australia) before use.

Table 3.1 Minimum inhibitory concentrations (MICs) for polymyxin B and mitotane against bacterial isolates examined in this study.

Bacterial isolate	MIC (mg/L)		
	Polymyxin B	**Mitotane**	**Mitotane in the presence of 2 mg/L polymyxin B**
A. baumannii ATCC 17978	0.25	>128	-
A. baumannii FADDI-AB225[PR]	16	>128	4
A. baumannii ATCC 19606	0.5	>128	-
A. baumannii FADDI-AB065[PR]	64	4	4
A. baumannii FADDI-AB180[MDR]	1	>128	-
A. baumannii FADDI-AB181[MDR, PR]	64	>128	4
P. aeruginosa ATCC 27853	0.5	>128	-
P. aeruginosa FADDI-PA070[MDR, PR]	64	>128	4
K. pneumoniae ATCC 13883	0.5	>128	-
K. pneumoniae FADDI-KP027[MDR, PR]	256	>128	4

[MDR], Multidrug-resistant: defined as non-susceptible to ≥1 treating agent in ≥3 antimicrobial categories (23).

[PR], Polymyxin resistant: defined as an MIC of ≥4 mg/L for *Acinetobacter* spp. and ≥8 mg/L for *P. aeruginosa* as per CLSI guideline (194); and >2 mg/L for Enterobacteriaceae as per EUCAST guidelines (197); and mitotane breakpoints are not available.

-, not performed.

3.3.2 Antimicrobial agents and susceptibility testing

Polymyxin B (Beta Pharma, China; Batch number 20120204) solutions were prepared in Milli-Q water (Millipore, North Ryde, Australia) and sterilised using a 0.20-µm cellulose acetate syringe filter (Millipore, Bedford, MA, USA). Mitotane (Sigma-Aldrich, Australia; Lot number BCBG9480V) solutions were prepared in dimethyl sulfoxide (Sigma-Aldrich, Australia). Stock solutions were stored at -20°C for no longer than one month. The MICs to polymyxin B and mitotane were determined for all isolates in three replicates on separate days using broth microdilution with cation-adjusted Mueller-Hinton broth (CAMHB; Oxoid, England; 20 - 25 mg/L Ca^{2+} and 10 - 12.5 mg/L Mg^{2+}) according to the Clinical and Laboratory Standards Institutes guidelines (194). Stock solutions of polymyxin B were diluted to the desired concentrations in CAMHB, while mitotane was initially diluted in dimethyl sulfoxide (DMSO) and subsequently in CAMHB to obtain the desired drug concentrations with a final level of

10% DMSO (v/v). The procedure to measure the MICs of polymyxin B and mitotane was adapted from the method in Chapter 2. Briefly, 100 µL of the bacterial suspension (10^6 cfu/mL) was combined with 100 µL of the polymyxin B solution or 50 µL of CAMHB plus 50 µL of mitotane solution in 96-well microtiter plates (Techno Plas, St Marys, SA, Australia). The plates were incubated at 37°C for 20 h and MICs were determined as the lowest drug concentrations that inhibited the visible growth of the bacteria. For polymyxin-resistant isolates, MICs of mitotane in the presence of 2 mg/L of polymyxin B were also determined. According to the CLSI guidelines polymyxin B MIC is ≤2 mg/L for polymyxin-susceptible *A. baumannii* and *P. aeruginosa*, ≥4 mg/L mg/L for polymyxin-resistant *A. baumannii*, and ≥8 mg/L for polymyxin-resistant *P. aeruginosa* (194). For *K. pneumoniae* where breakpoints have not yet been established by the CLSI, its susceptibility to polymyxin B was extrapolated from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) colistin breakpoints where susceptibility is defined as an MIC ≤2 mg/L and resistance an MIC of >2 mg/L (197).

3.3.3 Time-kill studies

Time-kill studies were conducted for all isolates based on the method from chapter 2. Briefly, bacteria were grown overnight in 20 mL CAMHB. The overnight broth cultures were transferred to 20 mL of fresh CAMHB at ~50-100 fold dilutions and incubated for an additional 3-4 h to generate log-phase culture at ~0.55 McFarland standard. The log-phase cultures were transferred to 20 mL of fresh CAMHB at ~100-fold dilution in borosilicate glass tubes for treatment to minimise loss of drug due to non-specific binding to the plastic. For the drug-containing tubes polymyxin B, mitotane, or both compounds were added to achieve final concentrations of 2 mg/L for polymyxin B and 4 mg/L for mitotane (the minimum concentration of mitotane identified by broth microdilution assay to inhibit to growth of polymyxin-resistant isolates in the presence of 2 mg/L polymyxin B). Samples (1 mL) were aseptically removed at 0, 0.5, 1, 2, 4, 6 and 24 h and inoculated onto nutrient agar plates for viable-cell counting. Colonies were counted after 24 h incubation at 37°C using a ProtoCOL colony counter (Synbiosis, Cambridge, UK). The combination of polymyxin B and mitotane was considered synergistic

if the bacterial killing was ≥2 $\log_{10}$ compared to the most active monotherapy. Changes to polymyxin B MICs were determined for all cultures that showed regrowth after 24 h to evaluate the emergence of polymyxin resistance.

3.3.4 Phase contrast, scanning electron, and transmission electron microscopy

Phase contrast microscopy, scanning electron microscopy (SEM), and transmission electron microscopy (TEM) were employed to examine the effect of the polymyxin B/mitotane combination on the cellular morphology of polymyxin-susceptible *A. baumannii* ATCC 17978 and polymyxin-resistant *A. baumannii* FADDI-AB225. Bacteria were subcultured and treated with 2 mg/L polymyxin B, 4 mg/L mitotane, or both antibiotics for 2 h in CAMHB as per the time-kill studies. For phase contrast microscopy, 20 µL of each culture was used to prepare wet samples for instant observation on a phase contrast microscope. For the SEM and TEM studies, samples were transferred to 50-mL polypropylene tubes (Greiner Bio-One, Frickenhausen, Germany) and centrifuged at 3220 × *g* for 10 min three times. Between centrifugation steps, supernatants were discarded and bacterial pellets resuspended and washed in 1 mL phosphate buffered saline (PBS). Following the final centrifugation step the supernatants were removed and bacterial pellets resuspended and fixed in 0.5 mL 2.5% glutaraldehyde in PBS. The tubes were left in a rocker shaker for 20 min at room temperature. Once fixed, tubes were centrifuged at 3220 × *g* for 10 min, the fixatives removed and bacterial pellets washed twice in 1 mL PBS as above. Pellets were finally resuspended in 1 mL PBS, and SEM and TEM were conducted at the Department of Botany, University of Melbourne, Australia.

3.3.5 Mouse burn wound infection model

A mouse burn wound infection model was employed to assess the *in vivo* antimicrobial activity of the polymyxin B/mitotane combination against polymyxin-resistant *A. baumannii* FADDI-AB225. Bacterial inoculums were prepared with early log-phase culture. After centrifugation at 3220 × *g* for 10 min, the supernatant was removed and bacterial cell pellets were suspended in 0.9% saline to approximately

10^9 cfu/mL. Bacterial samples (100 µL) were then loaded into 29-G 0.3-mL insulin syringes for inoculation of burn wounds. Drug solutions were prepared by initially dissolving mitotane in polyethylene glycol (PEG) 200 to ~4,096 mg/L and polymyxin B in 0.9% saline to ~1,536 mg/L. An equal amount of the two drug solutions was later combined to produce the combination solution with ~2,048 mg/L mitotane and 768 mg/L polymyxin B. For mitotane monotherapy, mitotane solution was combined with an equal volume of 0.9% saline. For polymyxin B monotherapy, polymyxin B was combined with an equal volume of PEG 200. For solvent controls, equal volumes of blank PEG 200 and 0.9% saline were combined. Prior to infection, female NIH Swiss mice (6-10 week-old, ~30 g body weight) were sedated with isoflurane and anesthesia was maintained throughout the entire procedure. Hair from the mouse dorsal skin was removed and the local skin area was injected with 100 µL of Bupivacaine (Marcaine 0.5%). A burn wound was established with a hot iron bolt from boiling water and bacteria injected into the burn eschar. After 2 h, different treatments were applied topically by evenly spreading 200 µL of the drug solutions across the wounds of groups of four mice. This study included 5 groups of 4 mice comprising blank control (no treatment), solvent control, polymyxin B monotherapy, mitotane monotherapy, and the combination (polymyxin B and mitotane). Each wound of the treated groups received 154 µg of polymyxin B (0.5%, w/w), 410 µg of mitotane (1.4%, w/w), or both. Four hours after treatment, mice were sacrificed and the burn wound skin tissues and the muscle tissue (~0.3 g) under the burn wounds were aseptically removed and placed separately into 8 mL of sterile saline in 50-mL Falcon tubes. Burn wound skin tissues were homogenised under sterile conditions and filtered using a filter bag (Bag Stomacher Filter Sterile, Pore Size 280 micrometer, 0.5 x 16 cm, Labtek Pty Ltd). Filtrate (1 mL) was then transferred into a sterile test tube for serial dilution and 100 µL was cultured onto nutrient agar for viable counting. Viable counts were performed on the next day following overnight incubation at 37^{o}C. Statistical significance for the bacterial killing of different treatment groups was calculated with one-way ANOVA and Tukey's multiple comparisons (Tukey's HSD).

This study was carried out in accordance with the recommendations of "Australian Code of Practice for the Care and Use of Animals for Scientific Purposes" and Monash Institute of Pharmaceutical Sciences Animal Ethics Committee. The protocol was approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee before the study started.

3.4 Results

3.4.1 MICs of polymyxin B and mitotane against polymyxin-susceptible and -resistant isolates of *A. baumannii, P. aeruginosa* and *K. pneumoniae*

The polymyxin B and mitotane MICs against all 10 Gram-negative isolates are shown in **Table 3.1**. Additionally, **Table 3.1** shows the MICs of mitotane in the presence of 2 mg/L polymyxin B against the polymyxin-resistant isolates. Apart from *A. baumannii* FADDI-AB065, mitotane monotherapy had no antimicrobial activity at concentrations up to 128 mg/L. However, in the presence of 2 mg/L polymyxin B, 4 mg/L of mitotane was effective at inhibiting growth of five polymyxin-resistant isolates (**Table 3.1**).

The changes to the polymyxin B MICs of ten examined isolates after overnight treatment with either polymyxin B monotherapy, mitotane monotherapy, or polymyxin B/mitotane combination are shown in **Table 3.2**. In the control group (overnight incubation in drug-free CAMHB), polymyxin B MICs of all isolates at 24 h were not affected as all values remained within two folds of the baseline MICs (342). After treatment with polymyxin B monotherapy at 2 mg/L, polymyxin B MICs of the polymyxin-resistant isolates at 24 h remained unchanged. However, with the three polymyxin-susceptible isolates that showed regrowth at 24 h, polymyxin B MICs of the 24-h samples increased significantly (≥32 times). Following mitotane monotherapy at 4 mg/L, polymyxin B MICs remained unchanged for all polymyxin-susceptible isolates and three polymyxin-resistant isolates; the polymyxin B MIC of polymyxin-resistant *A. baumannii* FADDI-AB065 at 24 h could not be determined, as it was highly susceptible to mitotane and showed no regrowth after 24 h. Interestingly, the polymyxin B MIC of polymyxin-resistant *A. baumannii* FADDI-AB225 was reduced significantly (32-fold lower than the

baseline) after 24-h exposure to mitotane. In the combination treatment group, the polymyxin B MICs did not change for all four polymyxin-resistant isolates that showed regrowth after 24 h.

Table 3.2 Changes in baseline polymyxin B MICs following overnight treatment with polymyxin B (PMB) monotherapy, mitotane (MIT) monotherapy, and polymyxin B/mitotane combination.

Bacterial isolates	Polymyxin B MICs relative to their baseline values			
	Control	PMB 2 mg/L	MIT 4 mg/L	PMB 2 mg/L + MIT 4 mg/L
A. baumannii ATCC 17978	2× MIC	NG	2× MIC	NG
A. baumannii FADDI-AB225	2× MIC	2× MIC	1/32× MIC	1× MIC
A. baumannii ATCC 19606	1× MIC	32× MIC	1× MIC	NG
A. baumannii FADDI-AB065	1× MIC	1× MIC	NG	NG
A. baumannii FADDI-AB180	1× MIC	32× MIC	1× MIC	NG
A. baumannii FADDI-AB181	1× MIC	1× MIC	2× MIC	1× MIC
P. aeruginosa ATCC 27853	2× MIC	NG	1× MIC	NG
P. aeruginosa FADDI-PA070	1× MIC	1× MIC	1× MIC	1× MIC
K. pneumoniae ATCC 13883	1/2× MIC	64× MIC	1/2× MIC	NG
K. pneumoniae FADDI-KP027	1× MIC	1× MIC	1× MIC	1× MIC

NG, no growth at 24 h.

3.4.2 Time-kill results for polymyxin B and mitotane against polymyxin-susceptible and -resistant isolates of *A. baumannii*, *P. aeruginosa* and *K. pneumoniae*

Time-kill profiles for polymyxin B and mitotane mono- and combination therapy are shown in **Figure 3.1**. Against the five polymyxin-susceptible isolates, polymyxin B monotherapy (2 mg/L) showed effective bacterial killing within 6 h with a minimum of ~3 $\log_{10}$ cfu/mL killing (FADDI-AB180) and ~6 $\log_{10}$ cfu/mL killing for the remaining susceptible isolates; however, regrowth to control values occurred by 24 h with three isolates (**Figure 3.1A**). There was no bacterial killing of polymyxin-susceptible isolates with mitotane monotherapy (4 mg/L), with growth comparable to that of controls (**Figure 3.1A**). With the combination bacterial counts for all five polymyxin-susceptible isolates were reduced to below the limit of detection within 0.5 - 1 h, with no viable colonies detected thereafter (**Figure 3.1A**). Against the five polymyxin-resistant isolates 2 mg/L polymyxin B monotherapy was ineffective with growth paralleling that of the controls (**Figure 3.1B**). Similarly, mitotane monotherapy

displayed no antimicrobial activity against four of the five isolates (**Figure 3.1B**). However, against *A. baumannii* FADDI-AB065 mitotane monotherapy reduced bacterial counts to below the level of detection within the first 0.5 h and prevented regrowth over 24 h. Combination treatment showed synergistic bacterial killing (i.e. >2 $\log_{10}$ reduction compared to the most active monotherapy) between 0.5 and 6 h with the remaining four isolates; interestingly, regrowth occurred at 24 h in all four cases and was close to control values in three cases (**Figure 3.1B**).

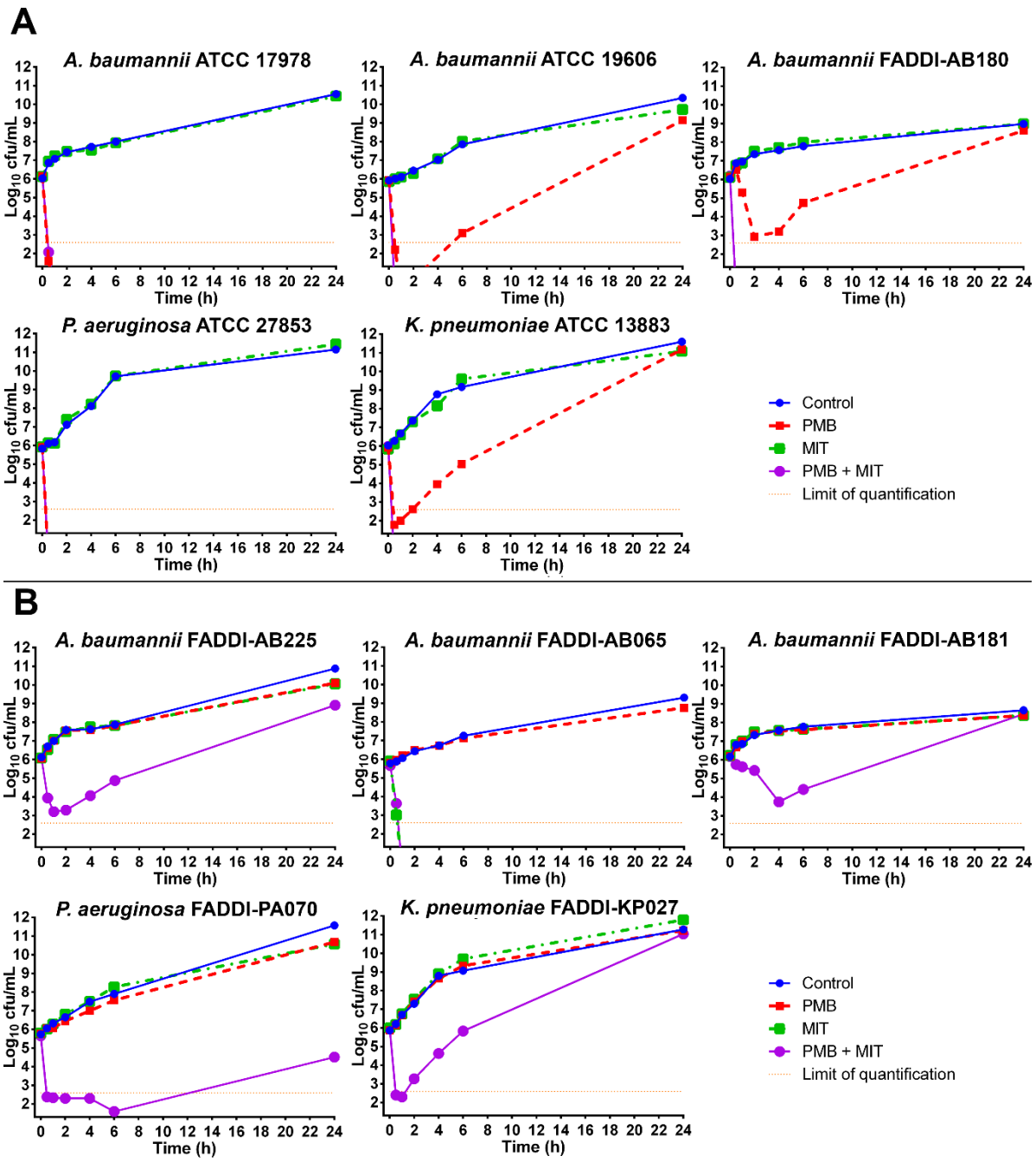

Figure 3.1 Time-kill kinetics of polymyxin B (PMB; 2 mg/L) and mitotane (MIT; 4 mg/L) monotherapy and combination therapy against five polymyxin-susceptible Gram-negative isolates (A) and five polymyxin-resistant Gram-negative isolates (B). The y-axis starts from the limit of detection and the limit of quantification is indicated by the orange dotted line.

3.4.3 Impact of polymyxin B and mitotane treatment on the cellular morphology of polymyxin-susceptible and -resistant *A. baumannii*

Figure 3.2 shows phase contrast microscopy, SEM and TEM images of polymyxin-susceptible *A. baumannii* ATCC 17978 following treatment with polymyxin B (2 mg/L), mitotane (4 mg/L), or both. Phase contrast microscopy images showed that polymyxin B (**Figure 3.2B**) or mitotane (**Figure 3.2C**) monotherapy had minimal impacts on the overall morphology of the bacterial cells compared to the control group (**Figure 3.2A**); the average cell length remained approximately 3 µm in all cases. However, more clumps of cells were observed with polymyxin B monotherapy (**Figure 3.2B**). In combination (**Figure 3.2D**), polymyxin B and mitotane resulted in significantly shorter cells compared to the other groups with the average cell length reduced to approximately 1 µm. From SEM, polymyxin B monotherapy (**Figure 3.2F**) affected the integrity of the cell surface in polymyxin-susceptible *A. baumannii*. Without treatment (**Figure 3.2E**) the bacterial surface appeared even and smooth, while the surface became uneven and rough following treatment with polymyxin B (**Figure 3.2F**). Mitotane monotherapy (**Figure 3.2G**) and polymyxin B/mitotane combination therapy (**Figure 3.2H**) had minimal impacts on the bacterial surface, although the cell length was confirmed to be much shorter. TEM results reveal that polymyxin B monotherapy (**Figure 3.2J**) caused membrane blebbing. Compared to the control group (**Figure 3.2I**), treatment with mitotane monotherapy (**Figure 3.2K**) had little impact on the bacterial surface. Similar to polymyxin B monotherapy, membrane blebbing was also observed for the treatment with polymyxin B/mitotane combination (**Figure 3.2L**). Additionally, TEM images showed that bacterial cells treated with the polymyxin B/mitotane combination were much shorter in length and most appeared to be undergoing a cell division cycle, with evident chromosomal segregation.

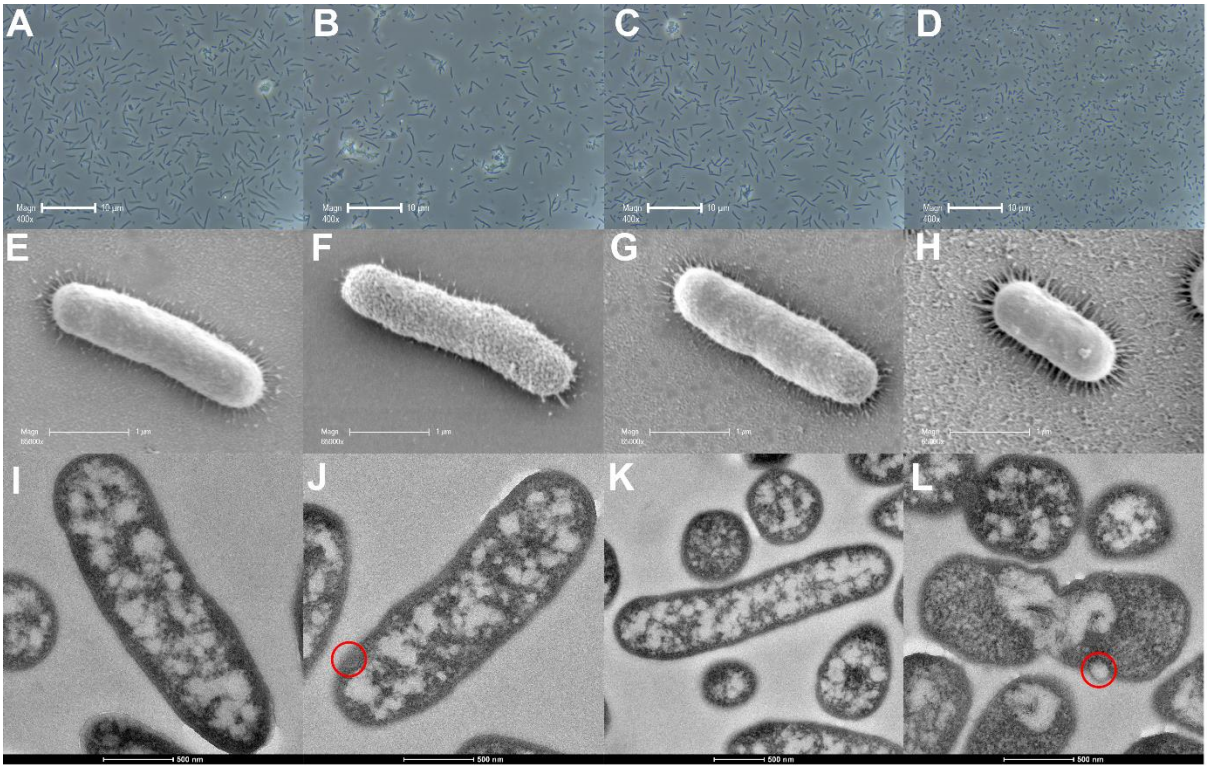

Figure 3.2 Images from phase contrast microscopy (A, B, C, D), scanning electron microscopy (E, F, G, H), and transmission electron microscopy (I, J, K, L) for polymyxin-susceptible *A. baumannii* ATCC 17978 treated with 2 mg/L polymyxin B (B, F, J), 4 mg/L mitotane (C, G, K), or both (D, H, L). A, E, and I represent the control condition. Membrane blebs are indicated by red circles.

Phase contrast microscopy, SEM and TEM images for polymyxin-resistant *A. baumannii* FADDI-AB225 treated with polymyxin B (2 mg/L), mitotane (4 mg/L), or both are shown in **Figure 3.3.** Similar to the results for polymyxin-susceptible *A. baumannii* ATCC 17978, phase contrast microscopy results showed no changes in bacterial size compared to the control group (**Figure 3.3A**) following treatment with polymyxin B (**Figure 3.3B**) and mitotane (**Figure 3.3C**) monotherapy, while the polymyxin B/mitotane combination (**Figure 3.3D**) led to a significant reduction in the cell length. For SEM, treatment with polymyxin B monotherapy (**Figure 3.3F**) did not affect the bacterial cell surface; however, the overall structure appeared distorted. Treatment with mitotane monotherapy (**Figure 3.3G**) affected the cell surface of polymyxin-resistant *A. baumannii* FADDI-AB225, as the

surface was more uneven and rough compared to the control group (**Figure 3.3E**). Combination therapy (**Figure 3.3H**) did not affect the membrane surface, although it led to substantial shortening of the cells. For TEM, similar results to polymyxin-susceptible isolates were once again observed. Membrane blebbing was evident in bacteria treated only with polymyxin B (**Figure 3.3J**), but not in those treated only with mitotane (**Figure 3.3K**). With the polymyxin B/mitotane combination (**Figure 3.3L**) most cells were substantially shorter compared to the control group (**Figure 3.3I**) and appeared to be going through cell division. Unlike the polymyxin-susceptible isolate, no membrane blebbing was observed with the combination in the polymyxin-resistant isolate.

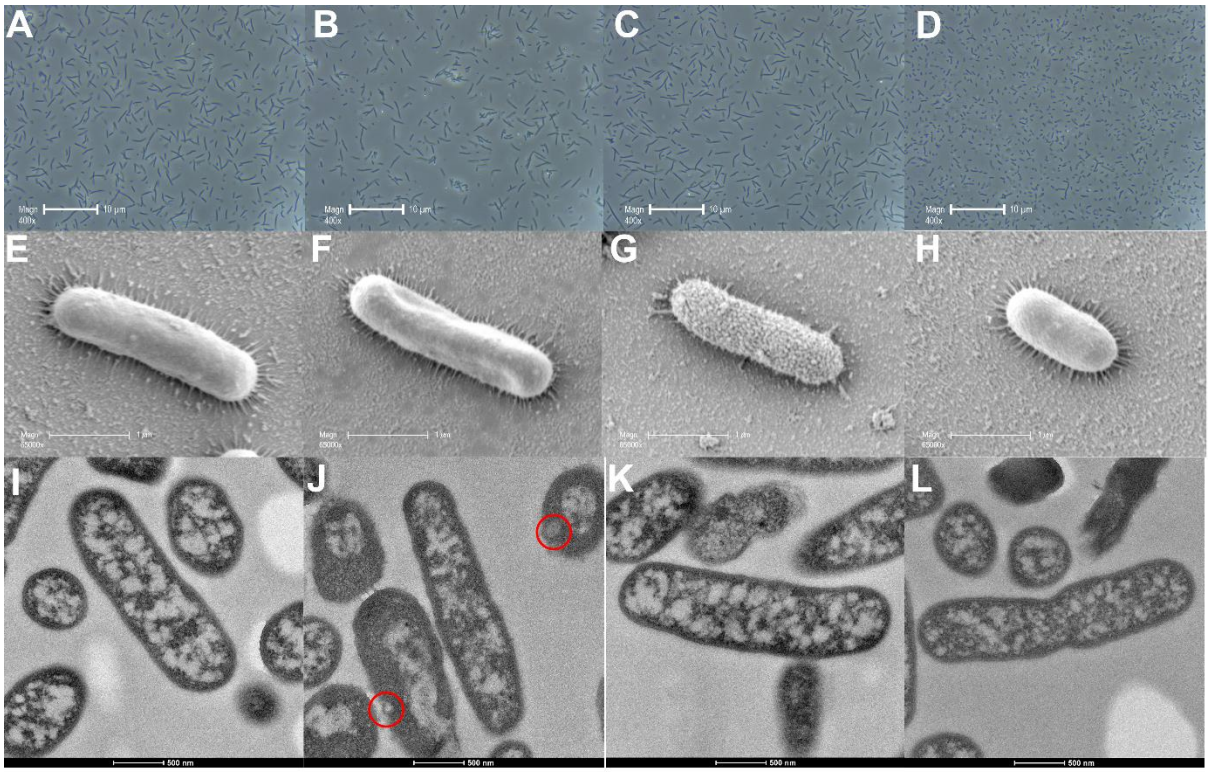

Figure 3.3 Images from phase contrast microscopy (A, B, C, D), scanning electron microscopy (E, F, G, H), and transmission electron microscopy (I, J, K, L) for polymyxin-resistant *A. baumannii* FADDI-AB225 treated with 2 mg/L polymyxin B (B, F, J), 4 mg/L mitotane (C, G, K), or both (D, H, L). A, E, and I represent the control condition. Membrane blebs are indicated by red circles.

3.4.4 In vivo antimicrobial activity of polymyxin B and mitotane against polymyxin-resistant *A. baumannii* FADDI-AB225 in a mouse burn wound infection model

Figure 3.4 shows the bacterial killing of polymyxin B (0.5%, w/w), mitotane (1.4%, w/w), and the polymyxin B/mitotane combination against polymyxin-resistant *A. baumannii* FADDI-AB225. One way ANOVA showed significant difference between the means of all groups ($p < 0.0001$). There was no significant difference in the bacterial load between the blank control (i.e. no treatment) and solvent control groups (mean $\log_{10}$ cfu/wound difference, -0.33; Tukey's HSD, $p > 0.05$), indicating the solvent possessed no major antimicrobial activity. Although this isolate was polymyxin-resistant, topical polymyxin B (0.5%, w/w) monotherapy significantly reduced the bacterial load (mean $\log_{10}$ cfu/wound difference, -1.44 *vs.* blank control; Tukey's HSD, $p \leq 0.0001$). However, there was no significant reduction in the bacterial load (mean $\log_{10}$ cfu/wound difference, -0.11 *vs.* blank control; Tukey's HSD, $p > 0.5$) with topical mitotane (1.4%, w/w) alone (**Figure 3.4**). Importantly, both agents used in combination produced a further significant reduction in the bacterial load compared to polymyxin B monotherapy (mean $\log_{10}$ cfu/wound difference, -0.74; Tukey's HSD, $p \leq 0.01$). Compared to the blank control group, the polymyxin B/mitotane combination resulted in a mean $\log_{10}$ cfu/wound difference of -2.19 (Tukey's HSD, $p \leq 0.0001$).

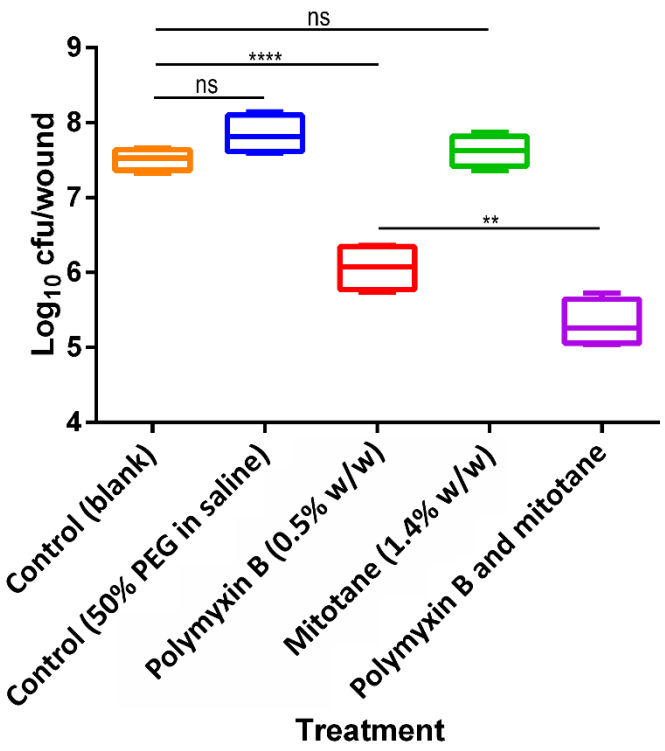

Figure 3.4 Efficacy of polymyxin B alone, mitotane alone and the combination against polymyxin-resistant *A. baumannii* FADDI-AB225 in a mouse wound infection model. Statistical significance was calculated with one-way ANOVA and Tukey's multiple comparisons (ns = $p > 0.5$, ** = $p \leq 0.01$, and **** = $p \leq 0.0001$). Box plots indicate upper and lower quartiles (top and bottom of box); median (line within box); and the spread of data (whiskers).

3.5 Discussion

This is the first study to investigate the potential utility of polymyxin B in combination with the FDA-approved antineoplastic mitotane to treat infections caused by polymyxin-resistant MDR Gram-negative pathogens. Mitotane is a derivative of the insecticide dichlorodiphenyl-trichloroethane and is currently used for the treatment of adrenocortical carcinoma (ACC) (343). The precise mechanism of action of mitotane in ACC is not well understood, but it has been shown to inhibit the activity of sterol-*O*-acyl-transferase and induce endoplasmic reticulum (ER) stress in ACC cells (344). This study

is the first to demonstrate its potential application for the treatment of Gram-negative infections when combined with polymyxin B.

To ensure the applicability of the combination of polymyxin B and mitotane to a diverse population of problematic Gram-negative bacteria, three Gram-negative bacterial species (*A. baumannii*, *P. aeruginosa* and *K. pneumoniae*) were selected for the initial *in vitro* antimicrobial activity evaluation. Isolates selected included MDR, carbapenem-resistant, and polymyxin-resistant strains with known different mechanisms of polymyxin resistance. *A. baumannii* and *P. aeruginosa* were selected, as they are frequently resistant to multiple classes of antibiotics and are currently considered by the World Health Organization as two of the top bacterial 'superbugs' requiring urgent antibiotic development (29). *K. pneumoniae* was also selected by the WHO due to the rapid emergence of carbapenem resistance (including New Delhi metallo-β-lactamase production) (345-347). The chosen drug concentrations of 2 mg/L for polymyxin B and 4 m/L for mitotane reflected clinically achievable concentrations of each agent (162, 348).

One of the major concerns surrounding the intravenous use of polymyxin B or colistin monotherapy for the treatment of infections caused by Gram-negative bacteria is the development of resistance *via* amplification of polymyxin-resistant subpopulations (136, 138, 140, 210, 217-222). Consequently, the use of antibiotic combination therapy represents a potential option to increase bacterial killing and prevent the emergence of polymyxin resistance as the combination may result in subpopulation or mechanistic synergy (252). Despite extensive bacterial killing by polymyxin B monotherapy against five polymyxin-susceptible isolates, regrowth with associated polymyxin resistance (the latter evident by significantly increased polymyxin B MICs compared to baseline values) subsequently occurred with three isolates (*A. baumannii* ATCC 19606, *A. baumannii* FADDI-AB180 and *K. pneumoniae* ATCC 13883) (**Figure 3.1A**). When used as monotherapy, mitotane only showed antimicrobial activity against one isolate (**Figure 3.1A**). However, the combination of polymyxin B and mitotane significantly improved the bacterial killing against the less susceptible isolates (i.e. those that were resistant to polymyxin B

or mitotane monotherapy, or showed regrowth at 24 h) (**Figure 3.1A**). The enhanced antimicrobial killing was indicated by the complete prevention of regrowth in all polymyxin-susceptible isolates after 24 h (**Figure 3.1A**) and >2 $\log_{10}$ cfu/mL reduction within the first 6 h treatment against the four polymyxin-resistant isolates compared to the more active monotherapy (**Figure 3.1B**). Although regrowth occurred in four of the five polymyxin-resistant isolates, the combination still enhanced initial bacterial killing which may assist with the bacterial clearance from the body. In the presence of immune cells, the bacteria will not have the opportunity to regrow. Furthermore, the lower number of bacteria minimises the risk of hyper immune response that can lead to sepsis shock. Since polymyxins are well-known for their ability to permeabilise the outer membrane of Gram-negative bacteria (349-351), a possible mechanism for the enhanced killing observed with the combination is permeabilization of the outer membrane by polymyxin B leading to the entry of mitotane into the bacterial cell. Indeed, polymyxin B and its derivative polymyxin B nonapeptide that lacks bactericidal activity had previously been shown to confer a similar effect for hydrophobic antibiotics against Gram-negative bacteria and yeasts (352, 353). Interestingly, mitotane monotherapy displayed substantial antimicrobial activity against LPS-deficient, polymyxin-resistant *A. baumannii* FADDI-AB065 (**Figure 3.1B**). LPS in the outer membrane of Gram-negative bacteria acts as a highly selective permeability barrier that protects Gram-negative bacteria from harmful substances (354). Consequently, it is possible that in the absence of LPS mitotane was able to enter the cells and exert its antimicrobial activity. Another notable finding is that mitotane monotherapy also lowered the polymyxin B MIC of polymyxin-resistant *A. baumannii* FADDI-AB225 (**Table 3.2**); however, it did not affect the polymyxin B MICs of the other polymyxin-resistant isolates. The mechanism for this phenomenon is currently unclear, although it may result from the expression of LPS variants by the different isolates; coincidently, it has been reported that *Moraxella catarrhalis* and *Salmonella typhimurium* with deep rough-type LPS displayed higher susceptibility to hydrophobic antimicrobial agents (355). Further mechanistic studies are warranted.

According to the SEM imaging results, it is possible that the polymyxin resistance in *A. baumannii* FADDI-AB225 altered their surface interaction with mitotane, as the outer membrane appeared disrupted (uneven and rough) following mitotane monotherapy in *A. baumannii* FADDI-AB225 (**Figure 3.3G**), but not *A. baumannii* ATCC 17978 **(Figure 3.2G**). Both the SEM and TEM images showed disruptive changes to the outer membrane of polymyxin-susceptible *A. baumannii* ATCC 17978 following polymyxin B monotherapy (**Figure 3.2F** and **3.2J**), which confirmed the known impact of polymyxin B on the outer membrane of Gram-negative bacteria. For the lipid A modified polymyxin-resistant *A. baumannii* FADDI-AB225, no disruptive effect on the surface membrane by polymyxin B monotherapy was observed with SEM (**Figure 3.3F**), most likely due to the modification of lipid A which resulted in minimal polymyxin B affinity. Membrane blebs, however, were still observed by TEM in *A. baumannii* FADDI-AB225 treated with polymyxin B monotherapy (**Figure 3.3J**), indicating blebbing may not necessarily result in cell death. Although monotherapy of mitotane or polymyxin B appeared to impact the outer membrane of polymyxin-resistant and -susceptible *A. baumannii*, the combination impacted the overall structure of both strains leading to an extensive shortening in the length of the bacteria (**Figure 3.2** and **3.3**). SEM images showed a smooth membrane surface on the shortened bacterial cells, suggesting that the combination prevented the formation of the rough surface, which could be an adaptive response to polymyxin B or mitotane monotherapy. Numerous incompletely separated cells revealed by TEM images (**Figure 3.2L** and **3.3L**) suggest a possible impact on the bacterial DNA replication.

In the mouse burn wound infection study, the combination displayed effective antimicrobial activity against polymyxin-resistant *A. baumannii*. The doses of 5 mg/kg for polymyxin B (subcutaneous median lethal dose in mice [LD_{50}] 59 mg/kg) and 14 mg/kg for mitotane (oral LD_{50} >4,000 mg/kg in mice, dermal LD_{50} not available) were selected, as they are safe in animals according to the literature. Based on the available LD_{50} limits of polymyxin B and mitotane, it is likely that much higher doses of both drugs can be used for topical combination therapy. Given the lack of an optimised topical formulation, it is possible that the *in vivo* efficacy of the combination in the current study is

underestimated. Nevertheless, the combination treatment was able to significantly reduce the number of polymyxin-resistant *A. baumannii*, compared to polymyxin B or mitotane monotherapy. As mitotane is an antineoplastic drug, care should be taken to avoid negative side effects.

3.6 Conclusions

This study is the first to reveal the synergistic activity of mitotane, an FDA-approved non-antibiotic drug, in combination with polymyxin B against problematic Gram-negative bacteria. Importantly, the combination also prevented the emergence of polymyxin resistance. As mitotane is currently used in humans, its repositioning for antimicrobial purposes may be easier than discovering novel antibacterial compounds against Gram-negative 'superbugs'. The synergistic antibacterial killing of polymyxin B with mitotane in animals raises hopes for the potential repositioning of mitotane against MDR Gram-negative bacteria and further clinical investigations are warranted.

CHAPTER FOUR: SYNERGISTIC KILLING OF POLYMYXIN B IN COMBINATION WITH THE ANTINEOPLASTIC DRUG MITOTANE AGAINST POLYMYXIN-SUSCEPTIBLE AND –RESISTANT *A. BAUMANNII*: A METABOLOMIC STUDY

4.1 Abstract

Polymyxins are currently used as the last-resort antibiotics against MDR *A. baumannii*. As resistance to polymyxins emerges in *A. baumannii* with monotherapy, combination therapy is often the only remaining treatment option. A novel approach is to employ the combination of polymyxin B with non-antibiotic drugs. In the present study, I employed non-targeted metabolomics to investigate the synergistic mechanism of polymyxin B in combination with the antineoplastic drug mitotane against polymyxin-susceptible and -resistant *A. baumannii*. The metabolomes of four *A. baumannii* strains were analysed following treatment with polymyxin B, mitotane and the combination. Polymyxin B monotherapy induced significant perturbation in glycerophospholipid metabolism and histidine degradation pathways in polymyxin-susceptible strains, and minimal perturbation in polymyxin-resistant strains. Mitotane monotherapy induced minimal perturbation in the polymyxin-susceptible strains but caused significant perturbation in glycerophospholipid metabolism and the pentose phosphate and histidine degradation pathways in the polymyxin-resistant strain which lacked LPS (FADDI-AB065). The polymyxin B - mitotane combination induced significant perturbation in all strains except the lipid A modified polymyxin-resistant FADDI-AB225 strain. For the polymyxin-susceptible strains, combination therapy significantly perturbed glycerophospholipid metabolism, the pentose phosphate pathway, citric acid cycle, pyrimidine ribonucleotide biogenesis, guanine ribonucleotide biogenesis, and the histidine degradation pathway. Against FADDI-AB065, the combination significantly perturbed only glycerophospholipid metabolism, the pentose phosphate pathway, citric acid cycle, and pyrimidine ribonucleotide biogenesis. Overall, these novel findings demonstrate that the disruption of the citric acid cycle and inhibition of nucleotide biogenesis are the key metabolic

features associated with synergistic bacterial killing by the combination against polymyxin-susceptible and -resistant *A. baumannii*.

4.2 Introduction

MDR *A.baumannii* has become a major global health threat (10, 25, 34, 36, 356). The incidence and severity of infections caused by *A. baumannii* has increased dramatically over the last two decades (38, 39, 42). A major contributing factor to the clinical significance of *A. baumannii* is its exceptional capacity to acquire antibiotic resistance determinants (36, 43). In 2013, the Centers for Disease Control and Prevention (CDC) reported approximately 63% of healthcare-associated *Acinetobacter* infections occurring in the United States were multidrug-resistant (MDR, i.e. non-susceptible to ≥1 treating agent in ≥3 antimicrobial categories) (23, 28). Consequently, MDR *A. baumannii* has been classified by the CDC as a "Serious threat" (28). More recently, carbapenem-resistant *A. baumannii* has been classified by the World Health Organization as one of the top priorities for research and development of new antibiotics, due to the current lack of novel antibiotic candidates in the drug development pipeline (29). Clearly, the development of novel therapeutic strategies to combat the threat of this deadly pathogen are urgently needed.

Polymyxin B and colistin are considered 'drugs of last resort' against MDR *A. baumannii* (81, 82). Although polymyxins are believed to cause cell death primarily by disorganizing the Gram-negative outer membrane *via* binding to lipopolysaccharide (LPS), the precise antibacterial killing mechanism is not completely understood (357). Worryingly, my research group and others have demonstrated that polymyxin resistance rapidly emerges in *A. baumannii* following polymyxin monotherapy (111, 128, 131, 149, 228, 341). *A. baumannii* becomes resistant to polymyxins by a reduction of the negative charge on the outer membrane (87), which can be achieved either through lipid A modification (with phosphoethanolamine [pEtN] and galactosamine [GalN] (111, 127, 358)) or loss of LPS (128).

Recently, the combination of an antibiotic (including polymyxins) and non-antibiotic drug has emerged as a potentially valuable and cost-effective approach to improve the clinical efficacy of currently available antibiotics against problematic MDR bacterial pathogens (285, 359-361). As polymyxins are able to permeabilise the outer membrane of *A. baumannii*, it is possible that in combination a polymyxin may help the non-antibiotic drug reach its intracellular target by facilitating entry into the bacterial cell (285, 359, 360). The study from Chapter 3 demonstrated that the combination of polymyxin B and the antineoplastic agent mitotane provided enhanced antimicrobial activity against MDR as well as polymyxin-resistant *A. baumannii*. Furthermore, the combination also prevented the emergence of polymyxin resistance in polymyxin-susceptible strains. Given the importance of the potential repositioning of mitotane to treat MDR *A. baumannii*, it is essential to understand the mechanisms by which the polymyxin B/mitotane combination achieves this enhanced bacterial killing and suppression of emergence of polymyxin resistance.

Metabolomics has emerged as a valuable tool for elucidating the mechanisms of drug action in bacterial physiology and drug discovery (362, 363). Notably, metabolomics provides snapshots of cellular biochemical networks and helps explain how bacteria respond to antibiotic treatment at the systems level (364-366). Moreover, understanding how bacteria respond to antibiotic treatment at the metabolic level is valuable for the discovery of novel antibiotic targets (364). Accordingly, the primary aim of this study was to use untargeted metabolomics to elucidate the mechanism(s) of the enhanced antimicrobial activity by the combination of polymyxin B and mitotane against *A. baumannii*.

4.3 Materials and methods

4.3.1 Drugs and bacterial isolates

Polymyxin B (Beta Pharma, China, Batch number 20120204) solutions were prepared in Milli-Q™ water (Millipore, Australia) and filtered through 0.22-μm syringe filters (Sartorius, Australia). Mitotane (Sigma-Aldrich, Australia, Lot number BCBG9480V) solutions were prepared in dimethyl sulfoxide

(Sigma-Aldrich, Australia). All other reagents were purchased from Sigma-Aldrich (Australia) and were of the highest commercial grade available. Polymyxin-susceptible *A. baumannii* ATCC 17978 (polymyxin B MIC = 0.25 mg/L) and ATCC 19606 (polymyxin B MIC = 0.5 mg/L) were obtained from the American Type Culture Collection (Rockville, MD, USA). *A. baumannii* FADDI-AB225 (formally designated ATCC 17978-R2) is polymyxin-resistant (polymyxin B MIC = 16 mg/L) with phosphoethanolamine-modified lipid A and *pmrB* mutation derived from *A. baumannii* ATCC 17978 (341). *A. baumannii* FADDI-AB065 (formally designated ATCC 19606R) is a polymyxin-resistant (polymyxin B MIC = 64 mg/L), LPS-deficient, *lpxA* mutant derived from the ATCC 19606 strain (128). Isolates were stored in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) in cryovials at -80°C. Before use, *A. baumannii* ATCC 17978 and ATCC 19606 were subcultured onto nutrient agar plates (Media Preparation Unit, University of Melbourne, Melbourne, VIC, Australia) and *A. baumannii* FADDI-AB225 and FADDI-AB065 were subcultured onto Mueller-Hinton plates supplemented with 10 mg/L of polymyxin B (Media Preparation Unit) to maintain the selection pressure.

4.3.2 Bacterial culture preparation for metabolomics experiments

To investigate the possible molecular mechanisms of polymyxin B and mitotane combination, I employed untargeted metabolomics to determine the changes in different metabolite levels in all *A. baumannii* strains following 2-h treatment with 2 mg/L polymyxin B, 4 mg/L mitotane, or the combination. For each *A. baumannii* strain, single colonies grown on nutrient or Mueller-Hinton agar were selected and grown overnight (16 - 18 h) in 20 mL CAMHB in 50 mL Falcon tubes (Thermo Fisher, Australia) incubated in a shaking water bath at 37°C (shaking speed, 180 rpm). Following overnight incubation, each culture was transferred to a 1000 mL conical flask with 250 mL of fresh CAMHB at ~50-100 fold dilutions. Flasks were incubated at 37°C with shaking at 180 rpm for ~3 - 4 h to log-phase (OD_{600} ~0.5). Cultures (50 mL) were transferred to four 500 mL conical flasks and solutions of polymyxin B, mitotane, or both added to three of four flasks to give a final concentration of 2 mg/L for

polymyxin B and 4 mg/L for mitotane; the remaining flask acted as a drug-free control. To prevent excessive bacterial killing, the starting bacterial inoculum used was ~10^8 cfu/mL. The flasks were further incubated at 37°C with shaking at 180 rpm. After 2 h, the OD_{600} reading for each flask was measured and normalised to ~0.5 with fresh CAMHB and 10 mL samples transferred to 15 mL Falcon tubes (Thermo Fisher, Australia) for metabolite extraction. To minimise inherent random variation, for each strain four biological samples were prepared for each treatment condition.

4.3.3 Metabolite extraction for metabolomic studies

Following bacterial culture preparation, extraction of metabolites was immediately performed to minimise further drug effects on metabolite levels. Samples were initially centrifuged at 3220 × *g* at 4˚C for 10 min. Supernatants were then removed and bacterial pellets washed twice in 2 mL of cold 0.9% NaCl followed by centrifugation at 3220 × *g* at 4˚C for 5 min to remove residual extracellular metabolites and medium components. The washed pellets were then resuspended with cold chloroform:methanol:water (CMW; 1:3:1, v/v) extraction solvent containing 1 µM each of the internal standards (CHAPS, CAPS, PIPES and TRIS). The selected internal standards are physicochemically diverse small molecules not naturally occurring in any microorganism. Samples were then thrice frozen in liquid nitrogen, thawed on ice and vortexed to release the intracellular metabolites. After the third cycle samples were centrifuged for 10 min at 3220 × *g* at 4˚C, whereby 300 µL of the supernatants was transferred to 1.5 mL Eppendorf tubes for immediate storage at -80°C. Prior to analysis samples were thawed and centrifuged at 14,000 × *g* at 4˚C for 10 min to remove the presence of any particles, and 200 µL transferred into the injection vial for LC-MS analysis (described below). An equal volume of each sample was combined and used as a pooled quality control sample (QC); namely, a sample that contains all the analytes that will be encountered during the analysis (367).

4.3.4 LC-MS analysis

Metabolites were detected with hydrophilic interaction liquid chromatography (HILIC) - high-resolution mass spectrometry (HRMS) using a Dionex high-performance liquid chromatography (HPLC) system (RSLCU3000, Thermo Fisher) with a ZIC-pHILIC column (5 µm, polymeric, 150 × 4.6 mm; SeQuant, Merck). The system was coupled to a Q-Exactive Orbitrap mass spectrometer (Thermo Fisher) operated in both positive and negative electro-spray ionization (ESI) mode at 35,000 resolution with a detection range of 85 to 1, 275 m/z. The LC solvents were (A) 20 mM ammonium carbonate and (B) acetonitrile, operated via a multi-step gradient system. The gradient system at 80% B and was reduced to 50% B over 15 min, then reduced from 50% B to 5% B over 3 min, followed by wash with 5% B for another 3 min, and finally 8 min re-equilibration with 80% B at a flow rate of 0.3 mL/min (368). The total run time was 32 min with an injection sample volume of 10 µL. All samples were analysed as a single LC-MS batch to reduce the batch-to-batch variation. Mixtures of pure standards containing over 250 metabolites were also included in the analysis to aid metabolite identification.

4.3.5 Data processing, bioinformatics and statistical analyses

Conversion of LC-MS raw data to metabolites was conducted using IDEOM (http:// mzmatch.sourceforge.net/ideom.php) free software (369), which initially employed ProteoWizard to convert raw LC-MS data to mzXML format and XCMS to pick peaks to convert to peakML files (370, 371). Mzmatch.R was subsequently used for the alignment of samples and the filtering of peaks using minimum detectable intensity of 100,000, relative standard deviation (RSD) of < 0.5 (reproducibility), and peak shape (codadw) of > 0.8. Mzmatch was also used to retrieve missing peaks and annotation of related peaks. Default IDEOM parameters were used to eliminate unwanted noise and artefact peaks. Loss or gain of a proton was corrected in negative and positive ESI mode, respectively, followed by putative identification of metabolites by the exact mass within 2 ppm. Retention times of authentic standards were used to confirm the identification of each metabolite (Level 1 identification based on

MSI standards). Other metabolites were putatively identified (Level 2 identification based on MSI standards) using exact mass and predicted retention time based on the Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, and LIPIDMAPS databases, using preference to bacterial metabolites annotated in EcoCyc. Raw peak intensity was used to quantify each metabolite. The free online tool MetaboAnalyst 3.0 was used for the statistical analysis. Briefly, putative metabolites with median RSD ≤ 0.2 (20%) within the QC group and IDEOM confidence level of ≥ 5 were incorporated into a table and uploaded to MetaboAnalyst. Data with > 50% missing values were removed and remaining missing values replaced with half the minimum positive value in the original data. Data were filtered using interquantile range (IQR), normalised by the median, $\log_2$ transformed and auto scaled. Principal component analysis was performed to identify and remove outliers. Outliers were defined as samples outside of ±2 standard deviations (SD) along the principal component 1 axis. One-way ANOVA was used to identify metabolites with significant level changes between all samples and Fisher's least square difference (LSD) to determine the metabolites with significant level changes between treatment and control groups. Statistically significant metabolites were selected using a false discovery rate of ≤ 0.05 for one-way ANOVA and $p \leq 0.05$ for Fisher's LSD. KEGG mapper was used to determine the pathway modules by statistically significant metabolites containing the KEGG compound numbers.

4.4 Results

4.4.1 Multivariate and univariate analyses of the metabolites affected by polymyxin B and mitotane in *A. baumannii*

Untargeted metabolomics analysis using HILIC-based high resolution accurate mass LC-MS allowed detection of 1769 putative metabolites in polymyxin-sensitive and –resistant strains of *A. baumannii* treated with polymyxin B and mitotane. The reproducibility of metabolite semi-quantitation was within acceptable limits based on the median relative standard deviation (RSD) from independent

biological replicates across the four *A. baumannii* strains, where the median RSD was 16% for all control groups and <20% for most treatment groups (**Table 4.1**) (372).

Table 4.1 Data precision of different treatment groups represented as the median relative standard deviation (RSD) for all assessed metabolites

	Median RSD (%)
***A. baumannii* ATCC 17978**	
Control	16
Polymyxin B	17
Mitotane	21
Combination	16
***A. baumannii* FADDI-AB225**	
Control	16
Polymyxin B	18
Mitotane	16
Combination	23
***A. baumannii* ATCC 19606**	
Control	16
Polymyxin B	23
Mitotane	14
Combination	14
***A. baumannii* FADDI-AB065**	
Control	16
Polymyxin B	15
Mitotane	16
Combination	14
PBQCs	14

Multivariate analysis was performed using PCA. Mitotane monotherapy clearly impacted the metabolome of FADDI-AB065 based on the first two principal components, but did not differentiate from controls for the other three tested strains (**Figure 4.1A**). Compared to polymyxin B and mitotane therapies, the combination produced more significant perturbation in the metabolomes of ATCC 17978 and FADDI-AB065 (**Figure 4.1A**). Minimal impact on the metabolome of FADDI-AB225 was observed for the combination (**Figure 4.1A**).

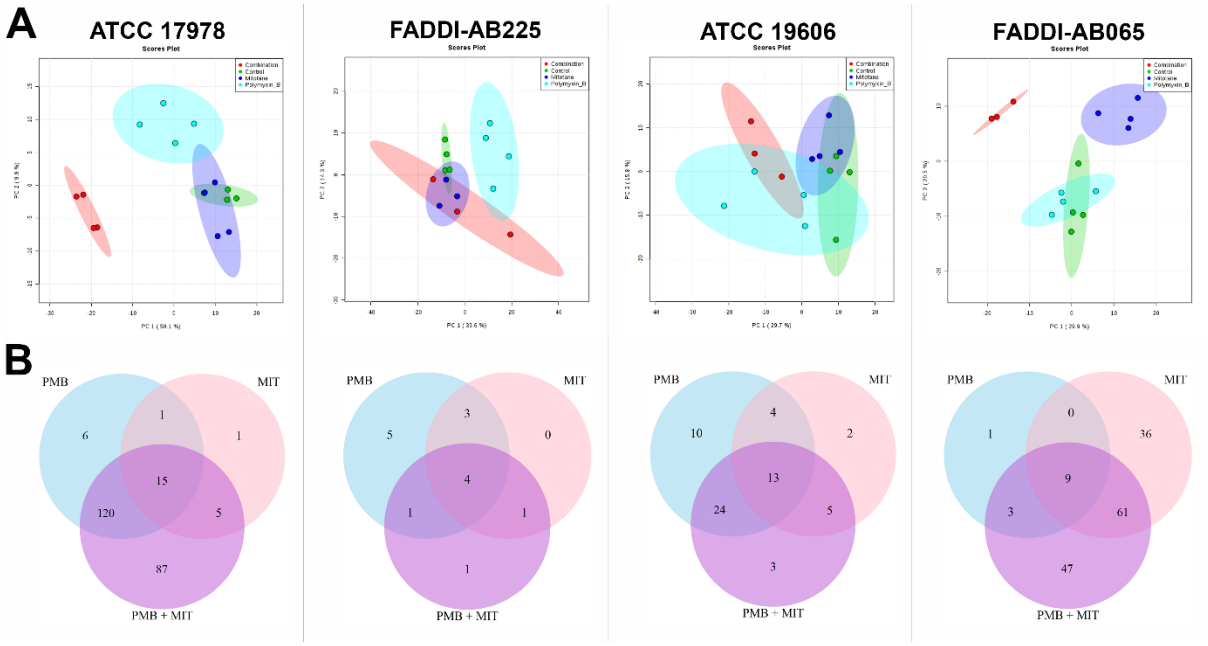

Figure 4.1 (**A**) PCA score plots showing metabolomic variance for polymyxin B (blue), mitotane (purple),combination (red) and untreated (green) samples for each *A. baumannii* strain along principal component 1 and principal component 2. (**B**) Venn diagrams showing the number of statistically significant metabolites affected by different treatments (one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$) in each *A. baumannii* strain. (PMB = polymyxin B; MIT = mitotane).

Univariate analysis was performed with one-way analysis of variance (ANOVA) followed by Fisher's least square difference (LDS). Using a statistical threshold of FDR ≤ 0.05 for one-way ANOVA and $p \leq 0.05$ for Fisher's LSD, polymyxin B monotherapy was identified to cause significant perturbations in a total of 142 metabolites in ATCC 17978, 51 in ATCC 19606, 13 in FADDI-AB225, and 13 in FADDI-AB065 (**Figure 4.1B**). For mitotane monotherapy, a total of 22 metabolites were perturbed in ATCC 17978, 24 in ATCC 19606, 8 in FADDI-AB225, and 106 in FADDI-AB065 (**Figure 4.1B**). The combination caused perturbations in a total of 227 metabolites in ATCC 17978, 45 in ATCC 19606, 7 in FADDI-AB225, and 120 in FADDI-AB065 (**Figure 4.1B**). Compared to mitotane monotherapy, polymyxin B monotherapy caused perturbation in more than twice the number of metabolites in the polymyxin-susceptible strains (ATCC 17978 and ATCC 19606). For the combination, over 50% of the perturbed metabolites in the polymyxin-susceptible strains were in common with those perturbed by polymyxin B and mitotane

monotherapy. The common perturbed metabolites between combination therapy and polymyxin B monotherapy were much higher than the common perturbed metabolites between the combination therapy and mitotane monotherapy (**Figure 4.1B**). Although mitotane monotherapy had little impact on the polymyxin-susceptible strains, it caused extensive metabolic changes in polymyxin-resistant FADDI-AB065, which lacks LPS (**Figure 4.1B**). Most of the perturbed metabolites caused by the combination in this strain, consequently, were in common with those perturbed by mitotane monotherapy (**Figure 4.1B**).

The statistically significant metabolites impacted (one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$) by different treatments in each *A. baumannii* strain were divided into seven different metabolite classes: amino acids, carbohydrates, energy, lipids, nucleotides, peptides, and others (the latter includes cofactors and vitamins, glycans, secondary metabolites and metabolites that could not be mapped into pathways). The number of metabolites impacted from each class that were higher or lower in abundance compared to the control group are shown in **Figure 4.2**. Details of all significantly impacted metabolites, including mass, retention time (RT), formula, putative identification, level of confidence (from IDEOM), map, pathway, fold-change (FC; based on raw intensity), and FDR are shown in Appendix 1 (**Tables A1.1 – A1.4)** for all four strains.

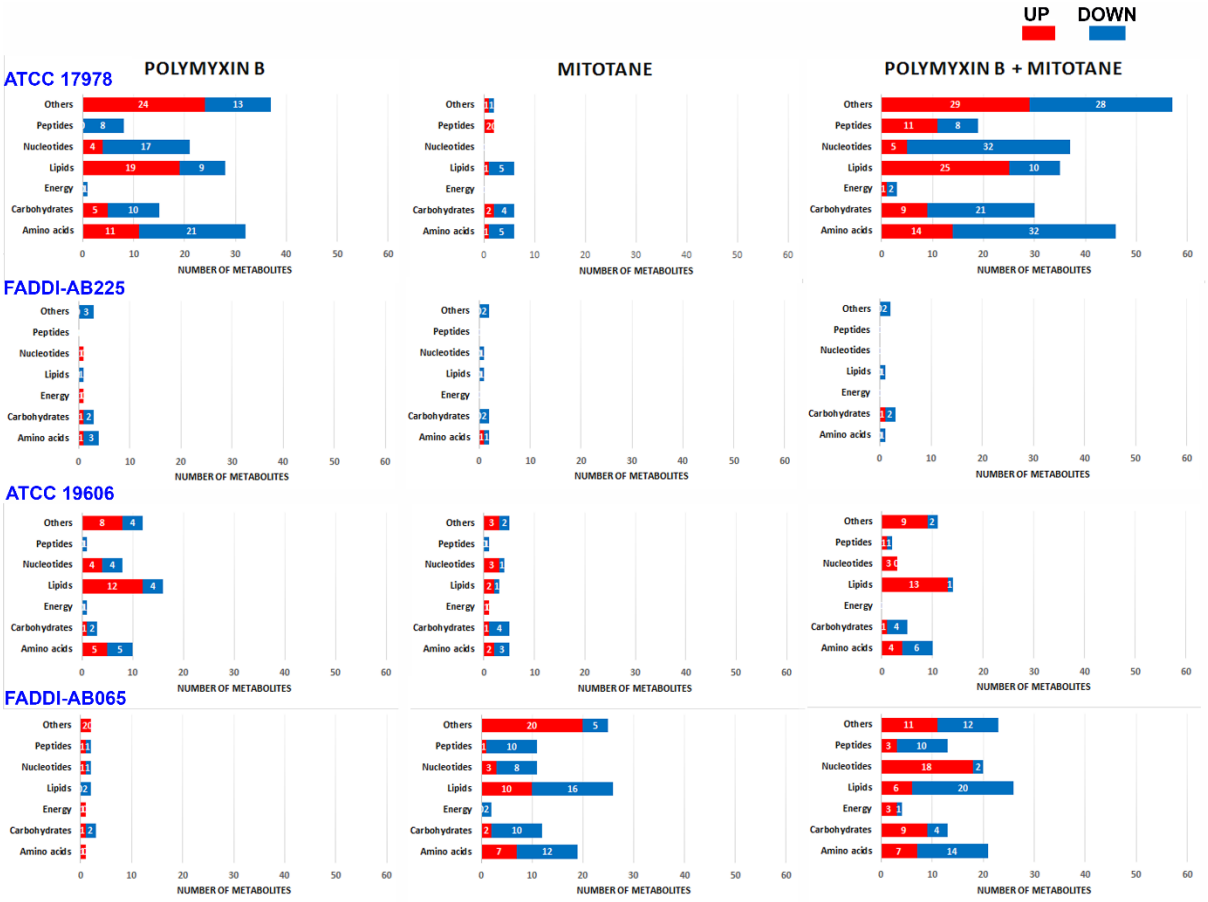

Figure 4.2 Bar graphs showing the number of significantly perturbed metabolites (ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$) in different metabolite classes following treatment with polymyxin B, mitotane, and the combination for polymyxin-susceptible *A. baumannii* ATCC 17978, polymyxin-resistant *A. baumannii* FADDI-AB225, polymyxin-susceptible *A. baumannii* ATCC 19606, and polymyxin-resistant *A. baumannii* FADDI-AB065. The class designated as 'Others' includes cofactors and vitamins, glycan, secondary metabolites and metabolites that could not be mapped into pathways based on existing bacterial metabolite databases.

4.4.2 Significantly impacted lipids and lipid metabolites

All glycerophospholipids (GPL) across four *A. baumannii* strains detected by LC-MS and their relative abundance (based on raw peak intensity) compared to the control groups are shown in **Figure 4.3**. In the polymyxin-susceptible strains, polymyxin B monotherapy induced significant changes in a wide

range of GPL while mitotane monotherapy induced minimal changes. Overall, polymyxin B monotherapy caused a higher level of GPL perturbation in ATCC 17978 than ATCC 19606. Compared to polymyxin B monotherapy, the combination substantially enhanced the perturbation of putative lysophospholipids PA(16:0), PC(14:0), PC(16:0), PC(18:0), PC(18:1), PI(16:0), and PI(18:0) in ATCC 17978 to greater than 2-fold change in the majority of cases. Against ATCC 19606, where the perturbation caused by polymyxin B monotherapy was lower than 2 fold-change in most cases, the combination did not significantly affect the GPL. In both polymyxin-resistant strains, polymyxin B monotherapy had minimal impact on the GLP while mitotane monotherapy significantly affected a wide range of GPL in FADDI-AB065. In FADDI-AB065, mitotane monotherapy caused over 2 fold reduction in putative glycerophospholipids PC(14:0), PC(16:0), PC(18:1), PG(34:3), PG(35:2), and PI(16:0). Interestingly, the combination did not enhance the lipid perturbation caused by mitotane monotherapy in FADDI-AB065.

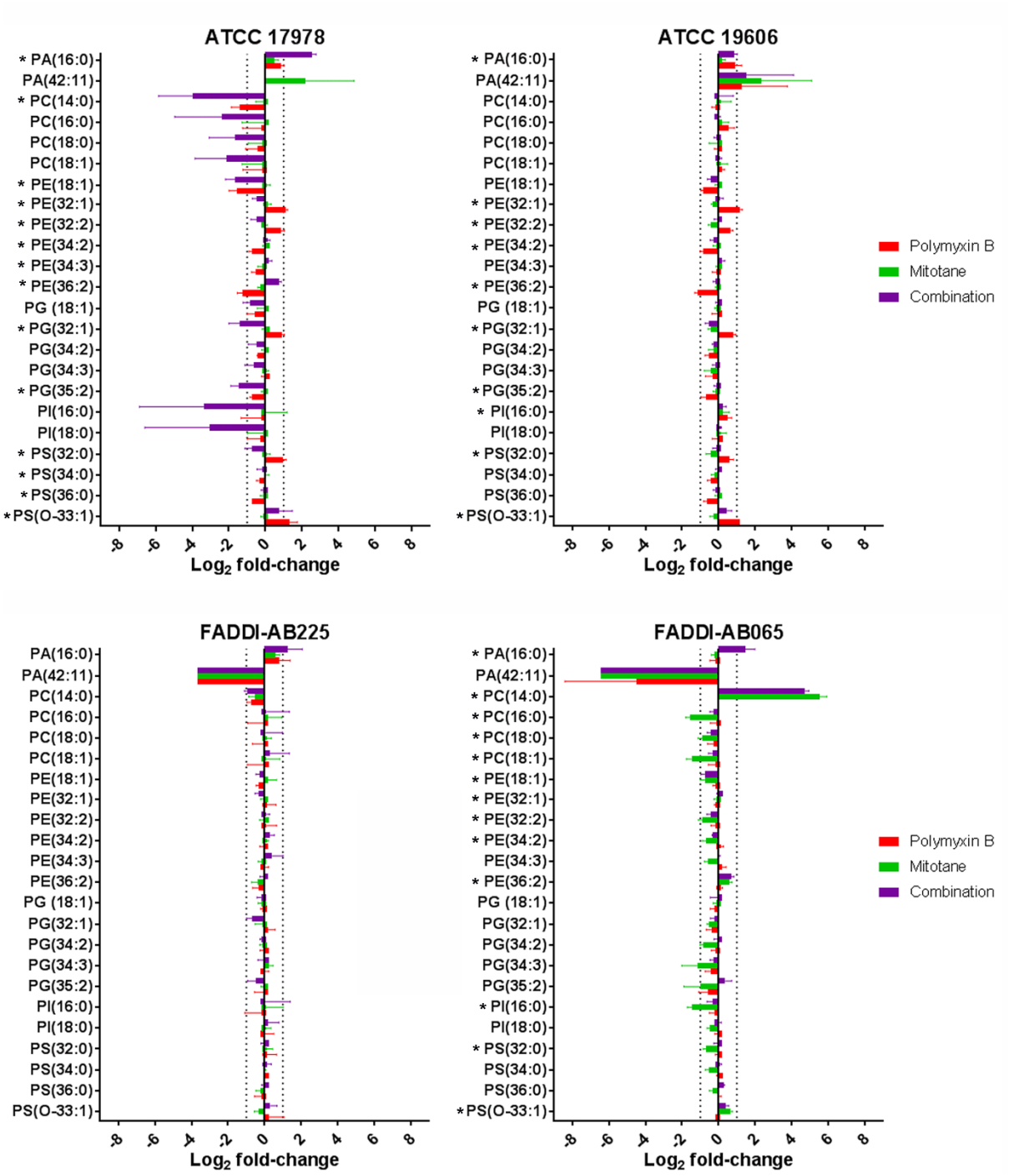

Figure 4.3 All detected glycerophospholipids in *A. baumannii* ATCC 17978, ATCC 19606, FADDI-AB065 and FADDI-AB225 following treatment with polymyxin B, mitotane and the combination. (* one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$, $\log_2$ fold-change ≥ |1| thresholds are indicated by vertical dotted lines). PA = phosphatidic acid, PC = phosphatidylcholine; PE = phosphatidylethanolamine; PG = phosphatidylglycerol; PI = phosphatidylinositol; PS = phosphatidylserine.

The statistically significant (ANOVA, FDR ≤ 0.05, Fisher's LSD, $p \leq 0.05$) fatty acyls impacted by polymyxin B monotherapy, mitotane monotherapy, or the combination are shown in **Figure 4.4**. In ATCC 17978, ATCC 19606 and FADDI-AB225, polymyxin B monotherapy induced significant perturbations in putative oleoyl-CoA, a metabolite involved in fatty acid metabolism. Based on the raw intensity, the relative abundance of oleoyl-CoA was over 2-fold lower in polymyxin-susceptible strains treated with polymyxin B monotherapy compared to the untreated group. For mitotane monotherapy, significant reduction of oleoyl-CoA was observed for FADDI-AB225 and its parent strain ATCC 17978. Compared to polymyxin B monotherapy, the combination caused a greater reduction of oeolyl-CoA in polymyxin-resistant FADDI-AB225. In addition, the combination also caused significant perturbation of putative oxidised fatty acids including putative Hydroxypentanoate in both polymyxin-susceptible strains, putative FA oxo(18:0) in ATCC 17978, and putative FA hydroxy(18:0) and putative FA oxo(19:0) in FADDI-AB065.

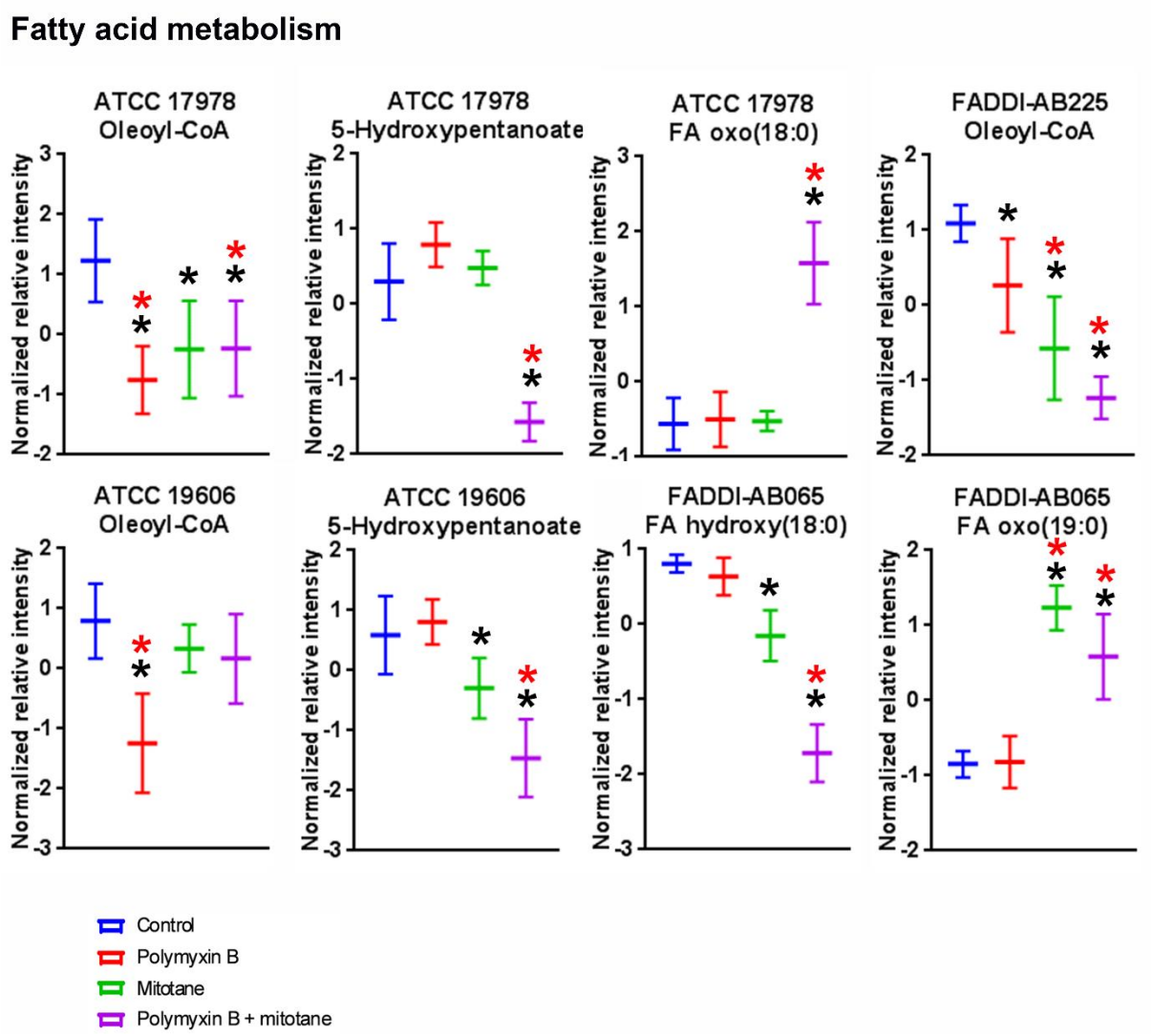

Figure 4.4 Putative fatty acyls from fatty acid metabolism in *A. baumannii* significantly impacted by polymyxin B, mitotane, and combination treatment. (Mean ± SD; * one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$; * $\log_2$ fold-change ≥ |1|).

The impact of polymyxin B and mitotane on the metabolites involved in glycerophospholipid metabolism in *A. baumannii* is shown in **Figure 4.5**. In ATCC 19606, polymyxin B monotherapy and combination therapy caused statistically significant perturbation in total putative PEs (the sum of all detected putative PE species) and acetylcholine, although the changes in relative abundance were less than 2-fold. Mitotane monotherapy had minimal impact on these metabolites in ATCC 19606. For ATCC 17978, in addition to total putative PEs and acetyl choline, polymyxin B monotherapy and combination therapy also substantially reduced putative *sn*-glycero-3-phosphoethanolamine (Log_2FC

= -2.42 and -2.83 respectively). Mitotane monotherapy only caused minor reduction of putative *sn*-glycero-3-phosphoethanolamine (Log_2FC = -0.47) in ATCC 17978. Against polymyxin-resistant FADDI-AB065, polymyxin B monotherapy did not impact any metabolites involved in glycerophospholipid metabolism. However, a total of six metabolites were significantly perturbed by mitotane monotherapy or combination therapy. Mitotane monotherapy caused significant perturbations in total putative PCs (the sum of all detected putative PC species) (Log_2FC = -1.25), total putative PEs (Log_2FC = -0.45), *sn*-glycero-3-phosphocholine (Log_2FC = -0.76), putative *sn*-glycero-3-phosphoethanolamine (Log_2FC = -1.71) and acetylcholine (Log_2FC = -0.04). The combination caused significant perturbations in total putative PEs (Log_2FC = -0.34), *sn*-glycero-3-phosphocholine (Log_2FC = -0.25), putative *sn*-glycero-3-phosphoethanolamine (Log_2FC = -1.62), and *sn*-glycerol-3-phosphate (Log_2FC = 1.55).

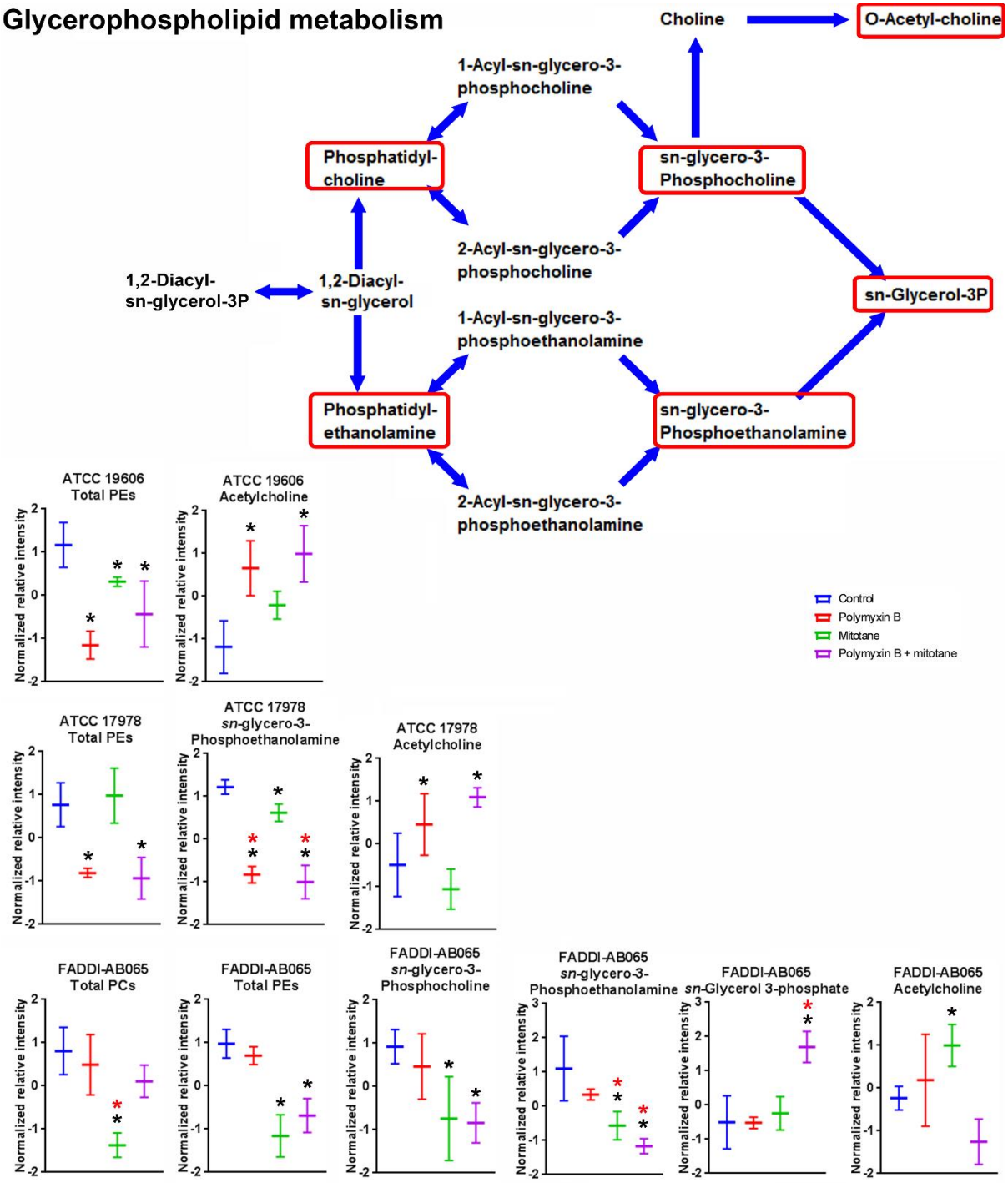

Figure 4.5 Metabolites involved in glycerophospholipid metabolism in *A. baumannii* significantly impacted by polymyxin B, mitotane, and the combination. Red boxes indicate statistically significant metabolites (Mean ± SD; * one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$; * $\log_2$ fold-change ≥ |1|).

4.4.3 Significantly impacted metabolites in the pentose phosphate pathway

Metabolites involved in the pentose phosphate pathway of A. baumannii were perturbed by polymyxin B and mitotane **(Figure 4.6)**. For ATCC 17978, polymyxin B monotherapy had no impact on metabolites of the pentose phosphate pathway. However, combination therapy caused a significant reduction in D-ribose-5-phosphate, putative D-sedoheptulose-7-phosphate, D-erythrose-4-phosphate, and 2-deoxy-D-ribose-5-phosphate (Log_2FC = -1.82, -3.09, -3.07, and -1.79, respectively). For FADDI-AB225, a significant reduction in D-gluconic acid was observed for polymyxin B and mitotane monotherapies (Log_2FC = -1.21 and -1.01, respectively) but not for combination therapy. For FADDI-AB065, mitotane monotherapy caused a statistically significant reduction of D-ribose-5-phosphate, putative D-Sedoheptulose-7-phosphate and D-erythrose-4-phosphate (≥2-fold change) (Log_2FC = -0.94, -1.93 and -2.74, respectively). Surprisingly, combination therapy only caused a significant reduction of D-gluconic acid (Log_2FC = -0.64).

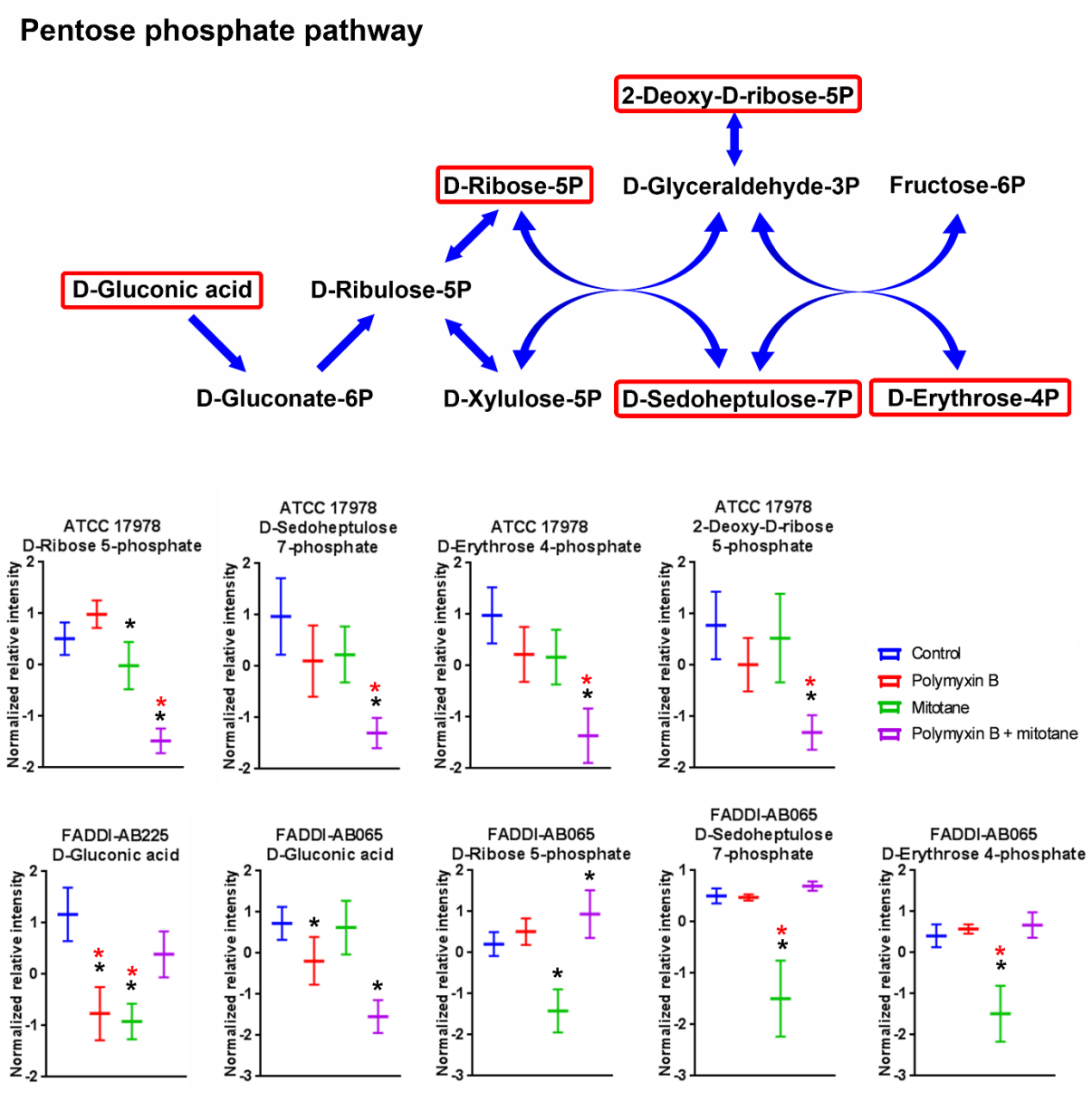

Figure 4.6 Metabolites in pentose phosphate pathway in *A. baumannii* significantly impacted by polymyxin B, mitotane, and the combination. Red boxes indicate statistically significant metabolites (Mean ± SD; * one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$; * $\log_2$ fold-change ≥ |1|).

4.4.4 Significantly impacted metabolites in the citric acid cycle

The impact of polymyxin B and mitotane on the citric acid cycle in *A. baumannii* is shown in **Figure 4.7**. In ATCC 17978, succinate was significantly reduced by both polymyxin B and mitotane monotherapies, however, the highest level of reduction was observed with combination treatment (Log_2FC = -2.49). In addition, the combination also caused a significant reduction in abundance in α-ketoglutarate and malate (Log_2FC = -2.01 and -1.47, respectively) in ATCC 17978. The abundance of malate was also reduced by combination treatment in FADDI-AB065 (Log_2FC = -0.82). Interestingly, in the same strain, malate abundance was increased by polymyxin B monotherapy (Log_2FC = 0.82).

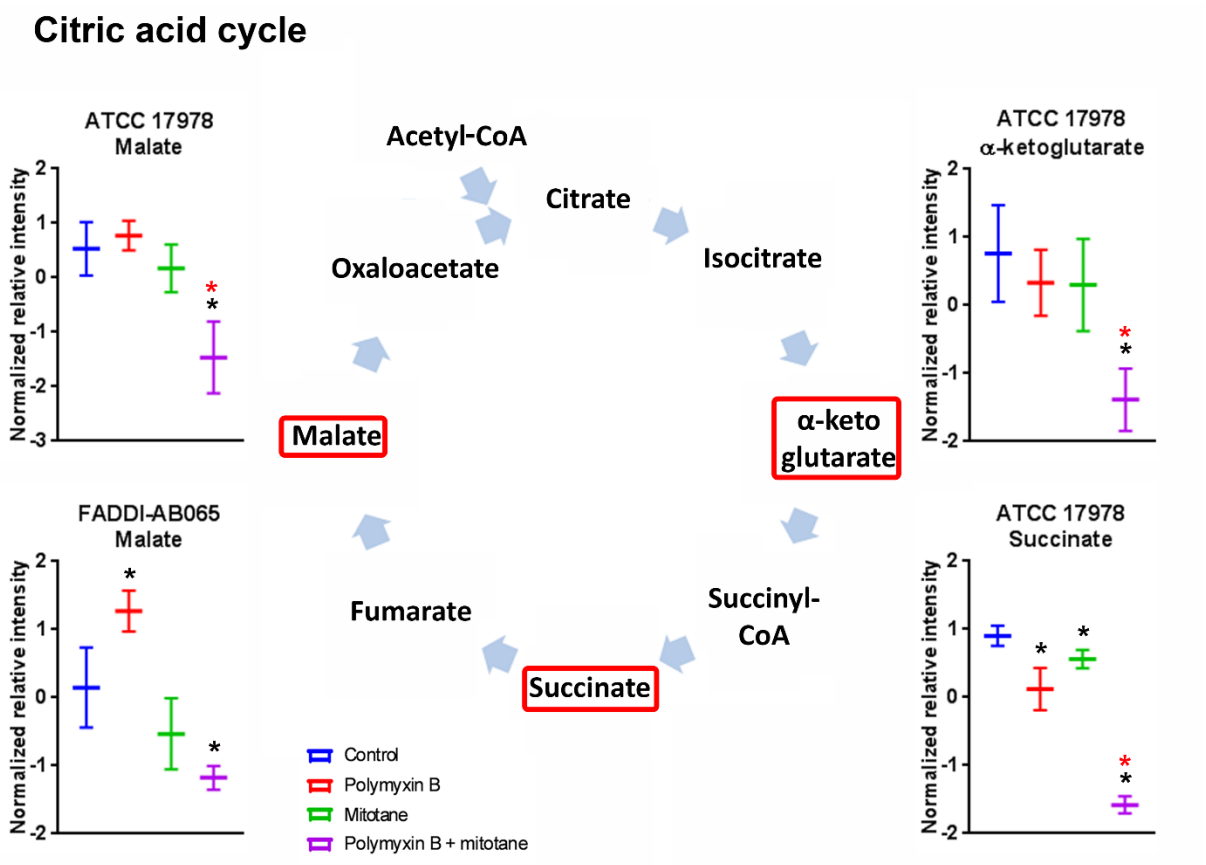

Figure 4.7 Metabolites in citric acid cycle in *A. baumannii* significantly impacted by polymyxin B, mitotane, and the combination. Red boxes indicate statistically significant metabolites (Mean ± SD; * one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$; * $\log_2$ fold-change ≥ |1|).

4.4.5 Significantly impacted metabolites in nucleotide metabolism

A high number of metabolites involved in nucleotide metabolism in *A. baumannii* were significantly impacted by treatment with polymyxin B and mitotane alone and in combination (**Tables A1.1 – A1.4**). In both ATCC 17978 and FADDI-AB065, the pyrimidine ribonucleotide biogenesis pathway was over represented (≥ 2 metabolites in the module affected) (**Figure 4.8**). In ATCC 17978, UMP was significantly reduced by both polymyxin B monotherapy and the combination (Log_2FC = -1.19 and -2.07, respectively); and the combination also reduced UDP and putative CDP (Log_2FC = -1.47 and -1.57, respectively). In FADDI-AB065, UDP was slightly reduced by mitotane monotherapy while it was increased by the combination therapy (Log_2FC = -0.04 and 0.87, respectively). Only the combination caused increases in UMP and putative CDP (Log_2FC = 1.74 and 1.33, respectively). A related pathway, guanine ribonucleotide biogenesis; was also over represented in ATCC 17978. In this pathway, GMP abundance was significantly reduced by polymyxin B monotherapy and the combination (Log_2FC = -1.28 and -3.22, respectively), with greater perturbation caused by the combination. Additionally, only the combination impacted putative xanthosine 5'-phosphate (XMP) and GDP (Log2FC = -1.40 and -0.05, respectively), with greater perturbation occurring for putative XMP.

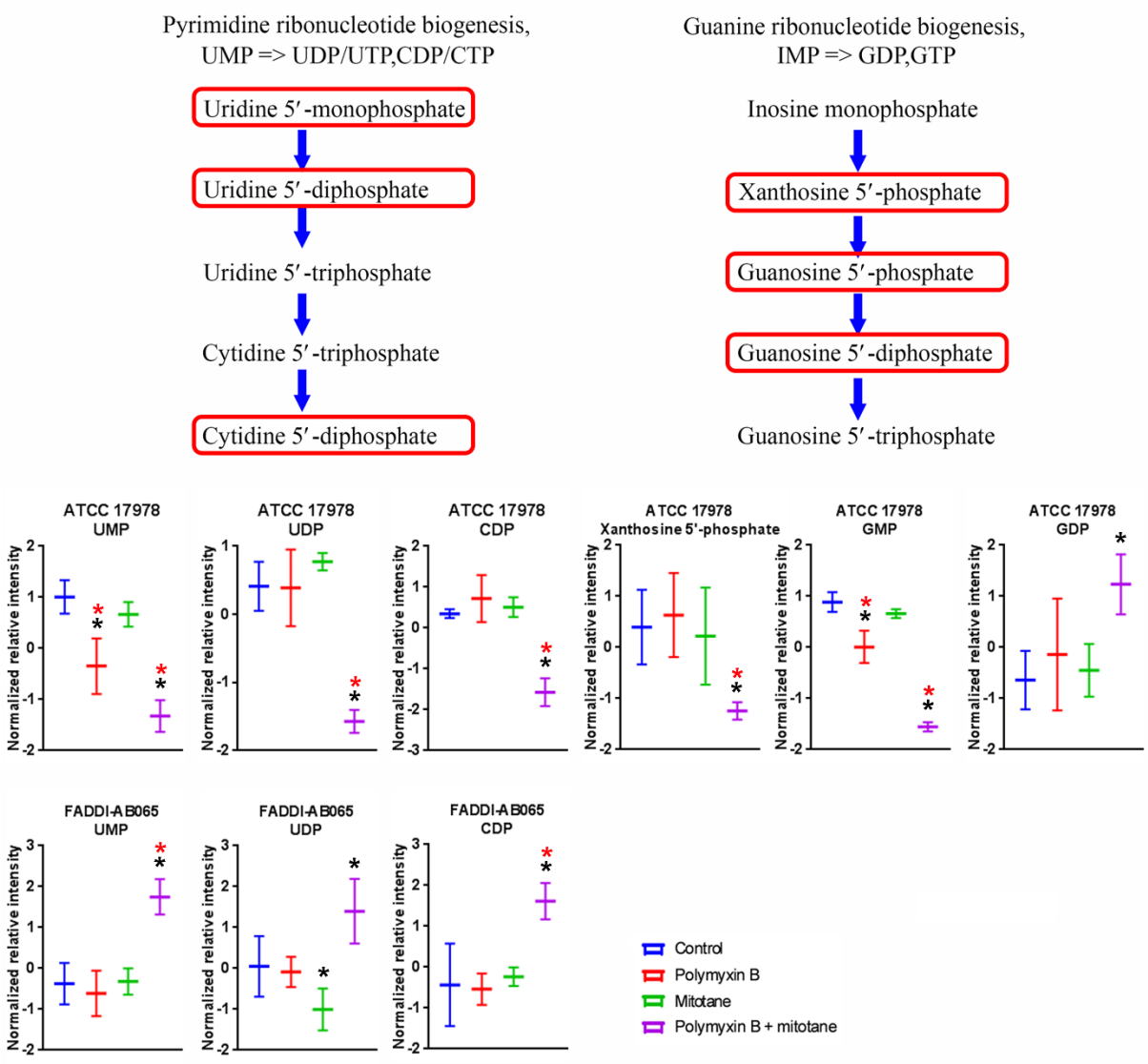

Figure 4.8 Metabolites in pyrimidine and guanine ribonucleotide biogenesis in *A. baumannii* significantly impacted by polymyxin B, mitotane, and the combination. Red boxes indicate statistically significant metabolites (Mean ± SD; * one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$; * $\log_2$ fold-change ≥ |1|).

4.4.6 Significantly impacted metabolites in amino acid metabolism

Treatment with polymyxin B and mitotane alone and in combination caused significant perturbations to a high number of metabolites involved in amino acid metabolism in *A. baumannii* (**Tables A1.1 – A1.4**). Across ATCC 17978, ATCC 19606 and FADDI-AB065, histidine degradation was over-represented (≥ 2 metabolites in the module affected) (**Figure 4.9).** In ATCC 17978, polymyxin B monotherapy and

combination treatment caused significant perturbations in putative urocanate (Log_2FC = 1.46 and 2.49, respectively), putative *N*-formimino-L-glutamate (Log_2FC = 1.27 and 2.29, respectively) and L-glutamate (Log_2FC = -1.25 and -4.44, respectively). The combination treatment, however, produced the highest level of perturbation in all three metabolites. In ATCC 19606, the intracellular concentration of putative urocanate was significantly increased by polymyxin B, mitotane, and combination treatment (Log_2FC = 0.11, 0.30 and 0.71, respectively), with the highest level of perturbation observed with the combination. Putative *N*-formimino-L-glutamate was significantly reduced by combination therapy (Log_2FC = -1.56). Interestingly, only mitotane monotherapy caused significant reduction in putative urocanate and putative *N*-formimino-L-glutamate in FADDI-AB065 (log_2FC = -1.13 and -1.75, respectively).

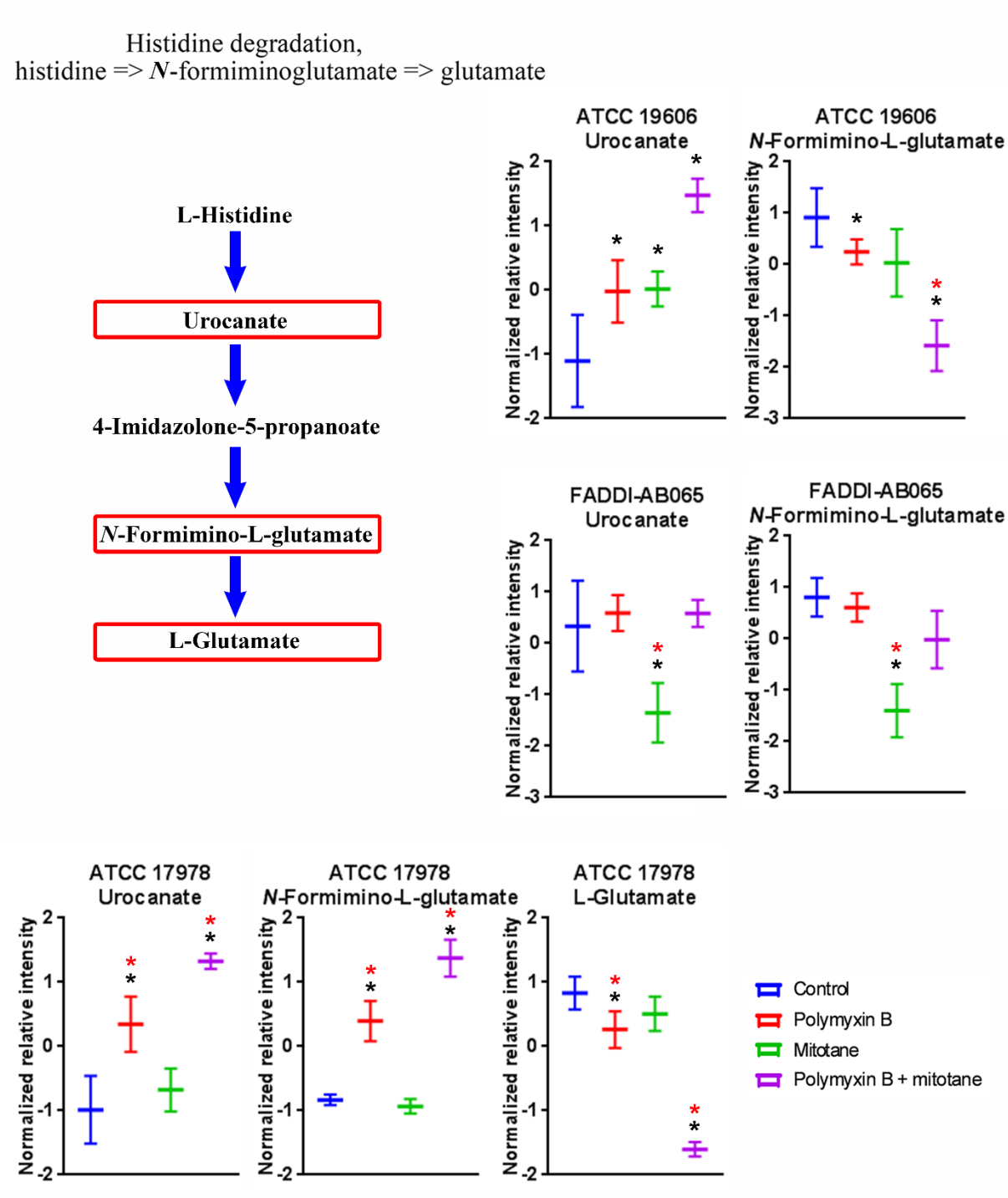

Figure 4.9 Metabolites in the histidine degradation pathway in *A. baumannii* significantly impacted by polymyxin B, mitotane, and the combination. Red boxes indicate statistically significant metabolites (Mean ± SD; * one-way ANOVA, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$; * $\log_2$ fold-change ≥ |1|).

4.5 Discussion

In recent years polymyxins have been revived as the agents of 'last resort' for treatment of MDR *A. baumannii* (81, 82). To improve the efficacy of polymyxins and prevent the emergence of polymyxin resistance, polymyxin combination therapy has been investigated against this organism (217, 259, 264, 265, 271). The combination of polymyxin B and the antineoplastic drug mitotane has been shown to produce synergistic bacterial killing and prevent polymyxin resistance in MDR *A. baumannii* in chapter 3. Mitotane is currently used for the treatment of adrenocortical carcinoma (ACC) where it is suspected to act by inhibiting the activity of sterol-O-acyl-transferase and inducing endoplasmic reticulum (ER) stress (344). However, the mechanism by which it acts to produce antimicrobial activity is unknown. Understanding the biochemical mechanism(s) by which polymyxin B and mitotane act synergistically against *A. baumannii* is essential for future repurposing of mitotane as an antimicrobial agent in combination with polymyxins. This report is the first to describe the potential biochemical mechanisms of action of this combination in *A. baumannii* using untargeted metabolomics.

To understand how polymyxin B and mitotane affect different polymyxin resistance mechanisms in *A. baumannii*, this study examined the impact of the combination against four *A. baumannii* strains: ATCC 19606 and its LPS-loss polymyxin-resistant derivative FADDI-AB065; and ATCC 17978 and its lipid A modified polymyxin-resistant derivative FADDI-AB225. To ensure the clinical relevance of these findings, the concentrations of polymyxin B (2 mg/L) and mitotane (4 mg/L) employed were within the clinically achievable range of concentrations of each agent (162, 348). A 2 h exposure to the antibiotics was selected for investigation as extensive bacterial killing normally occurs with polymyxins in *in vitro* studies across this time (131, 207, 373).

It is well established that polymyxins exert at least part of their antimicrobial activity through the disruption of the bacterial outer membrane (91, 93). Consequently, it was not unexpected that polymyxin B monotherapy impacted the membrane lipids of polymyxin-susceptible *A. baumannii* in the present study. Similar to a previous metabolomics study (106), pathway analysis revealed the

majority of the significantly perturbed metabolites caused by polymyxin B monotherapy were involved in fatty acid and glycerophospholipid metabolism (**Figures 4.4 and** 4.5). These findings were also in agreement with a previous transcriptomic study that showed *A. baumannii* altered the expression of genes that are primarily associated with outer membrane biogenesis, fatty acid metabolism and phospholipid trafficking after 1-h exposure to colistin (105). Promisingly, the combination caused substantial reduction of *sn*-glycero-3-phosphoethanolamine in polymyxin-susceptible and resistant strains while polymyxin B monotherapy only caused reduction of *sn*-glycero-3-phosphoethanolamine in the polymyxin-susceptible strain. This findings suggested that perturbation of *sn*-glycero-3-phosphoethanolamine in the polymyxin-resistant may be a bacterial killing mechanism of the combination.

In addition to the effect on the membrane lipids, pathway analysis also suggested that polymyxin B may affect the bacterial stress response through the degradation of L-histidine to L-glutamate. Since L-glutamate is an important metabolite involved in a wide range of bacterial metabolic processes including responses to acid and other stresses (374), a reduced level of L-glutamate can stifle the stress response and result in cell death. Furthermore, a low level of L-glutamate can also affect the level of L-proline, another contributor to stress survival (375, 376). In ATCC 17978, it is possible that polymyxin B monotherapy affected the enzymatic activity of urocanate reductase and imidazolonepropionase, which led to the accumulation of urocanate and *N*-formimino-L-glutamate and the subsequent reduction of L-glutamate (**Figure 4.9**). It is also possible that polymyxin B increased the activity of histidine ammonia-lyase in response to low glutamate level. Interestingly, polymyxin B caused a reduction in the concentration of *N*-formimino-L-glutamate in ATCC 19606, suggesting a different regulation of histidine in the two polymyxin-susceptible strains.

Remarkably, despite being a non-antibiotic, mitotane caused significant metabolic perturbation in the polymyxin-resistant strain lacking LPS (FADDI-AB065) (**Figure 4.1** and **Figure 4.2**). LPS is a key component of the outer membrane, a permeability barrier in Gram-negative bacteria (354); hence,

the loss of LPS likely enables hydrophobic mitotane to cross the outer membrane and access its intracellular target(s). Surprisingly, pathway analysis showed that mitotane monotherapy also affected glycerophospholipid metabolism and the histidine degradation pathway in the LPS-deficient strain. The significant reduction in *sn*-glycero-3-phosphoethanolamine and increase in acetylcholine suggests a possible interaction of mitotane with glycerophospholipid metabolism. Unlike that observed with polymyxin B, the levels of both uroconate and *N*-formimino-L-glutamate of the histidine degradation pathway were significantly reduced by mitotane monotherapy (**Figure 4.9**). It is possible that mitotane upregulated the histidine degradation pathway to produce additional essential L-glutamate for stress response (374).

Apart from its potential impact on membrane structure and the bacterial stress response, mitotane monotherapy also affected the pentose phosphate pathway in FADDI-AB065 (**Figure 4.6**). The pentose phosphate pathway is responsible for the production of NADPH during the oxidative phase and ribose during the non-oxidative phase, which are essential products for anabolic reactions and DNA/RNA synthesis, respectively (377, 378). As the metabolites affected by mitotane in FADDI-AB065 are involved in the non-oxidative phase of pentose phosphate pathway, it is likely that mitotane also affects DNA/RNA synthesis in *A. baumannii*. A previous study also identified that the pentose phosphate pathway was perturbed in *A. baumannii* by colistin monotherapy (106).

Despite enhanced bacterial killing observed with the polymyxin B/mitotane combination against polymyxin-resistant *A. baumannii* FADDI-AB225 in the previous study from chapter 3, only a minor number of metabolites in this strain were affected by all treatments. This narrow response to all treatments is likely because of the 100-fold higher inoculum used in the present metabolomics study. A higher inoculum was employed for metabolomics investigations to ensure the observed responses arose from the stress caused by antibiotic treatment and not from extensive bacterial killing. Previous studies have shown a pronounced inoculum effect for polymyxins, with reduced bacterial killing

observed at higher inocula (211). Similarly, the high inoculum used in this study might have decreased the antimicrobial activity of polymyxin B monotherapy and the combination treatment.

In combination, the findings showed polymyxin B and mitotane additionally affected the citric acid cycle and nucleotide metabolism. Given the significant reduction of the majority of the metabolites involved in the citric acid cycle, it is highly likely that the combination compromised energy production in *A. baumannii*. In relation to nucleotide metabolism, the findings were in line with the proposed effect of mitotane on RNA/DNA synthesis through perturbation of the pentose phosphate pathway. In addition to the observed effect of mitotane on the non-oxidative phase of pentose phosphate pathway, the combination also impacted the oxidative phase of pentose phosphate pathway. As this oxidative phase produces NADPH, the additional impact of the combination on the pentose phosphate pathway suggests a possible inhibition of fatty acid synthesis and membrane remodeling. Although polymyxin B and mitotane alone affected lipid metabolism, histidine degradation, and the pentose phosphate pathway, the combination showed superior perturbation of metabolites in most cases. Surprisingly, metabolites perturbed by the combination varied between different *A. baumannii* strains. This finding indicates that the responses to antibiotic treatment by bacteria are highly dynamic, even within the same species.

For antibiotic combination therapies, several models have been proposed to describe the mechanism of synergism, notably subpopulation synergy and mechanistic synergy (251, 252). Subpopulation synergy refers to the killing of the resistant subpopulations of one drug by the second drug, while mechanistic synergy refers to the targeting of different cellular pathways of each drug (252). From the present study, it is likely that the synergism of polymyxin B and mitotane is a result of both mechanisms. Since FADDI-AB065 is a polymyxin-resistant derivative of ATCC 19606 following colistin monotherapy (128), FADDI-AB065 represents a polymyxin-resistant subpopulation of ATCC 19606. Consequently, the susceptibility of FADDI-AB065 to mitotane monotherapy supports the subpopulation synergy model. Mechanistic synergy is suggested since much higher metabolic

perturbation was observed with combination therapy compared to polymyxin B and mitotane monotherapies. Additionally, given the lack of activity of mitotane alone compared to the enhanced activity in combination with polymyxin B, it is likely that bioavailability synergy, which refers to the increased intracellular availability of one drug due to the action of a second drug (379), contributed to the enhanced activity observed.

4.6 Conclusions

The present study is the first to investigate the synergistic killing mechanism of polymyxin B and mitotane in combination against *A. baumannii*. In addition to effects on lipid metabolism pathways identified in a previous metabolomic study (106), the histidine degradation pathway has been shown to be impacted by polymyxin B monotherapy. As monotherapy, mitotane impacted lipid metabolism, histidine degradation and the pentose phosphate pathway in an *A. baumannii* strain lacking LPS. In combination, citric acid cycle and nucleotide metabolism were impacted by the combination in all strains. The novel finding from this study is that polymyxin B treatment per se causes significant metabolic perturbations via the disorganization of cellular lipids and amino acid metabolism, specifically histidine degradation, all of which were further enhanced by mitotane leading ultimately to the depletion of nucleotides. Collectively, these findings suggest that the synergy between polymyxin B and mitotane results from a combination of subpopulation, mechanistic, and bioavailability synergy. This study provides valuable mechanistic insight into the synergistic antibacterial killing of polymyxin B and mitotane combinations, and is useful for the potential repositioning of mitotane for an antimicrobial indication in combination with polymyxins. Further studies are warranted to examine other non-antibiotic drugs as antimicrobial agents in combination with the outer-membrane permeabilizing polymyxins.

CHAPTER FIVE: INTERACTIONS BETWEEN A549 LUNG EPITHELIAL CELLS, *A. BAUMANNII* AND POLYMYXIN B: A TRANSCRIPTOMIC APPROACH

5.1 Abstract

A. baumannii is a major bacterial 'superbug' currently presenting a global health challenge. This highly antibiotic-resistant bacterium can cause life-threatening pneumonia in critically-ill patients. The present transcriptomics study investigated how *A. baumannii* and human lung epithelial cells interact at the molecular level when concomitantly exposed to polymyxin B. Simultaneous transcriptional profiling of A549 cells and *A. baumannii* ATCC 19606 from a host-pathogen-drug interaction was conducted with microarray and RNAseq, respectively. In A549 cells, *A. baumannii* caused upregulation of many inflammatory and immune responses and many potent proinflamatory cytokines, whereas treatment of the cells with 2 mg/L of polymyxin B alone did not induce significant transcriptional changes. In *A. baumannii*, A549 cells alone caused notable up-regulation of arginine and tyrosine degradation pathways, which are important for acid tolerance and energy production, respectively, and down-regulation of siderophore biosynthesis. Exposure to polymyxin B alone caused notable up-regulation of lipoprotein transport and, interestingly, the tightly-regulated histidine degradation pathway. Polymyxin B treatment also caused significant down-regulation of siderophore biosynthesis. Individually, putative *rcnB*, which involves in nickel/cobalt homeostasis, was the highest up-regulated gene caused by polymyxin B treatment in *A. baumannii* with and without the presence of A549 cells. Time-kill studies showed that an *rcnB* mutant was more susceptible to polymyxin B, while population analysis profiles (PAPs) revealed the same mutant treated with polymyxin B monotherapy led to a highly polymyxin-resistant mutant. The collective findings suggest that that arginine and tyrosine degradation in *A. baumannii* are major pathways involved in its infection against A549, and histidine degradation and nickel/cobalt homeostasis are potential novel pathways involved in polymyxin resistance in *A. baumannii*. This study showed that a systems approach for the host-pathogen-drug

interactions can enhance understanding of the progression of bacterial infection in the presence of antibiotics and is crucial for optimizing antibiotic use.

5.2 Introduction

A. baumannii are currently recognised as the one of the most medically important pathogens worldwide due to the resistance to multiple classes of antibiotics and the increasing incidence of infections caused by MDR isolates (29, 380). The majority of infections caused by this pathogen, in particular pneumonia, are in hospitalised patients (68, 380). Infections caused by *A. baumannii* are generally difficult to treat as the majority of *A. baumannii* isolates are MDR (28, 380). Due to the limited availability of effective antibiotics, polymyxins are often used as the last defense for the treatment of infections caused by these pathogens (81, 82). Worryingly, incidences of polymyxin resistance have emerged in *A. baumannii* following polymyxin therapies (311, 341, 381). As polymyxins are considered the last-line antibiotics for these bacteria (81), it is critical to preserve their utility and prevent the development of resistance.

Effective usage of antibiotics is necessary for successful prevention of antibiotic resistance. This can be achieved through better understanding of the host-pathogen interaction at the molecular level during infection. An example of this includes using the knowledge of the differences between a viral and a bacterial infection to decide whether or not to use antibiotics for treatment of pneumonia infections (382, 383). Indeed, until recently antibiotics are often used empirically for signs of pneumonia regardless of the causes (383-385). To overcome this problem, a recent study measured the global patterns of the host response to infection and identified interferon-induced protein 27 as a potential immune biomarker to discriminate patients with influenza infection from patients with bacterial infections (382). Likewise, understanding the bacteria global response during infection can be useful for the selection of more appropriate antibiotics and can potentially assist with the development of novel antimicrobial combination therapies through a more targeted approach. In this study, I aimed to understand the global responses of the host and pathogen during *Acinetobacter* pneumonia as well as how they respond to polymyxin B treatment.

5.3 Materials and methods

5.3.1 Bacterial isolates, cell culture, and antibiotic

The *A. baumannii* ATCC 19606 (polymyxin B MIC = 0.5 mg/L) used for the interaction study was obtained from the American Type Culture Collection (Rockville, MD, USA). This isolate was stored in tryptone soy broth (Oxoid) with 20% glycerol (Ajax Finechem, Seven Hills, NSW, Australia) in cryovials at -80°C and sub-cultured to nutrient agar plates (Media Preparation Unit, University of Melbourne, Melbourne, VIC, Australia) before use.

A. baumannii AB5075 and its *rcnB* mutant used for validation of the bacterial transcriptomic findings were obtained from the transposon insertion mutant library of the University of Washington (386). Before the experiment, *A. baumannii* AB5075 was sub-cultured onto plain lysogeny broth (LB; Thermo Fisher, Australia) agar while transposon inserted mutants were sub-cultured onto LB agar containing 10 mg/L of tetracycline to maintain selection pressure.

A549 cells (adenocarcinomic human alveolar basal epithelial cells) used for the interaction study were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were stored in liquid nitrogen and 1.5-2 × 10^6 cells were passaged every 2-3 days in T75 flasks (Corning, Sigma-Aldrich, NSW, Australia) with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen, Life Technologies, Victoria, Australia). The cell cultures were incubated at 37°C in a humidified atmosphere containing 5% CO_2. At the end of day 2-3, when the cell monolayer was around 90% confluence, the cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4; Invitrogen) and striped from the T75 flask by incubating with 2 mL of 0.25% Trypsin/0.53 mM EDTA (Thermo Fisher, Australia) at 37°C for around 5 min. Trypan Blue was used to stain the dead cells and the number of viable cells was estimated under a phase-contrast microscope.

Polymyxin B (Beta Pharma, China; Batch number 20120204) solutions were prepared in Milli-Q water (Millipore, North Ryde, Australia) and sterilised by passage through a 0.20-µm cellulose acetate

syringe filter (Millipore, Bedford, MA, USA). Stock solutions (1 mg/mL) were stored at -20°C for no longer than 1 month.

5.3.2 Host-pathogen-drug interaction model

To prepare the bacterial suspension for the interaction study, single colonies from a fresh culture of *A. baumannii* ATCC 19606 on nutrient agar plate were initially used to inoculate 20 mL of CAMHB in 50 mL Falcon tubes (Thermo Fisher, Australia). The CAMHB with the bacterial inoculation was then incubated overnight in a shaking water bath at 37°C (shaking speed, 180 rpm). On the next day, the broth culture was transferred to 20 mL of DMEM with 10% FBS at ~50 folds dilution and incubated at 37°C for 3-4 h to generate a log-phase culture (~0.55 McFarland; ~10^8 cfu/mL). Finally, the log-phase culture was diluted by two folds with fresh DMEM and 10% FBS.

To prepare the host epithelial cells for the interaction study, A549 cells (0.5-1×10^6 cells/well) were seeded in 6-well plates (Corning Costar, Sigma-Aldrich, Australia) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 for ~24 h to generate a cell monolayer with ~1×10^6 cell/well. At 24 h, the medium was removed and the cells were washed twice with phosphate-buffered saline (PBS) (pH 7.4; Invitrogen). To achieve a multiplicity of infection (MOI) 1:100, each well of cells was inoculated with 2 mL of bacterial suspension. To examine the responses of the host cells and *A. baumannii* to one another in the presence of 2 mg/L of polymyxin B, 4 µL of polymyxin B stock solution was also added to the co-culture system. To investigate the responses of the host cells to 2 mg/L of polymyxin B, each well of cells was inoculated with 2 mL of DMEM and 10% FBS and 4 µL of polymyxin B stock solution. To test the separate responses of *A. baumannii* to 2 mg/L of polymyxin B, the bacterial suspension (2 mL) was transferred to a blank well and 4 µL of polymyxin B stock solution was added. For the host cells control group, each well of cells was inoculated with 2 mL of DMEM and 10% FBS only. For the *A. baumannii* control group, only the bacterial suspension (2 mL) was transferred to a blank well. All cultures were subsequently incubated at 37°C in a humidified atmosphere containing 5% CO_2 for a further 2 h. Three biological replicates were prepared in total for each condition.

5.3.3 RNA samples preparation

The procedure for the extraction of the host and the bacterial RNA for transcriptomic investigation was adapted from an online protocol (387), which was a combination of Trizol (Ambion, Life Technologies, Australia) and RNeasy mini kit (Qiagen, Victoria, Australia). Briefly, A549 cells (with or without *A. baumannii*) were rapidly washed twice with cold PBS and 500 µL of Trizol was added for 5 min at room temperature. For *A. baumannii* without A549 cells, the cultures were centrifuged at 5000 × *g* for 5 min, the supernatant was discarded and 500 µL of Trizol was added to the bacterial pellet for 5 min at room temperature. To each sample, 100 µL of chloroform was subsequently added for a further 2 min at room temperature. Samples were centrifuged at 12000 × *g* for 15 min at 4 °C and the transparent upper phase (~300 µL) was transferred to a new Eppendorf tube. Equal volume (300 µL) of 70 % ethanol was added and mixed and the entire volume was transferred to an RNeasy Mini spin column. The content was spun down for 15 sec at 8000 × *g* and the flow-through was discarded. Buffer RW1 (700 µL) was added to the column and again spun down for 15 sec. Buffer RPE (500 µL) was added to the column and the previous step was repeated. Buffer RPE (500 µL) was added to the column and the column was spun down for 1 min. Once the flow-through was discarded, the column was again spun down for an additional 2 min at max speed (~18000 × *g*). The column was placed in a 1.5 mL Eppendorf tube and the RNA was eluted with 30 µL of RNase-free water by max speed centrifugation for 1 min.

To minimise the interference of the host RNA during the quantification step of the bacterial RNA, MicrobEnrich kit (Ambion, Thermo Fisher, Australia) was used to reduce the amount of human RNA (388). The procedure was performed according to the product handbook. Briefly, the RNA mixture was precipitated overnight at -20°C in 0.1 volume 5 M ammonium acetate and 3 volumes of 100% ethanol. The RNA sample was recovered by centrifugation, washed with cold 70% ethanol, re-pelleted and dissolved in 30 µL of TE (10 mM Tris-HCl pH 8, 1 mM EDTA) buffer. Binding buffer and Capture Oligo Mix was added to the RNA sample, heated to 70°C for 10 min, and incubated at 37°C for 1 h to

anneal with the mammalian RNA. Oligo MagBeads was then added to capture the annealed mammalian RNA and a magnetic stand was used to separate the enriched bacterial RNA from the mammalian RNA containing Oligo MagBeads. The enriched bacterial RNA was subsequently precipitated with cold 100% ethanol and resuspended in TE buffer.

5.3.4 Quantification of mRNA

The quantification of the host mRNA was provided by the Monash Health Translation Precinct (MHTP) Medical Genomic Facility with Agilent microarray using the Human Gene Expression v3. Briefly, Cyanine-3 (Cy3) labelled cRNA was prepared using the One-Color Low input Quick Amp labelling Kit (Agilent, Victoria, Australia) followed by RNeasy column purification. The Cy3 labeled cRNA was fragmented and hybridised with the Human Gene Expression v3 for 17 h at 65°C. After washings, the slides were scanned with DNA microarray scanner using one color scan settings for 8x60k array slides. The scanned images were analysed with Feature Extraction Software 11.0.1.1 (Agilent) using default parameters (protocol GE1-1100_Jul11 and Grid: (072363_D_F_20150612).

The quantification of the bacterial mRNA was provided by the MHTP Medical Genomic Facility with RNA sequencing using Illumina HiSeq1500. Briefly, ribosomal RNA (rRNA) was removed with RiboZero kit (Epicentre) and the cDNA library was constructed TruSeq Stranded Total RNA Library Prep Kit (Illumina). Single-read 100 bp sequences were generated on llumina HiSeq1500. To compensate for the low ratio of bacterial RNA to host RNA, a total of 25 million reads were generated for each sample.

5.3.5 Transcriptomic data analysis

The microarray data for genes expression of A549 cells was analysed using the interaction models: 2 × 2 factorial designs described by the R limma method (388) with the two factors being infection (i.e. exposure to *A. baumannii*) and treatment (i.e. exposure to polymyxin B). Briefly, all intensities were corrected with the background, intensities between arrays were normalised using the quantile method, and the average values were recorded for each group (389-391). Statistical significance of

differential gene expression was computed using the F-statistic with Benjamini Hochberg adjustment to control the false discovery rate (FDR). Differential gene expression was defined with the cutoff as Fold-change (FC) ≥ 2 and FDR ≤ 0.05. Differentially expressed genes containing the UniGene identifiers were analysed with the functional annotation tool from the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 for enrichment in Gene Ontology (GO) terms and KEGG pathways.

The RNA sequencing data for genes expression of *A. baumannii* was aligned to 19606 genome obtained from GenBank (GCA_000369385.1) using SubRead (392) with default settings. The counts of mapped reads were summarised by featureCounts (393). Data frame was filtered to contain transcripts with a minimum of 10 reads and normalised based on the size of the library. Differential gene expression were identified with Voom and Limma packages (388, 390, 394) using the 2 × 2 factorial design. Statistical significance of differential gene expression was computed using the F-statistic with Benjamini Hochberg adjustment to control the FDR. InterProScan was used to assign functions and GO terms to significant proteins sequences and BlastKOALA was used for the assignment of K numbers. Enrichment analysis of GO terms and KEGG pathways were conducted using Fisher's exact test ($p \leq 0.05$) and Benjamini Hochberg adjustment (FDR ≤ 0.05) to determine significance. Similarly, Biocyc was used to determine the enriched pathways of the differentially expressed genes.

5.3.6 Cytokines studies

To determine the effect of *A. baumannii* as well as polymyxin B on the cytokines activities of the host respiratory epithelial cells, the supernatant of all cultures from the interaction study after 2 h incubation was screened with the Human toll-like receptor (TLR) induced Cytokines II: Microbial-induced Multi-Analyte ELISArray Kit (Qiagen, VIC, Australia; Product no. 336161, Cat. no. MEH-008A). The kit consisted of 12 TLR-induced cytokines, which included tumor necrosis factor alpha (TNFα), interleukin-1-beta (IL-1β), interleukin-6 (IL-6), interleukin-12 (IL-12), interleukin-17A (IL-17A), interleukin-8 (IL-8 or CXCL8), monocyte chemoattractant protein-1 (MCP-1 or CCL2), Regulated on

Activation Normal T Cell Expressed and Secreted (RANTES), macrophage inflammatory protein 1 alpha (MIP-1α orCCL3), macrophage inflammatory protein 1 beta (MIP-1 β or CCL4), macrophage-derived chemokine (MDC/ CCL22), and Eotaxin (CCL11). The procedure was conducted according to the product handbook. Briefly, samples and control samples were added to appropriate wells and incubated at 37°C for 2 h. The plate was washed three times and detection antibody solution was added and incubated for 1 h. Avidin-horseradish peroxidase was then added following washings and incubated for 30 min. The plate was again washed and development solution was added and incubated in the dark for 15 min. Finally, stop solution was added and the optical density at 450 nm was recorded within 30 min.

5.3.7 Time-kill studies

Time kill assays were performed according to method previously described in **Section 2.3.5**. Briefly, *A. baumannii* AB5075 and its *rcnB* mutant derivative were grown overnight on nutrient agar plates (Media Preparation Unit). Single colonies were used to prepare an overnight broth cultures in CAMHB. The overnight broth cultures were dilute 100-fold in fresh CAMHB and incubated at 37°C in shaking water bath for approx. 3.5 h to create log-phase cultures. Polymyxin B stock solution was added to achieve a concentration of 0.5 mg/L. Serial samples (0.5 mL) were removed aseptically at 0, 1, 2, 4, 6 and 24 h and plated on nutrient agar plates for viable-cell counting.

5.3.8 Polymyxin population analysis profiles (PAPs) at baseline and 24 h following treatment with polymyxin B

PAPs assays were performed according to a previously described method (223). Briefly, AB5075 and the *rcnB* mutant at baseline and 24 h following treatment with polymyxin B were used to create cell suspensions (2 mL) of ~10^8 cfu/mL in 0.9% saline. The bacterial suspensions were serially diluted by 10-fold and plated onto Mueller-Hinton agar plates (Media Preparation Unit, University of Melbourne, Parkville, Australia) containing polymyxin B (0, 0.25, 0.5, 1, 2, 4 and 8 mg/L) using an automatic spiral

plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted after 24 h incubation at 37°C using a ProtoCOL colony counter (Synbiosis, Cambridge, UK).

5.4 Results

5.4.1 Differentially expressed genes in A549 cells in response to *A. baumannii* exposure, polymyxin B treatment, and both simultaneously

The total number of differentially expressed genes (DEGs) in A549 cells at 2 h post exposure to *A. baumannii*, polymyxin B (2 mg/L), or both and their overlaps are shown in **Figure 5.1**. Exposure to *A. baumannii* alone resulted in 685 significant differentially expressed genes in A549 cells (641 up-regulated and 44 down-regulated genes); these genes are listed in the Appendix 2 (**Table A2.1**). Similarly, 566 genes were differentially expressed (540 up-regulated and 26 down-regulated genes) following exposure to both *A. baumannii* and polymyxin B (see Appendix 2, **Table A2.2**); the majority of these (~90%) were in common with the DEGs in response to *A. baumannii* alone. No significant differentially expressed gene was observed when the A549 cells were exposed to 2 mg/L of polymyxin B alone (**Figure 5.1**).

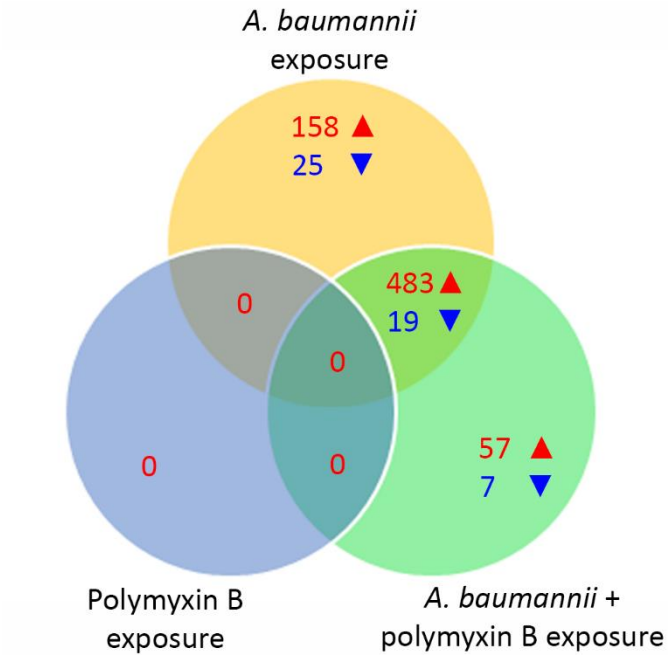

Figure 5.1 Venn diagram showing the total number and the overlap of differentially expressed genes at 2 h in A549 cells responding to *A. baumannii* ATCC 19606 infection, polymyxin B (2 mg/L) treatment, or both.

Enrichment analysis using the up-regulated genes of A549 cells exposed to *A. baumannii* alone showed a total of 40 GO terms KEGG pathways (see Appendix 2, **Table A2.3**). By using only the 10% of genes with the highest fold increase, the enriched GO biological processes were narrowed down to inflammatory response, immune response, cellular response to lipopolysaccharide, positive regulation of neutrophil chemotaxis, positive regulation of nitric oxide biosynthetic process, and chemokine-mediated signaling pathway. The two enriched GO molecular functions were subsequently identified to be chemokine activity and cytokine activity and the two GO cellular components were identified as extracellular space and extracellular region. Eight enriched KEGG pathways were identified and the most statistically significant pathway was TNF signaling pathway (**Table 5.1**).

Table 5.1 List of enriched GO terms, KEGG pathways and their relative genes identified in the 10% of genes with the highest fold increase in A549 cells infected with *A. baumannii* ATCC 19606.

GO term/KEGG pathway	Gene symbol	Fold Enrichment	FDR
GO biological process			
GO:0006954~inflammatory response	IL6, NFKBIZ, NFKBID, TNFAIP3, IL1A, CXCL2, TNF, CCL20, PTX3, CXCL3, CXCL1, IL1B, CXCL8	18.99	8.99E-10
GO:0006955~immune response	IL6, CCL20, CSF2, LTB, CXCL3, CXCL1, IL1A, CXCL2, TNF, IL1B, CXCL8	14.66	1.66E-06
GO:0071222~cellular response to lipopolysaccharide	IL6, CCL20, CSF2, TNFAIP3, ICAM1, TNF, CXCL8	30.60	1.05E-04
GO:0090023~positive regulation of neutrophil chemotaxis	CXCL3, CXCL1, CXCL2, IL1B, CXCL8	116.58	1.09E-04
GO:0045429~positive regulation of nitric oxide biosynthetic process	IL6, PTX3, ICAM1, TNF, IL1B	62.17	1.49E-03
GO:0070098~chemokine-mediated signalling pathway	CCL20, CXCL3, CXCL1, CXCL2, CXCL8	39.41	9.44E-03
GO molecular function			
GO:0008009~chemokine activity	CCL20, CXCL3, CXCL1, CXCL2, CXCL8	53.70	1.97E-03
GO:0005125~cytokine activity	IL6, CSF2, LTB, IL1A, TNF, IL1B	17.94	1.67E-02
GO cellular component			
GO:0005615~extracellular space	IL6, CSF2, IL1A, ICAM4, CXCL2, TNF, CCL20, PTX3, LTB, ANGPTL4, CXCL3, STC2, HS.658118, CXCL1, ICAM1, IL1B, CXCL8	7.33	4.19E-08
GO:0005576~extracellular region	IL6, CSF2, IL1A, CXCL2, TNF, CCL20, PTX3, ANGPTL4, CXCL3, EFNA1, CXCL1, STC2, IL1B, CXCL8	5.12	4.84E-04

KEGG pathway			
hsa04668:TNF signalling pathway	IL6, CCL20, CSF2, CXCL3, CXCL1, TNFAIP3, ICAM1, CXCL2, TNF, IL1B	29.63	8.95E-09
hsa05323:Rheumatoid arthritis	IL6, CCL20, CSF2, LTB, IL1A, ICAM1, TNF, IL1B, CXCL8	32.12	9.47E-08
hsa05132:Salmonella infection	IL6, CSF2, CXCL3, CXCL1, IL1A, CXCL2, IL1B, CXCL8	30.27	3.01E-06
hsa05134:Legionellosis	IL6, CXCL3, CXCL1, CXCL2, TNF, IL1B, CXCL8	40.72	9.03E-06
hsa04060:Cytokine-cytokine receptor interaction	IL6, CCL20, CSF2, LTB, IL1A, TNF, IL1B, CXCL8	10.92	3.43E-03
hsa04064:NF-kappa B signalling pathway	LTB, TNFAIP3, ICAM1, TNF, IL1B, CXCL8	21.66	5.20E-03
hsa05144:Malaria	IL6, ICAM1, TNF, IL1B, CXCL8	32.05	1.30E-02
hsa04621:NOD-like receptor signalling pathway	IL6, TNFAIP3, TNF, IL1B, CXCL8	28.55	2.07E-02

Among the up-regulated genes, the gene with the highest fold increase was CCL20 (chemokine (C-C motif) ligand 20) [$log_2FC = 6.67$, FDR = 1.59E-06], which encodes for a small chemotactic cytokine that regulates the migration of immune cells to the infection sites. Other highly up-regulated genes encoding for chemokines were CXCL8 (chemokine (C-X-C motif) ligand 8) [$log_2FC = 5.58$, FDR = 5.02E-07], CXCL3 [$log_2FC = 4.85$, FDR = 1.87E-06], CXCL2 [$log_2FC = 4.61$, FDR = 6.06E-07], and CXCL1 [$log_2FC = 4.36$, FDR = 5.12E-06]. Apart from the chemotactic cytokines, other notable up-regulated genes included CSF2 (colony stimulating factor 2) [$log_2FC = 4.99$, FDR = 8.18E-06], which controls the production, differentiation, and function of granulocytes and macrophages, TNF (tumour necrosis factor, regulating a wide spectrum of biological processes (395)) [$log_2FC = 4.73$, FDR = 6.73E-07], and ICAM4 (intercellular adhesion molecule 4, involved in inflammation, immune responses and in

intracellular signalling events (396)) [log_2FC = 4.97, FDR = 9.01E-05] and ICAM1 [log_2FC = 4.54, FDR = 2.00E-05].

GO term enrichment analysis was conducted with all the down-regulated genes containing the UniGene identifiers in A549 cells exposed to *A. baumannii* alone. The enrichment identified protein refolding as the significant GO biological process [Fold enrichment = 159.88, FDR = 2.41E-03], cadherin binding involved in cell-cell adhesion as the significant GO molecular function [Fold enrichment = 13.13, FDR = 1.11E-02], and cell-cell adherens junction as the significant GO cellular component [Fold enrichment = 14.09, FDR = 6.96E-03]. Interestingly, KEGG pathway enrichment analysis identified the Epstein-Barr virus infection pathway to be significant [Fold enrichment = 12.84, FDR = 4.86E-02]. The gene with the highest fold decrease was HSPA1B (heat shock 70kDa protein 1B) [log_2FC = -3.34, FDR = 3.43E-05], a class of proteins that stabilises existing proteins against aggregation and mediates the folding of newly translated proteins (397). Other identified down-regulated genes encoding for heat shock proteins were HSPA1A [log_2FC = -3.25, FDR = 3.02E-05], HSPA8 [log_2FC = -1.63, FDR = 5.10E-04], and HSPA2 [log_2FC = -1.15, FDR = 1.20E-04].

For the differentially expressed genes of A549 cells exposed to both *A. baumannii* and 2 mg/L of polymyxin B, the up-regulated genes were enriched for a total of 33 GO terms and KEGG pathways similar to the response of A549 cells to *A. baumannii* alone (see Appendix 2, **Table A2.4**). Likewise, when only the 10% of genes with the highest fold increase were considered, similar enriched GO terms and KEGG pathways were identified for A549 cells exposed to both *A. baumannii* and 2 mg/L of polymyxin B compared to A549 cells to *A. baumannii* alone (see Appendix 2, **Table A2.5**). Unsurprisingly, the list of highly up-regulated genes was similar with the responses of A549 cells to *A. baumannii* alone, with CCL20 again the most up-regulated gene [log_2FC = 6.63, FDR = 1.61E-06]. The down-regulated genes were significantly enriched in protein refolding [FDR = 2.83E-04] and negative regulation of inclusion body assembly [FDR = 3.90E-02] for GO biological process, C3HC4-type RING finger domain binding [FDR = 1.30E-02] for GO molecular function, and legionellosis [FDR = 2.00E-02]

and Epstein-Barr virus infection [FDR = 2.90E-02] for KEGG pathway. No GO cellular component was significantly enriched. HSPA1B [log_2FC = -3.41, FDR = 3.38E-05] and HSPA1A [log_2FC = -3.27, FDR = 3.18E-05] were the two genes with the highest fold decrease.

5.4.2 TLR-induced cytokines production

To investigate the effect of *A. baumannii*, polymyxin B, or both on the production of TLR-induced cytokines in A549 cells, an ELISA panel consisting of 12 human TLR-induced cytokines was used to screen all exposure conditions. The ELISA results are shown in **Table 5.2**. Exposure to *A. baumannii* resulted in the production of 3 TLR-induced cytokines. The detected cytokine with the highest concentration observed was Interleukin-8 (IL-8/CXCL8), which is responsible for recruiting neutrophils and other granulocytes to the site of infection and inducing phagocytosis of foreign objects (398). Monocyte Chemotactic Protein-1 (MCP-1/CCL2), which recruits monocytes, memory T cells, and dendritic cells to the sites of inflammation, had the second highest observed concentration (398). The cytokine with the lowest detected concentration was Regulated on Activation, Normal T Cell Expressed and Secreted protein (RANTES/CCL5), which plays an active role in recruiting leukocytes, including T cells, eosinophils, and basophil, into inflammatory sites (398). The absorbance value of RANTES/CCL5, however, was slightly below the recommended detection limit of the ELISA kit, which is less than two times the negative control absorbance value. Interestingly, IL-8 and MCP-1 were also detected in A549 cells in the absence of *A. baumannii,* but only at much lower concentrations. Exposure to 2 mg/L of polymyxin B alone did not cause significant production of TLR-induced cytokines in A549 cells.

Table 5.2 Effect of *A. baumannii* ATCC 19606 and polymyxin B on 12 TLR-induced cytokines in A549 cells. The numbers indicate corrected OD_{450} values using an ELISA kit.

Condition	TNFα	IL1B	IL6	IL12	IL17A	IL8	MCP-1	RANTES	MIP-1A	MIP-1B	MDC	Eotaxin
Negative control	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
A549 cells	0.004	-0.035	-0.012	-0.006	-0.040	0.099	0.233	-0.007	0.005	-0.053	0.011	-0.022
A549 cells + polymyxin B	-0.006	-0.032	-0.009	0.003	-0.026	0.141	0.239	0.010	0.000	-0.045	-0.009	-0.013
A. baumannii	-0.011	-0.035	-0.012	-0.007	-0.030	0.000	-0.008	0.003	0.002	-0.060	-0.011	-0.026
***A. baumannii* + polymyxin B**	-0.017	-0.033	-0.010	0.005	-0.020	0.020	0.000	0.000	0.004	-0.015	-0.001	-0.024
A549 cells + *A. baumannii*	-0.015	-0.030	0.002	0.008	-0.030	0.761	0.280	0.058	0.003	-0.056	0.005	-0.022
A549 cells + *A. baumannii* + polymyxin B	-0.014	-0.028	-0.001	0.004	-0.024	0.673	0.338	0.050	0.001	-0.057	0.001	-0.026
Positive control	2.058	3.255	2.365	3.029	3.119	1.781	1.861	2.764	3.022	2.174	2.344	2.976
Recommended limit of detection	0.097	0.096	0.067	0.056	0.091	0.067	0.086	0.078	0.063	0.309	0.088	0.109

To validate the ELISA results, the microarray intensities of the DEGs in A549 cells following exposure to *A. baumannii*, polymyxin B, or both were used in a plot for comparison (**Figure 5.2**). In addition to IL-8, MCP-1, and RANTES, the microarray results also showed up-regulation of four additional cytokines, that involved in the acute phase reaction and systemic inflammation, tumor necrosis factor alpha (TNFα) (398); various cellular activities (e.g. cell proliferation, differentiation, and apoptosis, interleukin-1β (IL-1β) (398)); stimulation of immune response to fight infection, interleukin-6 (IL-6) (399); and recruitment of T lymphocytes and neutrophils, Eotaxin-2 (CCL24) (398, 400).

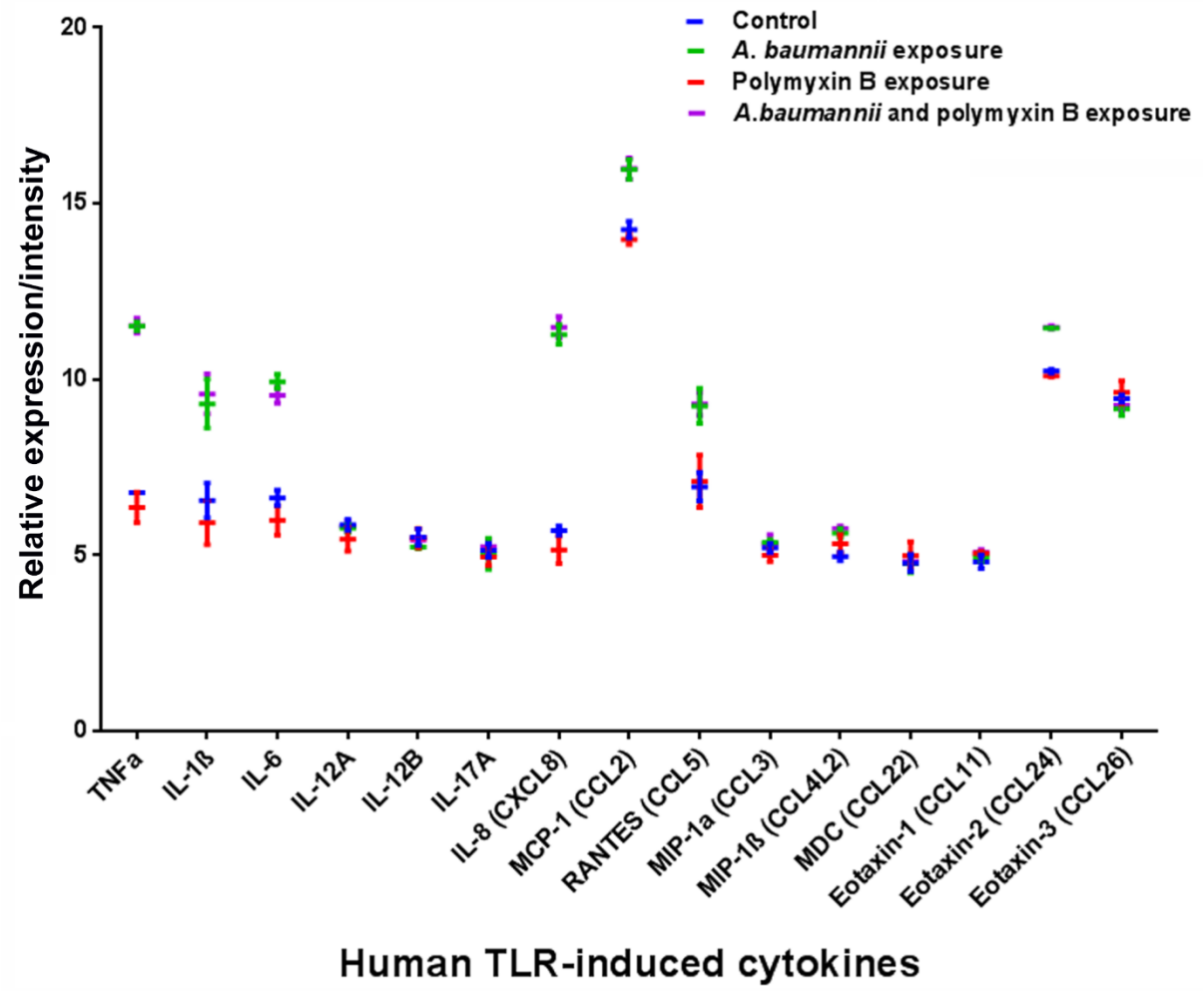

Figure 5.2 TLR-induced cytokines that were differentially expressed in A549 cells following infection with *A. baumannii* ATCC 19606, polymyxin B treatment (2 mg/L), or both. Data are normalized and presented as mean ± SD (n = 3).

5.4.3 Differentially expressed genes in *A. baumannii* in response to A549 cells exposure and polymyxin B treatment

Multivariate analysis results showing the clustering of samples for each condition are demonstrated in **Figure 5.3**. The PCA score plot revealed that the transcriptomic responses of *A. baumannii* were unique for different exposures. Compared to the control group, the groups exposed to A549 cells only, polymyxin B only, and both A549 cells and polymyxin B simultaneously, were separated by principal component 2 (PC2), principal component 1 (PC1), and both PC1 and PC2, respectively (**Figure 5.3A**). PC2 was responsible for c.a. 14% of the variance, while PC1 was responsible for 70% of the variance (**Figure 5.3B**). The results showed high reproducibility as the replicate samples from each condition clustered closely together.

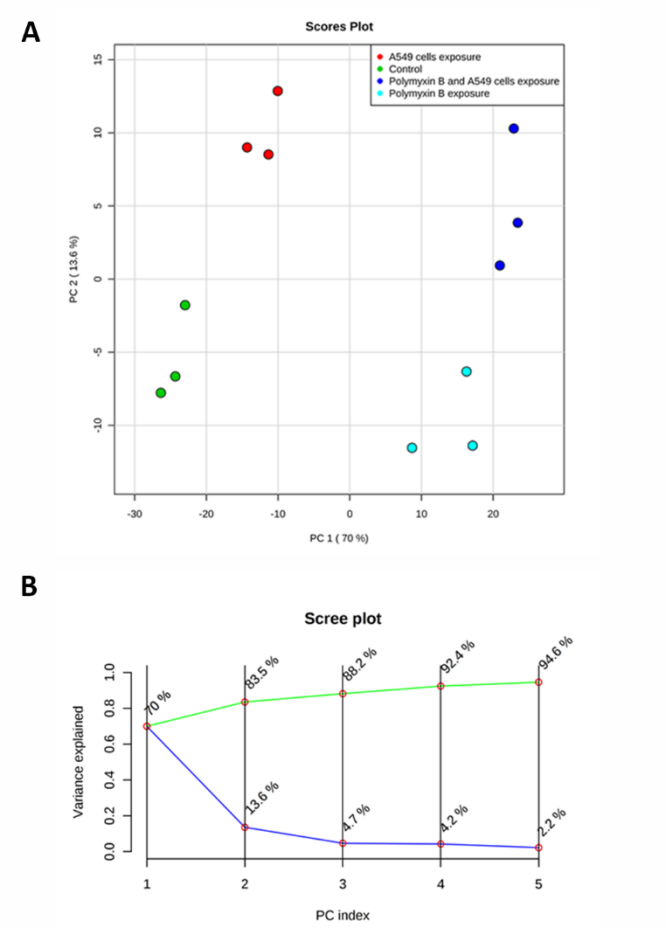

Figure 5.3 (**A**) PCA score plots of the A549 cell – *A. baumannii* – polymyxin B interactions showing separation along principal component 1 and principal component 2. (**B**) Scree plot showing the percentage of variance for each principal component (blue line) and the collective percentage of variance (green line).

The total numbers and the overlap of the DEGs, identified by F-statistic ($p \leq 0.05$) with Benjamini Hochberg adjustment (FDR ≤ 0.05), in *A. baumannii* at 2 h post exposure to A549 cells or polymyxin B (2 mg/L) treatment are shown in **Figure 5.4**. Additionally, **Figure 5.4** shows the numbers of DEGs resulting from additional exposure to polymyxin B in *A. baumannii* ATCC 19606 already exposed to A549 cells (i.e. exposure to both A549 cells and polymyxin B vs exposure to A549 cells alone). All significant DEGs, including BioCyc gene ID, gene product, FDR and $\log_2$ fold-change are provided in the Appendix 2 (**Tables A2.6 – A2.8)** for all three conditions.

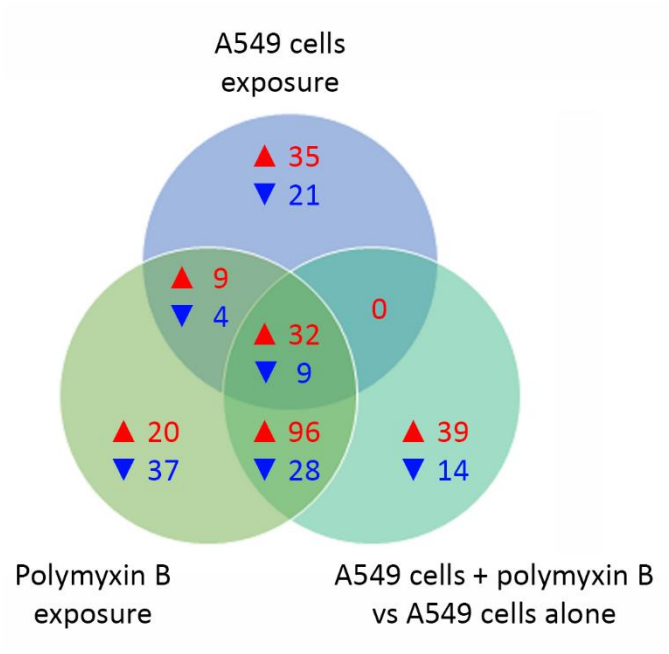

Figure 5.4 Venn diagram showing the total number and the overlap of differentially expressed genes at 2 h in *A. baumannii* ATCC 19606 responding to A549 cells, polymyxin B treatment (2 mg/L), and *A. baumannii* ATCC 19606 co-cultured with A549 cells responding to polymyxin B treatment.

Exposure to A549 cells resulted in 110 significant (FDR ≤ 0.05, $\log_2$ fold-change > |1|) DEGs (76 up-regulated and 34 down-regulated genes) in *A. baumannii* ATCC 19606 (**Figure 5.4** and **Table A2.6**). Among the up-regulated genes, the gene with the highest fold increase was HMPREF0010_01868 [$\log_2$FC = 3.75, FDR = 1.74E-04], which encodes for a conserved hypothetical protein (**Table A2.6**). HMPREF0010_03356 [$\log_2$FC = 3.22, FDR = 2.07E-05], encoding for another conserved hypothetical protein but with a possible role in nickel/cobalt transport, was the second most up-regulated gene (**Table A2.6**). Interestingly, among the down-regulated genes, the gene with the highest fold reduction was HMPREF0010_02293 [$\log_2$FC = -2.46, FDR = 2.69E-04], which encodes for vulnibactin utilization protein (ViuB) involved in intracellular removal of iron from iron-vulnibactin complex (**Table A2.6**).

The collective roles of all the DEGs of ATCC 19606 in response to A549 cells described by GO terms and KEGG pathways are shown in **Table 5.3**. GO terms analysis of the up-regulated genes in ATCC 19606 responding to A549 cells showed high enrichment in aromatic amino acid family metabolic

process, iron ion transport, and cellular iron ion homeostasis for the GO biological processes, ferric iron binding for the GO molecular function, and periplasmic space for the GO cellular component. KEGG pathway analysis showed enrichment in the metabolism of D-glutamine and D-glutamate; arginine and proline; tyrosine; alanine, aspartate and glutamate. Down-regulated genes were highly enriched in cellular response to phosphate starvation, siderophore biosynthetic process, and siderophore transport for the GO biological processes. Enriched GO molecular functions include 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase, (2,3-dihydroxybenzoyl)adenylate synthase, isochorismate synthase, oxidoreductase and ligase activities. No enriched KEGG pathway was observed with the down-regulated genes.

Table 5.3 GO biological processes, GO molecular functions, GO cellular components, and KEGG pathways enriched by the differentially expressed genes in *A. baumannii* ATCC 19606 cells in the presence of A549 cells. Processes in red were up-regulated and blue were down-regulated.

GO term/KEGG pathway	Fold-change	FDR
GO biological process		
GO:0009072~aromatic amino acid family metabolic process	162.76	4.40E-04
GO:0006826~iron ion transport	108.51	6.28E-03
GO:0006879~cellular iron ion homeostasis	108.51	6.28E-03
GO:0006525~arginine metabolic process	54.25	2.45E-02
GO:0006527~arginine catabolic process	54.25	2.45E-02
GO:0045454~cell redox homeostasis	10.17	4.33E-02
GO:0006520~cellular amino acid metabolic process	9.57	4.47E-02
GO:0019290~siderophore biosynthetic process	151.31	7.42E-05
GO:0015891~siderophore transport	44.83	7.42E-05
GO:0006810~transport	3.90	2.87E-02
GO:0016036~cellular response to phosphate starvation	100.88	3.12E-02
GO molecular function		
GO:0008199~ferric iron binding	36.17	3.47E-02
GO:0004872~receptor activity	33.63	3.22E-05
GO:0005506~iron ion binding	20.18	7.42E-05
GO:0008667~2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase activity	100.88	3.12E-02
GO:0008668~(2,3-dihydroxybenzoyl)adenylate synthase activity	100.88	3.12E-02
GO:0008909~isochorismate synthase activity	100.88	3.12E-02

GO:0016706~oxidoreductase activity	100.88	3.12E-02
GO:0016879~ligase activity, forming carbon-nitrogen bonds	100.88	3.12E-02
GO cellular component		
GO:0042597~periplasmic space	32.55	6.28E-03
KEGG pathway		
ko00330~Arginine and proline metabolism	29.12	4.90E-05
ko00350~Tyrosine metabolism	19.44	9.07E-03
ko00471~D-Glutamine and D-glutamate metabolism	29.19	2.85E-02
ko00250~Alanine, aspartate and glutamate metabolism	7.95	4.12E-02

Pathway analysis through BioCyc showed the up-regulated genes in *A. baumannii* in response to A549 cells exposure were enriched for amino acid degradation [FDR = 5.98E-05], which included arginine degradation II and tyrosine degradation I pathways. The flow chart for arginine degradation II pathway consisting of the significantly up-regulated genes, their products and relative expression levels, is shown in **Figure 5.5A**. Exposure to A549 cells caused up-regulation of all the genes required in the arginine degradation pathway II. HMPREF0010_02575 [$\log_2$FC = 1.40, FDR = 6.63E-03], HMPREF0010_02577 [$\log_2$FC = 1.49, FDR = 4.68E-03], HMPREF0010_02574 [$\log_2$FC = 1.57, FDR = 5.17E-04], HMPREF0010_02576 [$\log_2$FC = 1.55, FDR = 4.34E-03], and HMPREF0010_02578 [$\log_2$FC = 1.13, FDR = 1.98E-02], encode for arginine *N*-succinyltransferase, succinylarginine dihydrolase, ornithine-oxo-acid transaminase, succinylglutamic semialdehyde dehydrogenase, and succinylglutamate desuccinylase, respectively.

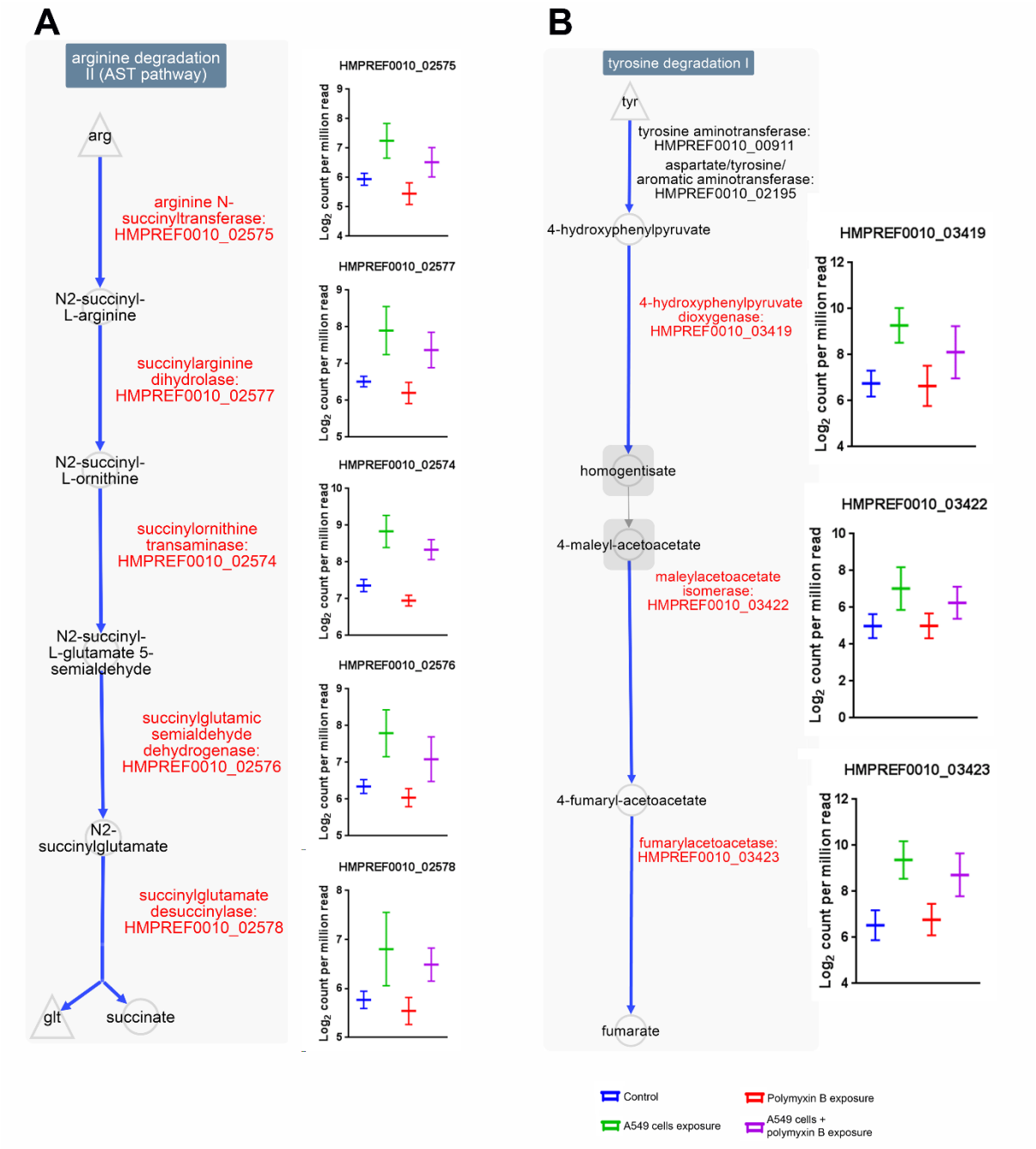

Figure 5.5 **(A)** Arginine degradation II and **(B)** tyrosine degradation I pathways in *A. baumannii* ATCC 19606 exposed to A549 cells. Significantly up-regulated genes are highlighted red and their relative expression level in different exposure groups are shown by the plots to their right. Data are normalised and presented as mean ± SD (n = 3).

Figure 5.5B shows the flow chart for tyrosine degradation I pathway, including the significantly up-regulated genes, their products and relative expression levels. Exposure to A549 cells caused up-regulation of three of four genes required in the tyrosine degradation I pathway; HMPREF0010_03419

[encoding for 4-hydroxyphenylpyruvate dioxygenase; log_2FC = 2.65, FDR = 6.03E-03], HMPREF0010_03422 [encoding for maleylacetoacetate isomerase; log_2FC = 2.14, FDR = 2.62E-02], and HMPREF0010_03423 [encoding for fumarylacetoacetase; log_2FC = 2.97, FDR = 2.25E-03].

For the down-regulated genes of *A. baumannii* in response to A549 cell exposure, BioCyc pathway analysis showed enrichment for siderophore biosynthesis [FDR = 2.27E-04], more specifically, acinetobactin/vibriobactin biosynthesis [FDR = 1.30E-04]. Significantly down-regulated genes, including their products, and relative expression levels, are shown in **Figure 5.6A**. The down-regulated genes involved in acinetobactin/vibriobactin biosynthesis affected were HMPREF0010_02312 [log_2FC = -2.23, FDR = 1.01E-03], HMPREF0010_00620 [log_2FC = -2.13, FDR = 2.60E-04], and HMPREF0010_02304 [log_2FC = -1.86, FDR = 1.24E-02].

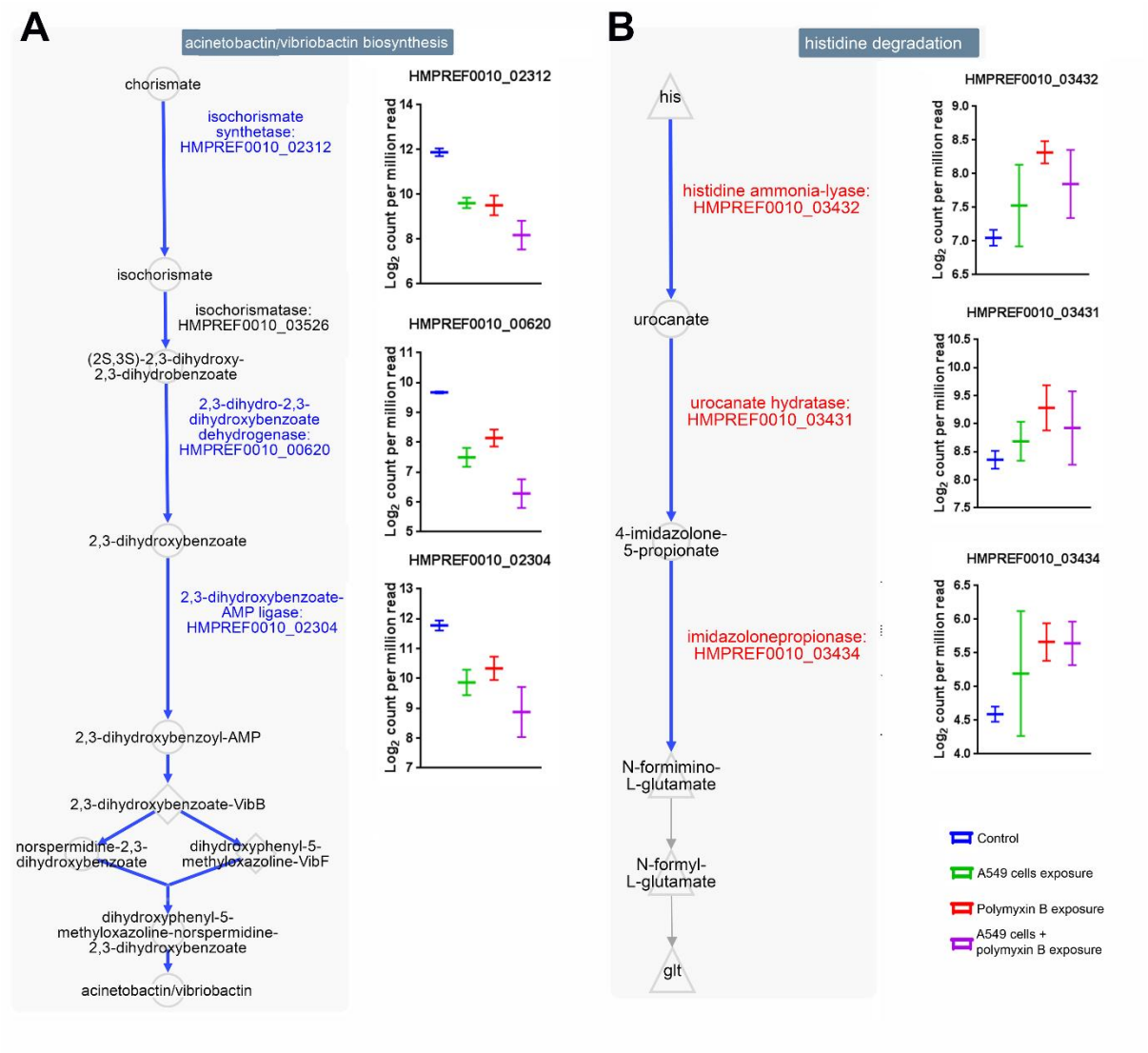

Figure 5.6 **(A)** Acinetobactin/vibriobactin biosynthesis pathway significantly affected in *A. baumannii* ATCC 19606 following exposure to A549 cells and **(B)** histidine degradation pathway following exposure to 2 mg/L of polymyxin B. Significantly down-regulated genes are highlighted blue and up-regulated genes are highlighted red; their relative expression level in different exposure groups are shown by plots to their right. Data are normalised and presented as mean ± SD (n = 3).

In ATCC 19606 treated with polymyxin B (2 mg/L), a total of 235 DEGs were identified (157 up-regulated and 78 down-regulated genes) (**Figure 5.4** and **Table A2.7**). Among the up-regulated genes, both of the two most up-regulated genes encoded for conserved hypothetical proteins with a possible role in nickel/cobalt transport; HMPREF0010_03356 [log_2FC = 9.69, FDR = 1.01E-10] and

HMPREF0010_02733 [log_2FC = 9.21, FDR = 2.88E-11] (**Table A2.7**). Interestingly, HMPREF0010_03356 was also the second most up-regulated gene identified in ATCC 19606 exposed to A549 cells (**Table A2.7**). Among the down-regulated genes, the gene with the highest fold change was HMPREF0010_02312 [log_2FC = -2.20, FDR = 4.63E-04], which encodes for the enzyme isochorismate synthetase involved in the biosynthesis of the siderophore enterobactin (**Table A2.7**).

The collective roles of all the differentially expressed genes of ATCC 19606 in response to A549 cells described by GO terms and KEGG pathways are shown in **Table 5.4.** The up-regulated genes showed enrichment in lipoprotein transport for the GO biological process, lipoprotein transporter activity for the GO molecular function, and periplasmic space and membrane for the GO cellular components. KEGG pathway analysis with the up-regulated genes showed high enrichment in amyotrophic lateral sclerosis, chlorocyclohexane and chlorobenzene degradation, toluene degradation, and cationic antimicrobial peptide. Down-regulated genes showed high enrichment in carboxylic acid metabolic process and siderophore transport for the biological process, isochorismatase activity, potassium-transporting ATPase activity, receptor activity, and iron ion binding molecular functions. KEGG pathway analysis with the down-regulated genes showed high enrichment in the biosynthesis of siderophore group nonribosomal peptides.

Table 5.4 GO biological processes, GO molecular functions, GO cellular components, and KEGG pathways enriched by the differentially expressed genes in *A. baumannii* ATCC 19606 in response to 2 mg/L of polymyxin B treatment. Processes in red were up-regulated and blue were down-regulated.

GO term/KEGG pathway	Fold-change	FDR
GO biological process		
GO:0042953~lipoprotein transport	66.86	5.94E-03
GO:0015891~siderophore transport	30.25	8.45E-05
GO:0019752~carboxylic acid metabolic process	32.26	3.30E-02
GO molecular function		
GO:0042954~lipoprotein transporter activity	66.86	5.94E-03
GO:0004872~receptor activity	26.06	3.24E-06
GO:0005506~iron ion binding	9.68	4.78E-03
GO:0008908~isochorismatase activity	96.79	5.15E-03
GO:0008556~potassium-transporting ATPase activity	96.79	1.22E-02
GO cellular component		
GO:0042597~periplasmic space	22.29	1.02E-02
GO:0016020~membrane	2.19	1.75E-02
KEGG pathway		
ko01503~Cationic antimicrobial peptide	27.30	4.73E-03
ko01501~beta-Lactam resistance	10.92	2.23E-02
ko05014~Amyotrophic lateral sclerosis	54.68	2.23E-02
ko02010~ABC transporters	4.06	2.99E-02
ko00361~Chlorocyclohexane and chlorobenzene degradation	54.68	3.27E-02
ko00623~Toluene degradation	54.68	3.27E-02
ko01053~Biosynthesis of siderophore group nonribosomal peptides	246.20	8.99E-05

Biocyc pathway analysis of the DEGs of ATCC 19606 responding to polymyxin B treatment only identified enrichment in the up-regulated genes, which was for histidine degradation pathway [FDR = 2.29E-02]. Significantly up-regulated genes, including their products and relative expression levels, are shown in **Figure 5.6B.** The up-regulated genes involved in histidine degradation pathway were HMPREF0010_03432 [Log_2FC = 1.43, FDR = 1.01E-03], HMPREF0010_03431 [Log_2FC = 1.08, FDR = 1.82E-02], and HMPREF0010_03434 [Log_2FC = 1.24, FDR = 2.25E-03].

In ATCC 19606 already exposed to A549 cells, additional exposure to polymyxin B resulted in 218 DEGs (167 up-regulated and 51 down-regulated genes) (**Figure 5.4** and **Table A2.8**). Similar to the response to polymyxin B treatment by ATCC 19606 alone, the two most up-regulated genes were encoded for conserved hypothetical proteins with a possible role in nickel/cobalt transport ; HMPREF0010_03356 [log_2FC = 6.88, FDR = 1.66E-09] and HMPREF0010_02733 [log_2FC = 6.17, FDR = 1.02E-09] (**Table A2.8**). For the down-regulated genes, the gene with the highest fold change was HMPREF0010_02300 [log_2FC = -2.63, FDR = 1.55E-03], which encodes for beta-alanine degradation protein (BauB) (**Table A2.8**).

The enriched GO terms and KEGG pathways that describe the collective roles of all DEGs in response to polymyxin B treatment by ATCC 19606 already exposed to A549 cells are shown in **Table 5.5**. Again, similar to the response by ATCC 19606 alone to polymyxin B treatment, GO terms analysis of the up-regulated genes showed enrichment in lipoprotein transport for the biological process and lipoprotein transporter activity for the molecular function. No enrichment for GO cellular component and KEGG pathway was identified. The down-regulated genes showed high enrichment for many GO biological and molecular processes. Highly enriched GO biological processes were siderophore transport, carboxylic acid and asparagine metabolic processes. For the GO molecular functions, isochorismatase, asparaginase, histidine decarboxylase, isocitrate lyase, and 2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase were highly enriched. KEGG pathway analysis with the down-regulated genes again showed high enrichment in the biosynthesis of siderophore group nonribosomal peptides. Pathway analysis by Biocyc showed no significant enrichment for all the DEGs in this group.

Table 5.5 GO biological processes, GO molecular functions, GO cellular components, and KEGG pathways enriched by the differentially expressed genes in *A. baumannii* ATCC 19606 in response to 2 mg/L of polymyxin B treatment and A549 cells. Processes in red were up-regulated and blue were down-regulated.

GO term/KEGG pathway	Fold-change	FDR
GO biological process		
GO:0042953~lipoprotein transport	63.99	6.74E-03
GO:0015891~siderophore transport	79.76	3.02E-09
GO:0006810~transport	4.53	7.96E-04
GO:0019752~carboxylic acid metabolic process	45.57	1.30E-02
GO:0006528~asparagine metabolic process	68.36	4.74E-02
GO molecular function		
GO:0042954~lipoprotein transporter activity	63.99	6.74E-03
GO:0004872~receptor activity	45.57	3.02E-09
GO:0005506~iron ion binding	20.81	1.98E-06
GO:0008908~isochorismatase activity	136.72	1.87E-03
GO:0004067~asparaginase activity	68.36	4.74E-02
GO:0004398~histidine decarboxylase activity	68.36	4.74E-02
GO:0004451~isocitrate lyase activity	68.36	4.74E-02
GO:0008667~2,3-dihydro-2,3-dihydroxybenzoate dehydrogenase activity	68.36	4.74E-02
GO:0008909~isochorismate synthase activity	68.36	4.74E-02
GO:0042626~ATPase activity, coupled to transmembrane movement of substances	17.09	4.74E-02
GO cellular component		
GO:0009279~cell outer membrane	20.51	5.57E-03
KEGG pathway		
ko01053~Biosynthesis of siderophore group nonribosomal peptides	204.67	1.51E-04
ko02020~Two-component system	5.35	4.93E-02

5.4.4 Time-kill results for polymyxin B against *A. baumannii* AB5075 and rcnB mutant

To investigate the significance of *rcnB* in *A. baumannii* on polymyxin susceptibility, a time-kill assay was conducted with *A. baumannii* AB5075 and its *rcnB* mutant derived from a transposon insertion. The killing kinetics of polymyxin B at 0.5 mg/L against these isolates are shown in the Appendix 2 **(Figure A2.1)**. In both the mutant and wild-type strain, similar growth curves were observed over 24

h in the absence of polymyxin B. In the presence of 0.5 mg/L of polymyxin B, similar bacterial killing was observed over the first 4 h (~4 $\log_{10}$ cfu/mL killing). Interestingly, greater bacterial killing of the *rcnB* mutant occurred at 6 h than the wild-type strain (~3 $\log_{10}$ cfu/mL killing). Although t-test returned a *p*-value of 0.13, bacterial killing at 6 h was consistently higher (> 20-fold) against the *rcnB* mutant. For both isolates, similar regrowth was observed at 24 h with polymyxin B monotherapy.

To further investigate the association of *rcnB* to polymyxin resistance in *A. baumannii*, PAPs were conducted for *A. baumannii* AB5075 and its *rcnB* mutant derivative at baseline (0 h) and 24 h following treatment with polymyxin B (0.5 mg/L). The proportion of colonies growing in the presence of varying concentrations of polymyxin B for AB0575 and the *rcnB* mutant are shown in the Appendix 2 **(Figure A2.2)**. The proportion of colonies growing at each concentration was similar for both strains at baseline. Interestingly, the growth proportion of *rcnB* mutant at 0.5 mg/L of polymyxin B at baseline was significantly lower than the wild-type (10^2 fold). Similarly, the reduced growth proportion of *rcnB* mutant compared to the wild-type was also observed at 0.5 mg/L of polymyxin B post 24 h without treatment. Surprisingly, following 24 h treatment with 0.5 mg/L of polymyxin B, the growth proportion of the *rcnB* mutant were consistently and significantly higher compared to AB5075 at polymyxin B concentrations of 1 mg/L and higher. In the presence of 8 mg/L of polymyxin B in the PAPs plates, the growth proportion *rcnB* mutant was approximately 10^6 folds higher compared to the AB5075.

The colony morphology of *rcnB* mutants after 24 h treatment with polymyxin B on the PAPs plates is shown in the Appendix 2 (**Figure A2.3)**. At this time the colonies of the *rcnB* mutant were extremely small on plates with low polymyxin B concentrations and much larger on plates with high polymyxin B concentrations.

5.5 Discussion

Currently, polymyxins are considered the 'last resort' antibiotics for treatment of important nosocomial infections caused by *A. baumannii*, including pneumonia (81, 82). It is therefore essential

that polymyxins are used effectively to prevent the development of polymyxin resistance in these bacteria. One possible means to achieve this is to understand the complex interaction between the host and pathogen during an infection and treatment with polymyxins. Knowledge of the molecular networks of the host and pathogen provides better understanding of the infection pathogenesis and may contribute to the development of rational therapeutic interventions e.g. adjustment of polymyxin dosages as infection progresses.

The A549 cells transcriptomic data showed that *A. baumannii* was the sole inducer of the transcriptomic changes for A549 cells in this host-pathogen-drug interaction model. Minimal difference was observed between the transcriptomic responses to *A. baumannii* alone and *A. baumannii* plus polymyxin B (**Figure 5.1**). The finding of no transcriptomic changes caused by 2 mg/L of polymyxin B A549 cells shows that 2 mg/L polymyxins was less toxic to A549 cells, which is in line with a previous toxicity study that showed that the viability of A549 cells was not affected even at polymyxin B concentrations of ~325 mg/L (401).

The significant up-regulation of pro-inflammatory and immune responses caused by *A. baumannii* in A549 cells (**Table 5.1**) are consistent with the host responses to the bacterial infection (398). Among the up-regulated genes, CCL20 displayed the highest level of increase in A549 cells (**Table A2.1**). CCL20 plays an important role at the mucosal surfaces under homeostatic and inflammatory conditions and is responsible for the recruitment of dendritic cells (DC), effector/memory T-cells and B-cells (398, 402). Interestingly, CCL20 was previously found to possess antimicrobial activity against *E. coli* and *S. aureus* (403). *A. baumannii* also caused high up-regulation of potent pro-inflammatory cytokines including IL-1, IL-6, and TNF (**Table A2.1**). As these cytokines can lead to heightened inflammatory responses (398, 404), it is possible that immune mediated tissue damage contributes to the pathogenesis of pneumonia caused by *A. baumannii*. On the other hand, cell-cell adhesion involving cell-cell adherens junction was significantly down-regulated in A549 cells by *A. baumannii*. Although the down-regulation of cell-cell adhesion has been implicated to assist with the migration of

leucocytes to inflamed tissues (405), other studies have suggested that bacteria can disrupt the cell junction to gain access to other parts of the body (406-408). Consequently, it is possible that the down-regulation of cell-cell adherens also contributes to the pathogenesis of pneumonia caused by *A. baumannii*. The investigation of TLR-induced cytokines by the Human TLR-induced Cytokines II: Microbial-induced Multi-Analyte ELISArray Kit did not detect TNFα, IL-1β, and IL-6 at 2 h (**Table 5.5.2**). In accordance with previous findings (409, 410), the present results indicate that there is a possible delay in the translation of these cytokines. Both IL-8 and CCL2 (MCP-1) enzymes were detected by ELISA in A549 with and without *A. baumannii* (**Table 5.5.2**). The presence of IL-8 and CCL2 in the absence of *A. baumannii* indicates an important role for these enzymes in the respiratory epithelial cells.

The analysis of *A. baumannii* transcriptomic data in response to A549 cells showed that iron ion transport was significantly up-regulated while siderophore biosynthesis and siderophore transport pathways were significantly down-regulated (**Table 5.3**). The ability to achieve effective iron homeostasis is essential for the growth of bacteria (411). Under physiological conditions, iron can exist as the soluble reduced Fe^{2+} ferrous form and the insoluble oxidised Fe^{3+} ferric form (411). Due to their insolubility, ferric ions are bound to different groups of proteins during transportation and storage in animals (412). Consequently, bacteria causing infection need to be able to obtain iron from their hosts (412). In bacteria, ferrous iron is transported via iron transport systems under iron-sufficient conditions while ferric iron is transported in complex with siderophores (195, 413). These findings suggest that the iron ions were not limited and may not be required for the initial phase of infection. Similarly, a previous interaction study showed that the initial interaction of *A. baumannii* ATCC 19606 with A549 cells was independent of the production of BasD and BauA proteins, which are required for acinetobactin biosynthesis and transport, respectively (414). Siderophore transport and isochorismatase activity, which participates in the biosynthesis of siderophore group, were also down-regulated in *A. baumannii* treated with polymyxin B (**Table 5.5.4**). Although the significance of this

observation is currently unclear, it is possible that *A. baumannii* re-directed the energy from the siderophore transport process to other more critical pathways for survival.

Exposure to A549 cells also caused upregulation of arginine and tyrosine degradation pathways in *A. baumannii* (**Figure 5.5**). Arginine is a conditionally essential amino acid in humans as it can be synthesised or absorbed from the diet (415). Arginine is involved in the synthesis of innate immune product nitric oxide and is readily convertible to other amino acids like proline and glutamate (415) which have been shown to be important in stress survival in bacteria (375, 376). Consequently, the up-regulation of the arginine degradation pathway shows that *A. baumannii* potentially breaks down arginine to use as a source of energy and nitrogen, and to prevent the production of nitric oxide by the host cells. In the tyrosine degradation pathway, fumarate and acetoacetate are the two end products (416). Since acetoacetate can be converted into acetyl-CoA, together with fumarate, the tyrosine degradation pathway essentially gives rise two substrates of the citric acid cycle. It is therefore likely that breaking down tyrosine is a means of *A. baumannii* acquiring energy during infection. Additionally, *A. baumannii* could be generating additional acetyl-CoA for fatty acid synthesis.

In *A. baumannii* alone and *A. baumannii* exposed to A549 cells, treatment with polymyxin B caused significant up-regulation of lipoprotein transport activity (**Tables 4** and **5**) which involves the directed movement of protein-lipid complexes. In *A. baumannii*, treatment with polymyxin monotherapy has been shown to produce polymyxin resistance through modification of lipid A or loss of LPS (128, 341). It is therefore likely that the observed up-regulated lipoprotein transport activity is a sign of outer membrane remodeling in *A. baumannii*. These findings are in accord with a previous study that investigated the transcriptomic response of *A. baumannii* to colistin and its combination (105).

Significant upregulation of histidine degradation pathway was also observed in *A. baumannii* treated with polymyxin B (**Figure 5.6B**). The histidine ultilization (Hut) system is highly regulated and conserved in bacteria. Bacteria breakdown histidine to produce ammonia, glutamate, and a one-carbon compound (formate or formamide) when required (417). As glutamate is the end product of

the histidine degradation pathway and has an important role in in stress survival in bacteria (375, 376), up-regulation of histidine degradation in *A. baumannii* in response to polymyxin B is most likely related to stress tolerance. Consistently, the histidine degradation pathway was found to be significantly perturbed in *A. baumannii* treated with polymyxin B in the metabolomic study in Chapter 4.

In *A. baumannii* treated with polymyxin B, the levels of expression of HMPREF0010_03356 [Log_2FC = 9.69, FDR = 1.01E-10] and HMPREF0010_02733 [Log_2FC = 9.21, FDR = 2.88E-11] (**Table A2.7**) were significantly higher compared to the remaining up-regulated genes. Both genes encoded for the same hypothetical protein with similar identity to nickel/cobalt homeostasis protein RcnB. Currently, little is known about this protein. A study showed that RcnB is a periplasmic protein that is essential for maintaining intracellular nickel and cobalt concentrations in *Escherichia coli* (418). Interestingly, RcnB was also found to be upregulated in *E. coli* when challenged with three cationic amphipathic antimicrobial peptides (AMPs) of natural origin; magainin 2, LL-37, and pleurocidin (419). Promisingly, the results from the time-kill assay with the *rcnB* mutant and the subsequent PAP assay implicated a role of RcnB in polymyxin resistance. The mutation appeared to have no impact on the biological fitness *of A. baumannii* as both isolates showed similar growth (**Figure S2.1**). Although the *rcnB* mutant was more susceptible to polymyxin B treatment (**Figure S2.1**), colonies that regrew were highly resistant to polymyxin B (**Figure S2.2B**). Additionally colonies that regrew appeared to grow better in the presence of polymyxin B (**Figure S2.3**), although the reason for this is currently unclear. The collective findings highly support the involvement RcnB in in the development of polymyxin resistance.

5.6 Conclusions

This is the first study to investigate the simultaneous transcriptomic responses of both A549 cells and *A. baumannii* in response to polymyxin B treatment. Significant findings include the potential independence on siderophores by *A. baumannii* to establish early infection in A549 cells, the potential involvement of tyrosine and arginine degradations in infections caused *A. baumannii*, and the likelihood of immune mediated tissue injury being the pathogenesis of *Acinetobacter* pneumonia. The

most significant finding is the involvement of the histidine degradation pathway and a putative RcnB protein in the development of polymyxin resistance in *A. baumannii*. In summary, this study provides key mechanistic information on the complex interactions among *A. baumannii,* lung epithelial cells and polymyxin treatment; and new insights to the pathogenesis as well as potential pathways for novel antimicrobial therapies.

CHAPTER SIX: CONCLUSIONS AND FUTURE DIRECTIONS

MDR Gram-negative pathogens are increasingly recognised as a significant unmet burden for the healthcare system globally. In recent years, MDR Gram-negatives have become widespread while concomitantly the number of antibiotics approved for the treatment of infections caused by these pathogens has dramatically reduced (10, 25). This has eventuated in a situation of higher healthcare costs, morbidity, and mortality (10, 11, 28). Among these pathogens, the CDC has classified MDR *A. baumannii* as a "Serious threat" (28) and the WHO has assigned top priority for research and development of new antibiotics for carbapenem-resistant *A. baumannii* (29). Currently, polymyxins are effective last-line drugs for the treatment of MDR Gram-negative bacteria, including *A. baumannii* (81). Unfortunately, recent PK and PD data have indicated that polymyxin monotherapy can rapidly lead to the development of polymyxin resistance (111, 149, 219-222, 341). This discerning situation, has led to the clinicians implementing approaches such as using polymyxins in combination with other antibiotics to improve bacterial killing and prevent the emergence of polymyxin resistance. Encouragingly, the combination of an antibiotic with FDA approved non-antibiotic drugs has emerged as a potential cost effective means to expedite the discovery of new treatments to combat the rapid emergence of these problematic pathogens (285).

In the first experimental chapter of the thesis (Chapter 2), I investigated the potential benefit of combining polymyxin B with a non-antibiotic drug for the treatment of various MDR Gram-negatives, including polymyxin-resistant, Gram-negative bacteria. From a library of 1,248 non-antibiotic drugs approved by the FDA for use abroad or undergoing phase 2 clinical trials, a total of 66 non-antibiotic drugs showed potential synergy with polymxin B. For these 66 non-antibiotic drugs, bacterial killing was observed against polymyxin-resistant *A. baumannii*, *P. aeruginosa* or *K. pneumoniae* in the presence of 2 mg/L of polymyxin B. In addition to synergistic bacterial killing, follow up investigations using combinations of polymyxin B and a non-antibiotic anthelminthic closantel against eight *A. baumannii* strains indicated that the combination of a polymyxin and non-antibiotic drug may also

prevent the emergence of polymyxin resistance in polymyxin-susceptible *A. baumannii* strains. Time-kill assays with polymyxin B at 2 mg/L and closantel at 4 and 16 mg/L successfully prevented the regrowth of the polymyxin-resistant subpopulation in polymyxin-susceptible *A. baumannii* strains. Against strains of MDR and polymyxin-resistant *A. baumannii*, all combinations of polymyxin B (2mg/L) and closantel (2, 4, and 16 mg/L) showed superior bacterial killing during the first 6 h, with the greatest killing occurring with 2 mg/L polymyxin B and 16 mg/L closantel (2 - 5 $\log_{10}$ additional killing between 2 to 6 h compared to polymyxin B monotherapy). Despite regrowth occurring with combination therapies in MDR and polymyxin-resistant *A. baumannii* strains at 24 h, the extensive bacterial killing was observed during the first 6 h; and as such this treatment may be effective in facilitating clearance of bacteria by the host's immune system. Overall, these findings provided an important foundation for the further investigation of potential repositioning of non-antibiotic drugs in combination with polymyxins for antimicrobial purposes.

A significant advantage of drug repositioning is the increased drug safety and reduced pharmacokinetic and dosage uncertainties (278). For veterinary drugs, such as closantel, despite limitation in human usage, information of its PK in animals is still superior to no information at all. Although variation in PK may occur when drugs are used in combination, the existing pharmacological information of the drugs are highly useful for optimising their dosages.To take advantage of the benefit that drug repositioning provides, in Chapter 3, I evaluated the *in vitro* and *in vivo* synergistic antimicrobial activity of the combination of polymyxin B and the FDA-approved antineoplastic drug mitotane, which is currently indicated for the treatment of adrenocortical carcinoma. Promisingly, time-kill assays revealed that the combination of polymyxin B (2 mg/L) and mitotane (4 mg/L) at these clinically relevant concentrations produced superior bacterial killing compared to either drug alone against not only *A. baumannii*, but also *P. aeruginosa* and *K. pneumoniae*, including MDR and polymyxin-resistant strains. Bacterial killing by the combination was rapid, with 2 - 5 $\log_{10}$ killing occurred against most strains in the first 0.5 h. Furthermore, the combination prevented the emergence of polymyxin resistance in all polymyxin-susceptible strains. Being a non-antibiotic drug,

mitotane monotherapy was ineffective against most Gram-negative bacteria. Interestingly, however, mitotane monotherapy was effective against the LPS-deficient polymyxin-resistant *A. baumannii* FADDI-AB065. This led to the hypothesis that the loss of LPS weakened the highly selective permeability barrier in Gram-negative bacteria and allowed mitotane to enter the cells and exert its antimicrobial activity. The *in vivo* bacterial killing of polymyxin B and mitotane combination was evaluated with a mouse burn wound infection model against polymyxin-resistant *A. baumannii* FADDI-AB225. The resistance of this isolate to polymyxins is due to modification of lipid A, a common method of polymyxin-resistance in Gram-negative bacteria (110, 111). Through topical application, the combination of polymyxin B (0.5% w/w) and mitotane (1.4% w/w) also produced superior killing of FADDI-AB225 (mean $\log_{10}$ cfu/wound difference, - 2.19) compared to polymyxin B (mean $\log_{10}$ cfu/wound difference, -1.44) and mitotane (mean $\log_{10}$ cfu/wound difference, - 0.11) monotherapies. This finding highlights the potential for the repositioning of non-antibiotic drugs, in particular mitotane, in combination with polymyxins to treat infections caused by MDR Gram-negative bacteria and to prevent the emergence of polymyxin resistance. Given the enhanced bacterial killing observed from polymyxin B and mitotane combinations against MDR and polymyxin-resistant Gram-negative bacteria, further investigations into the mechanisms underpinning this synergy were warranted. This was the focus of experimental Chapter 4.

Increasingly, systems-level approaches incorporating transcriptomics and metabolomics is emerging as a valuable approach for the investigation of antimicrobial mechanisms and resistance development (105, 106, 130, 296-298). In addition to identifying the drug-target interaction, system-oriented research can also provide a better understanding of the specific sequences of events following the binding of an antibiotic to its target (291, 292). In light of this, metabolomics was used to investigate the synergistic killing of polymyxin B and mitotane combinations against four *A. baumannii* strains; ATCC 19606 and its derivative LPS-deficient polymyxin-resistant FADDI-AB065, and ATCC 17978 and its derivative lipid A modified polymyxin-resistant FADDI-AB225. In line with the current understanding of the mode of action of polymyxins, the results showed the killing of polymyxin-

susceptible strains involved the destabilization of the Gram-negative bacteria outer membrane. This was evident by the significant perturbation of glycerophospholipid metabolism in polymyxin-susceptible *A. baumannii* by polymyxin B monotherapy. Additionally, the study also identified, for the first time, that polymyxin B monotherapy also affected the histidine degradation pathway in *A. baumannii*. The significance of the histidine degradation pathway in relation to bacterial killing by polymyxin B is currently unclear. However, it is likely to be a bacterial stress response to antibiotic treatment. In accordance with the antimicrobial activity of mitotane observed in Chapter 3, mitotane monotherapy only caused significant metabolic perturbation in LPS-deficient FADDI-AB065. The findings showed that in addition to glycerophospholipid metabolism and the histidine degradation pathway; mitotane monotherapy also affected the non-oxidative phase of the pentose phosphate pathway. As the non-oxidative phase of the pentose phosphate pathway is responsible for the generation of ribose, the findings indicated that the antimicrobial activity of mitotane may involve the inhibition of DNA synthesis. Indeed, when polymyxin B and mitotane were used in combination, the metabolic perturbation extended to also affect pyrimidine ribonucleotide biogenesis, guanine ribonucleotide biogenesis, and the citric acid cycle. Consequently, the antimicrobial mechanism of the combination potentially involved inhibition of DNA synthesis as well as energy production. The hypothesis of DNA synthesis inhibition as the antimicrobial mechanism of the combination is in line with the microscopy results in Chapter 3 which showed treatment with combination therapy led to extensive shortening of the bacterial cells and incomplete cell division. Given that common pathways were affected by polymyxin B and mitotane monotherapies, it was hypothesised that the targeting of the same pathway may have contributed to the observed synergistic effect of the combination. Subsequently, the studies in experimental Chapter 5 were conducted to further understand the interactions between *A. baumannii* and host cells during polymyxin B therapy.

Given that one of the most significant infections caused by *A. baumannii* is pneumonia in critically-ill patients (68, 380) and that polymyxins are the last-resort drugs for the treatment of MDR *A. baumannii* (81), a host-pathogen-drug interaction model consisting of A549 cells, *A. baumannii* ATCC19606, and

polymyxin B was employed in Chapter 5. Microarray and RNA sequencing were utilised to capture the global responses of both host and pathogen. The transcriptomic findings from the host suggested that immune-mediated tissue damage was the major contributor to the pathogenesis of *Acinetobacter* pneumonia as significant up-regulation of inflammatory and immune responses, including many potent pro-inflammatory cytokines, were identified in A549 cells exposed to *A. baumannii*. Down-regulation of cell-cell adhesion in the host cells is also suspected to be involved in the pathogenesis of *Acinetobacter* pneumonia as the process may enable bacteria to gain access to other parts of the body via entering between these cells. The results from treatment with polymyxin B showed that A549 cells are highly resistant to the effects of polymyxins as 2 mg/L of polymyxin B did not affect their transcriptomes. The transcriptomic findings from the pathogen showed that the arginine and tyrosine degradation pathways are important for the bacteria to establish infection. Interestingly, siderophore biosynthesis was down-regulated in *A. baumannii* exposed to A549 cells, suggesting siderophores are non-essential for the initial phase of infection. The transcriptomic changes of *A. baumannii* in response to 2 mg/L of polymyxin B were in line with the metabolomics findings in Chapter 4. Treatment with polymyxin B caused significant up-regulation of lipoprotein transport and the histidine degradation pathway. From these findings, it is highly possible that the histidine degradation pathway is an important target of the polymyxins. Furthermore, RcnB protein, which is involved in nickel/cobalt homeostasis, was also identified as potential novel target for polymyxins. *A. baumannii* with *rcnB* mutation developed high level of polymyxin resistance following polymyxin B monotherapy. The findings from Chapter 5 not only provided important information for a better understanding of the progression of *Acinetobacter* pneumonia in the presence of polymyxin B, but are also useful for the selection of non-antibiotic drugs for use in combination with polymyxins for treatment of problematic *A. baumannii* infections.

The work presented in this thesis can be expanded through additional studies. For combinations involving polymyxins and non-antibiotics, formulation studies may be conducted for the non-antibiotic drugs that showed synergistic activity with polymyxin B. Such studies can examine modifying the

pharmacokinetics of the non-antibiotic drugs to fit the profiles of antibiotics, which include water solubility, bioavailability, and clearance rate. Regarding the *in vivo* efficacy of polymyxin B and mitotane combinations, additional animal studies using different infection models would be useful for evaluation against different forms of infection. As mitotane is highly hydrophobic, adjusting its hydrophobicity is highly desirable as that would reduce mitotane distribution in fatty tissue and increase its rate of elimination. For the meantime, mitotane may be administered orally or topically together with polymyxin B for the treatment of XDR-GNB. Given the clinical significance of *A. baumannii* and the high rates of pneumonia and bacteraemia cause by this organism, the efficacy of the combination against these pathogens in animal pneumonia and bacteraemia models are warranted. For a better understanding of disease progression caused by *A. baumannii* infections and the benefit of combination therapies, additional comparative transcriptomic studies would be highly beneficial. Such comparative transcriptomic studies could include different combinations of drug concentrations, different strains of *A. baumannii*, different sample collection time-points, and different human cell lines. By using multiple criteria, such results can better describe the disease progression over time rather than for a single time point. Another research area that may prove useful is validating the transcriptomic findings in *A. baumannii* associated with disease progression and polymyxin resistance using a library of *A. baumannii* mutants. The use of different mutants will allow definitive identification of genes that are significant for causing disease and developing polymyxin resistance. The last research area that may help to expand the findings in this thesis is the use of the collected metabolomics and transcriptomic data, here and elsewhere, to generate an *in silico* model of *A. baumannii* to identify targets for the development of novel combinations of non-antibiotic drugs and polymyxins.

In conclusion, the current thesis provided new findings of (1) treatment of MDR and polymyxin-resistant Gram-negative bacteria including *A. baumannii* with novel combinations of polymyxin B and non-antibiotic drugs, (2) the potential repositioning of mitotane for use in combination with polymyxin B for treatment of polymyxin-resistant *A. baumannii*, (3) the synergistic mechanism of polymyxin B

and mitotane combinations against *A. baumannii*, and (4) the simultaneous global transcriptomic responses of the A549 cells and *A. baumannii* during an infection and treatment with polymyxin B. These findings are important for the understanding of the pathogenesis of *A. baumannii* and its polymyxin resistance mechanism, as well as better treatment of MDR Gram-negative bacteria with novel antimicrobial combination therapies.

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APPENDIX 1: ADDITIONAL SUPPORTING DATA FOR CHAPTER 4

Table A1.1. Significant metabolites identified following exposure to polymyxin B and mitotane alone and in combination in *A. baumannii* ATCC 17978. Significant fold-changes are highlighted in bold and italic. One-way ANOVA for multiple comparison, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$.

MASS	RT	FORMULA	METABOLITE	CONFIDENCE	MAP	PATHWAY	Log_2-fold change			FDR
							Polmyxin B	Mitotane	Combination	
117.08	11.18	$C_5H_{11}NO_2$	Betaine	10	Amino Acid Metabolism	Glycine, serine and threonine metabolism	-0.75	-0.11	***-5.29***	7.58E-09
161.07	14.36	$C_6H_{11}NO_4$	L-2-Aminoadipate	9	Amino Acid Metabolism	Lysine biosynthesis; Lysine degradation; Penicillin and cephalosporin biosynthesis	***-1.28***	0.07	***-4.41***	2.77E-08
161.11	13.16	$C_7H_{15}NO_3$	L-Carnitine	10	Amino Acid Metabolism	Lysine degradation	***-1.69***	-0.22	***-3.18***	6.02E-08
174.06	14.21	$C_6H_{10}N_2O_4$	*N*-Formimino-L-glutamate	7	Amino Acid Metabolism	Histidine metabolism	***1.27***	-0.01	***2.29***	2.97E-07
147.05	14.29	$C_5H_9NO_4$	L-Glutamate	10	Amino Acid Metabolism	Arginine and proline metabolism; Glutamate metabolism; Histidine metabolism; D-Glutamine and D-glutamate metabolism; Glutathione metabolism_Butanoate metabolism_C5-Branched dibasic acid metabolism_Porphyrin and chlorophyll metabolism_Nitrogen metabolism	***-1.25***	-0.41	***-4.44***	5.80E-07
129.04	14.61	$C_5H_7NO_3$	5-Oxoproline	7	Amino Acid Metabolism	Glutathione metabolism	***-1.41***	-0.45	***-4.95***	1.27E-06
203.08	16.94	$C_8H_{13}NO_5$	N2-Acetyl-L-aminoadipate	5	Amino Acid Metabolism	Lysine biosynthesis	***-1.60***	0.18	***-2.40***	2.55E-06
102.03	7.47	$C_4H_6O_3$	Succinate semialdehyde	6	Amino Acid Metabolism	Glutamate metabolism; Tyrosine metabolism; Butanoate metabolism; Vitamin B6 metabolism	0.39	0.29	***1.40***	6.98E-06
131.07	14.42	$C_4H_9N_3O_2$	Creatine	5	Amino Acid Metabolism	Glycine, serine and threonine metabolism; Arginine and proline metabolism	-0.66	0.15	***-1.59***	6.98E-06
230.11	9.33	$C_{13}H_{14}N_2O_2$	(1xi,3xi)-1,2,3,4-Tetrahydro-1-methyl-beta-carboline-3-carboxylic acid	7	Amino Acid Metabolism	Undefined	***-1.43***	0.10	***-1.10***	6.98E-06
274.13	15.01	$C_{10}H_{18}N_4O_5$	N2-Succinyl-L-arginine	8	Amino Acid Metabolism	Arginine and proline metabolism	-0.92	0.62	***-3.51***	7.58E-06
113.05	9.00	$C_5H_7NO_2$	1-Pyrroline-2-carboxylate	7	Amino Acid Metabolism	Arginine and proline metabolism; D-Arginine and D-ornithine metabolism	-0.12	0.04	***2.21***	8.82E-06
133.04	14.70	$C_4H_7NO_4$	L-Aspartate	10	Amino Acid Metabolism	Alanine and aspartate metabolism; Arginine and proline metabolism; Glycine, serine and threonine metabolism; Lysine biosynthesis; Arginine and proline metabolism; Histidine metabolism; beta-Alanine metabolism; Cyanoamino acid metabolism; Carbon fixation	0.16	0.28	0.04	8.89E-06

190.10	23.12	$C_7H_{14}N_2O_4$	LL-2,6-Diaminoheptanedioate	6	Amino Acid Metabolism	Lysine biosynthesis	-0.64	0.08	***-1.39***	2.04E-05
173.08	14.01	$C_6H_{11}N_3O_3$	5-Guanidino-2-oxopentanoate	5	Amino Acid Metabolism	D-Arginine and D-ornithine metabolism	***-1.02***	-0.26	***-2.04***	2.18E-05
104.01	15.16	$C_3H_4O_4$	Hydroxypyruvate	6	Amino Acid Metabolism	Glycine, serine and threonine metabolism; Glyoxylate and dicarboxylate metabolism	0.28	0.07	0.67	3.15E-05
242.10	11.80	$C_9H_{14}N_4O_4$	(S,S)-Nt-Histidinylalanine	5	Amino Acid Metabolism	Undefined	-0.62	0.04	***-1.80***	4.19E-05
138.04	10.37	$C_6H_6N_2O_2$	Urocanate	7	Amino Acid Metabolism	Histidine metabolism	***1.46***	0.52	***2.49***	4.80E-05
130.06	4.93	$C_6H_{10}O_3$	4-Methyl-2-oxopentanoate	10	Amino Acid Metabolism	Valine, leucine and isoleucine degradation; Valine, leucine and isoleucine biosynthesis	-0.27	0.17	0.45	1.46E-04
132.09	15.53	$C_5H_{12}N_2O_2$	D-Ornithine	7	Amino Acid Metabolism	D-Arginine and D-ornithine metabolism	-0.59	0.23	***-1.86***	1.78E-04
160.08	11.13	$C_6H_{12}N_2O_3$	D-Alanyl-D-alanine	6	Amino Acid Metabolism	D-Alanine metabolism; Peptidoglycan biosynthesis	-0.54	0.39	***-1.87***	2.00E-04
116.05	7.45	$C_5H_8O_3$	5-Oxopentanoate	5	Amino Acid Metabolism	Lysine degradation	-0.01	0.10	0.13	3.32E-04
301.06	14.31	$C_8H_{16}NO_9P$	*N*-Acetyl-D-glucosamine 6-phosphate	8	Amino Acid Metabolism	Glutamate metabolism; Aminosugars metabolism	***-1.38***	-0.67	***-2.05***	3.39E-04
258.09	13.56	$C_{10}H_{14}N_2O_6$	(1-Ribosylimidazole)-4-acetate	5	Amino Acid Metabolism	Histidine metabolism	-0.85	0.02	-0.65	3.47E-04
145.04	10.14	$C_5H_7NO_4$	2-Oxoglutaramate	5	Amino Acid Metabolism	Glutamate metabolism	-0.98	-0.92	***-3.21***	3.74E-04
384.12	13.78	$C_{14}H_{20}N_6O_5S$	S-Adenosyl-L-homocysteine	8	Amino Acid Metabolism	Methionine metabolism	-0.45	0.10	***-1.26***	3.93E-04
188.12	14.86	$C_8H_{16}N_2O_3$	N2-Acetyl-L-lysine	7	Amino Acid Metabolism	Lysine biosynthesis	-0.47	0.48	***-1.46***	4.71E-04
150.07	5.05	$C_9H_{10}O_2$	Phenylpropanoate	6	Amino Acid Metabolism	Phenylalanine metabolism	0.23	0.10	0.29	6.96E-04
137.08	28.46	$C_8H_{11}NO$	Tyramine	7	Amino Acid Metabolism	Tyrosine metabolism; Alkaloid biosynthesis I	-0.01	-0.11	-0.08	7.00E-04
243.09	11.15	$C_9H_{13}N_3O_5$	gamma-Glutamyl-beta-cyanoalanine	5	Amino Acid Metabolism	Cyanoamino acid metabolism	-0.65	0.02	***-1.34***	9.88E-04
199.10	11.93	$C_8H_{13}N_3O_3$	gamma-Glutamyl-	5	Amino Acid Metabolism	Cyanoamino acid metabolism	-0.78	-0.02	***-1.36***	1.12E-03

182.06	8.55	$C_9H_{10}O_4$	beta-aminopropionitrile 3-(2,3-Dihydroxyphenyl)propanoate	8	Amino Acid Metabolism	Phenylalanine metabolism	-0.82	0.16	***-1.53***	1.56E-03
185.08	11.53	$C_7H_{11}N_3O_3$	(S)-*N*-(4,5-Dihydro-1-methyl-4-oxo-1H-imidazol-2-yl)alanine	5	Amino Acid Metabolism	Undefined	-0.64	0.06	***-1.33***	1.73E-03
103.06	13.72	$C_4H_9NO_2$	*N*,*N*-Dimethylglycine	7	Amino Acid Metabolism	Glycine, serine and threonine metabolism	-0.58	-0.96	-0.38	2.70E-03
175.10	15.88	$C_6H_{13}N_3O_3$	L-Citrulline	8	Amino Acid Metabolism	Arginine and proline metabolism	-0.80	0.13	***-1.74***	5.39E-03
226.11	15.51	$C_9H_{14}N_4O_3$	Carnosine	5	Amino Acid Metabolism	Alanine and aspartate metabolism; Histidine metabolism; beta-Alanine metabolism	-0.52	0.14	***-1.01***	8.65E-03
172.05	11.69	$C_6H_8N_2O_4$	Hydantoin-5-propionate	5	Amino Acid Metabolism	Histidine metabolism	-0.87	-0.03	***-1.49***	1.71E-02
232.11	13.18	$C_9H_{16}N_2O_5$	gamma-Glutamyl-gamma-aminobutyrate	6	Amino Acid Metabolism	Arginine and proline metabolism	-0.87	-0.21	***-1.94***	2.36E-02
197.12	12.15	$C_9H_{15}N_3O_2$	Hercynine	5	Amino Acid Metabolism	Histidine metabolism	-0.71	-0.03	***-1.14***	3.72E-02
113.06	9.65	$C_4H_7N_3O$	Creatinine	7	Amino Acid Metabolism	Arginine and proline metabolism	0.37	0.56	0.26	3.81E-02
149.05	11.85	$C_5H_{11}NO_2S$	D-Methionine	7	Amino Acid Metabolism	Undefined	-0.56	0.20	-0.93	4.39E-02
188.08	10.28	$C_7H_{12}N_2O_4$	*N*-Acetylglutamine	9	Amino Acid Metabolism	Undefined	***-1.11***	-0.12	***-2.67***	6.98E-06
174.10	13.55	$C_7H_{14}N_2O_3$	N5-Ethyl-L-glutamine	5	Amino Acid Metabolism	Undefined	-0.93	0.39	***-1.78***	4.65E-05
103.06	11.30	$C_4H_9NO_2$	(R)-3-Amino-2-methylpropanoate	5	Amino Acid Metabolism	Undefined	-0.05	0.08	-0.01	7.71E-05
103.06	10.14	$C_4H_9NO_2$	*N*-methylalanine	5	Amino Acid Metabolism	Undefined	-0.03	0.09	0.02	1.91E-04
102.03	14.57	$C_4H_6O_3$	(R)-methylmalonate-semialdehyde	7	Amino Acid Metabolism	Undefined	-0.21	-0.10	-0.23	9.88E-04

202.06	11.99	$C_7H_{10}N_2O_5$	N3-fumaramoyl-L-2,3-diaminopropanoate	5	Amino Acid Metabolism	Undefined	-0.73	-0.02	***-1.26***	1.54E-03
226.10	8.13	$C_{10}H_{14}N_2O_4$	carbidopa	5	Amino Acid Metabolism	Undefined	-0.80	0.17	***-1.07***	7.56E-03
156.04	7.49	$C_7H_8O_4$	2,3-Dihydro-2,3-dihydroxybenzoate	8	Biosynthesis of Polyketides and Nonribosomal Peptides	Biosynthesis of siderophore group nonribosomal peptides	-0.14	-0.13	-0.17	3.96E-02
416.11	7.52	$C_{21}H_{20}O_9$	Daidzin	5	Biosynthesis of Secondary Metabolites	Isoflavonoid biosynthesis	***1.09***	0.18	0.77	7.23E-04
270.05	7.50	$C_{15}H_{10}O_5$	Apigenin	7	Biosynthesis of Secondary Metabolites	Flavonoid biosynthesis; Isoflavonoid biosynthesis	0.98	0.18	***1.20***	3.35E-03
194.08	10.29	$C_8H_{10}N_4O_2$	Caffeine	5	Biosynthesis of Secondary Metabolites	Caffeine metabolism	-0.48	0.31	-0.94	6.70E-03
200.08	9.10	$C_8H_{12}N_2O_4$	Dihydroclavaminic acid	5	Biosynthesis of Secondary Metabolites	Clavulanic acid biosynthesis	-0.64	0.17	***-1.02***	9.14E-03
503.18	16.96	$C_{18}H_{33}NO_{15}$	beta-D-Galactopyranosyl-(1-4)-2-amino-2-deoxy-beta-D-glucopyranosyl-(1-6)-D-mannose	7	Carbohydrate Metabolism	Undefined	-0.91	0.35	***-5.83***	3.42E-09
118.03	14.61	$C_4H_6O_4$	Succinate	8	Carbohydrate Metabolism	Citrate cycle (TCA cycle); Oxidative phosphorylation; Glutamate metabolism; Alanine and aspartate metabolism; Tyrosine metabolism; Phenylalanine metabolism; gamma-Hexachlorocyclohexane degradation; Glyoxylate and dicarboxylate metabolism	-0.94	-0.16	***-2.49***	8.22E-08
342.12	15.77	$C_{12}H_{22}O_{11}$	Lactose	10	Carbohydrate Metabolism	Galactose metabolism	***-1.79***	0.68	***-3.92***	1.11E-07
607.08	14.65	$C_{17}H_{27}N_3O_{17}P_2$	UDP-*N*-acetyl-D-glucosamine	10	Carbohydrate Metabolism	Aminosugars metabolism; Lipopolysaccharide biosynthesis; Peptidoglycan biosynthesis	***-1.03***	-0.32	***-3.75***	2.94E-07
230.02	15.29	$C_5H_{11}O_8P$	D-Ribose 5-phosphate	8	Carbohydrate Metabolism	Pentose phosphate pathway; Purine metabolism; Carbon fixation	-0.11	-0.23	***-1.82***	1.27E-05
259.05	15.95	$C_6H_{14}NO_8P$	alpha-D-Glucosamine 1-phosphate	8	Carbohydrate Metabolism	Aminosugars metabolism	***-1.21***	-0.94	***-5.13***	4.95E-05
166.05	11.39	$C_5H_{10}O_6$	L-Lyxonate	5	Carbohydrate Metabolism	Pentose and glucuronate interconversions; Ascorbate and aldarate metabolism	0.04	0.09	0.08	5.11E-05

130.03	14.09	$C_5H_6O_4$	2,5-Dioxopentanoate	7	Carbohydrate Metabolism	Ascorbate and aldarate metabolism; Arginine and proline metabolism	-0.92	-0.13	***-2.10***	6.65E-05
566.05	15.85	$C_{15}H_{24}N_2O_{17}P_2$	UDP-glucose	10	Carbohydrate Metabolism	Pentose and glucuronate interconversions; Galactose metabolism; Ascorbate and aldarate metabolism; Pyrimidine metabolism; Starch and sucrose metabolism; Nucleotide sugars metabolism; Glycerolipid metabolism; Zeatin biosynthesis; Biosynthesis of ansamycins	***-1.16***	-0.24	***-3.62***	1.75E-04
166.05	12.43	$C_5H_{10}O_6$	D-Xylonate	5	Carbohydrate Metabolism	Pentose and glucuronate interconversions	-0.02	0.12	0.03	1.78E-04
180.06	16.28	$C_6H_{12}O_6$	D-Galactose	8	Carbohydrate Metabolism	Galactose metabolism	0.03	0.07	0.04	2.81E-04
134.02	15.46	$C_4H_6O_5$	(S)-Malate	10	Carbohydrate Metabolism	Citrate cycle (TCA cycle); Glutamate metabolism; Alanine and aspartate metabolism; Pyruvate metabolism; Glyoxylate and dicarboxylate metabolism; Carbon fixation; Reductive carboxylate cycle (CO2 fixation)	-0.26	-0.04	***-1.47***	4.55E-04
255.71	5.19	$C_{21}H_{36}N_7O_{16}P_3S$	CoA	8	Carbohydrate Metabolism	Citrate cycle (TCA cycle); Fatty acid metabolism; Pantothenate and CoA biosynthesis	***-1.72***	-0.73	***-2.59***	4.71E-04
185.99	16.45	$C_3H_7O_7P$	3-Phospho-D-glycerate	10	Carbohydrate Metabolism	Glycolysis / Gluconeogenesis; Glycine, serine and threonine metabolism; Glycerolipid metabolism; Glyoxylate and dicarboxylate metabolism; Carbon fixation	-0.89	-0.04	***-2.25***	6.96E-04
200.01	15.77	$C_4H_9O_7P$	D-Erythrose 4-phosphate	8	Carbohydrate Metabolism	Pentose phosphate pathway; Phenylalanine, tyrosine and tryptophan biosynthesis; Carbon fixation; Vitamin B6 metabolism	***-1.18***	-0.78	***-3.07***	1.12E-03
106.03	16.37	$C_3H_6O_4$	D-Glycerate	6	Carbohydrate Metabolism	Glycine, serine and threonine metabolism; Glycerolipid metabolism; Glyoxylate and dicarboxylate metabolism	-0.82	-0.01	***-1.92***	1.54E-03
621.06	17.20	$C_{17}H_{25}N_3O_{18}P_2$	UDP-*N*-acetyl-D-mannosaminouronate	8	Carbohydrate Metabolism	Aminosugars metabolism	-0.86	0.20	***-1.73***	1.54E-03
260.03	15.64	$C_6H_{13}O_9P$	D-Glucose 6-phosphate	10	Carbohydrate Metabolism	Starch and sucrose metabolism; Streptomycin biosynthesis; Inositol phosphate metabolism	-0.90	-0.74	***-2.96***	1.65E-03
166.05	12.88	$C_5H_{10}O_6$	D-Arabinonate	7	Carbohydrate Metabolism	D-arabinose degradation III	-0.09	0.15	0.06	1.66E-03
146.02	14.99	$C_5H_6O_5$	α-ketoglutarate	10	Carbohydrate Metabolism	Citrate cycle (TCA cycle); Ascorbate and aldarate metabolism; Glutamate metabolism; Alanine and aspartate metabolism; Lysine biosynthesis; Histidine metabolism; D-Glutamine and D-glutamate metabolism; Glyoxylate and dicarboxylate metabolism	-0.69	-0.23	***-2.01***	2.84E-03

290.04	15.77	$C_7H_{15}O_{10}P$	D-Sedoheptulose 7-phosphate	6	Carbohydrate Metabolism	Pentose phosphate pathway; Carbon fixation	***-1.31***	-0.76	***-3.09***	2.91E-03
504.17	16.32	$C_{18}H_{32}O_{16}$	Maltotriose	10	Carbohydrate Metabolism	glycogen degradation I	-0.59	0.12	-0.34	3.18E-03
214.02	13.32	$C_5H_{11}O_7P$	2-Deoxy-D-ribose 5-phosphate	8	Carbohydrate Metabolism	Pentose phosphate pathway	-0.82	-0.03	***-1.79***	4.58E-03
162.05	15.57	$C_6H_{10}O_5$	2-Dehydro-3-deoxy-L-rhamnonate	8	Carbohydrate Metabolism	Fructose and mannose metabolism	-0.06	0.07	-0.31	1.52E-02
180.06	13.88	$C_6H_{12}O_6$	D-Glucose	10	Carbohydrate Metabolism	Glycolysis / Gluconeogenesis; Pentose phosphate pathway; Galactose metabolism; Starch and sucrose metabolism; Streptomycin biosynthesis; Indole and ipecac alkaloid biosynthesis	0.27	0.31	0.22	2.51E-02
210.04	16.42	$C_6H_{10}O_8$	D-Glucarate	8	Carbohydrate Metabolism	Ascorbate and aldarate metabolism	-0.40	0.44	-0.02	2.65E-02
179.08	13.28	$C_6H_{13}NO_5$	D-Galactosamine	7	Carbohydrate Metabolism	Galactose metabolism	-0.65	-0.24	***-1.23***	3.95E-02
545.20	16.94	$C_{20}H_{35}NO_{16}$	alpha-D-Galactosyl-*N*-acetyllactosamine	5	Carbohydrate Metabolism	Undefined	***-1.82***	0.15	***-5.73***	1.99E-09
189.10	12.26	$C_8H_{15}NO_4$	Castanospermine	5	Carbohydrate Metabolism	Undefined	-0.30	-0.15	-0.25	7.16E-04
120.04	15.29	$C_4H_8O_4$	D-Erythrulose	5	Carbohydrate Metabolism	Undefined	-0.74	0.49	-0.71	2.98E-03
662.09	15.84	$C_{19}H_{28}N_4O_{18}P_2$	UDP-2,3-diacetamido-2,3-dideoxy-alpha-D-glucuronate	5	Carbohydrate Metabolism	Undefined	-0.78	-0.49	***-2.25***	4.03E-03
663.11	13.82	$C_{21}H_{27}N_7O_{14}P_2$	NAD^+	10	Energy Metabolism	Oxidative phosphorylation; Glutamate metabolism; Nicotinate and nicotinamide metabolism	***-1.04***	-0.01	***-1.93***	1.35E-05
97.98	12.44	H_3O_4P	Orthophosphate	9	Energy Metabolism	Oxidative phosphorylation; Photosynthesis; Peptidoglycan biosynthesis	-0.09	0.03	-0.06	3.19E-03
210.07	13.72	$C_7H_{14}O_7$	Sedoheptulose	7	Energy Metabolism	Carbon fixation	-0.73	-0.66	***-2.26***	1.04E-02
215.06	15.43	$C_5H_{14}NO_6P$	*sn*-glycero-3-Phosphoethanolamine	7	Lipid Metabolism	Glycerophospholipid metabolism; Ether lipid metabolism	***-2.42***	-0.47	***-2.83***	1.05E-06
145.11	28.45	$C_7H_{15}NO_2$	Acetylcholine	7	Lipid Metabolism	Glycerophospholipid metabolism	0.01	-0.12	-0.02	2.18E-03

515.68	5.20	$C_{39}H_{68}N_7O_{17}P_3S$	Oleoyl-CoA	5	Lipid Metabolism	Biosynthesis of unsaturated fatty acids	***-1.82***	-0.91	***-1.65***	1.98E-02
242.02	15.46	$C_6H_{11}O_8P$	D-*myo*-Inositol 1,2-cyclic phosphate	5	Lipid Metabolism	Undefined	***-1.00***	-0.79	***-2.75***	2.27E-03
548.48	3.76	$C_{35}H_{64}O_4$	Cohibin A	5	Lipids: Fatty Acyls	Undefined	0.90	-0.03	-0.95	2.70E-06
118.06	7.48	$C_5H_{10}O_3$	5-Hydroxypentanoate	7	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.05	0.26	***-2.25***	1.51E-05
298.25	3.95	$C_{18}H_{34}O_3$	FA oxo(18:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	-0.28	0.15	***1.83***	3.93E-05
186.16	4.17	$C_{11}H_{22}O_2$	FA (11:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.13	0.22	0.21	6.48E-05
116.05	6.77	$C_5H_8O_3$	3-Oxopentanoic acid	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.10	0.24	0.34	1.36E-04
158.13	4.36	$C_9H_{18}O_2$	FA methyl(8:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	-0.03	0.10	-0.02	1.68E-04
103.06	12.13	$C_4H_9NO_2$	(S)-2-Aminobutanoate	7	Lipids: Fatty Acyls	Amino fatty acids	0.01	0.02	0.05	1.83E-04
200.18	4.10	$C_{12}H_{24}O_2$	Dodecanoic acid	9	Lipids: Fatty Acyls	Fatty acid biosynthesis	-0.08	0.09	0.22	2.29E-04
116.08	5.13	$C_6H_{12}O_2$	Hexanoic acid	9	Lipids: Fatty Acyls	OxidationofVeryLongChainFattyAcids	0.07	0.35	0.10	2.83E-04
103.06	11.79	$C_4H_9NO_2$	FA amino(4:0)	5	Lipids: Fatty Acyls	Amino Fatty Acids	0.01	0.09	0.03	4.81E-04
171.16	4.68	$C_{10}H_{21}NO$	decanamide	5	Lipids: Fatty Acyls	Fatty amides	0.41	0.34	0.16	5.23E-04
270.22	4.06	$C_{16}H_{30}O_3$	FA oxo(16:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.14	0.32	0.32	2.52E-03
256.24	3.95	$C_{16}H_{32}O_2$	FA(16:0)	7	Lipids: Fatty Acyls	Fatty acid biosynthesis; Fatty acid elongation in mitochondria; Fatty acid metabolism; Biosynthesis of unsaturated fatty acids	-0.53	-0.23	-0.12	1.02E-02
574.50	3.74	$C_{37}H_{66}O_4$	Montecristin	5	Lipids: Fatty Acyls	Undefined	-0.37	-0.05	-0.14	1.04E-02
102.07	7.47	$C_5H_{10}O_2$	3-Methylbutanoic acid	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.00	0.08	-0.13	1.62E-02
266.19	3.92	$C_{16}H_{26}O_3$	FA trimethyl(13:2)	7	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.32	-0.02	0.19	3.20E-02
214.19	4.06	$C_{13}H_{26}O_2$	FA methyl(12:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	-0.27	0.17	-0.12	4.52E-02
602.53	3.73	$C_{39}H_{70}O_4$	1-(14-methyl-pentadecanoyl)-2-(8-[3]-	5	Lipids: Glycerolipids	Diradylglycerols	-0.72	-0.17	-0.10	4.85E-04

			ladderane-octanyl)-*sn*-glycerol							
743.55	4.12	$C_{41}H_{78}NO_8P$	PE(36:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	***-1.23***	-0.27	0.75	5.80E-07
410.24	7.50	$C_{19}H_{39}O_7P$	PA(16:0)	7	Lipids: Glycerophospholipids	Glycerophosphates	0.86	0.52	***2.56***	6.76E-07
689.50	4.24	$C_{37}H_{72}NO_8P$	PE(32:1)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	***1.12***	0.17	-0.45	1.56E-06
715.52	4.18	$C_{39}H_{74}NO_8P$	PE(34:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	-0.73	-0.02	0.12	1.95E-05
687.48	4.23	$C_{37}H_{70}NO_8P$	PE(32:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	0.86	0.02	-0.47	2.23E-05
713.50	4.17	$C_{39}H_{72}NO_8P$	PE(34:3)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	-0.53	-0.14	0.20	2.53E-05
720.49	3.76	$C_{38}H_{73}O_{10}P$	PG(32:1)	5	Lipids: Glycerophospholipids	Glycerophosphoglycerols	0.92	-0.01	***-1.32***	2.88E-05
735.50	3.76	$C_{38}H_{74}NO_{10}P$	PS(32:0)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	0.96	0.09	-0.67	3.65E-05
733.52	4.28	$C_{39}H_{76}NO_9P$	PS(O-33:1)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	***1.35***	-0.11	0.86	3.99E-04
791.57	3.72	$C_{42}H_{82}NO_{10}P$	PS(36:0)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	-0.70	-0.13	-0.13	2.24E-03
467.30	4.92	$C_{22}H_{46}NO_7P$	PC(14:0)	5	Lipids: Glycerophospholipids	Glycerophosphocholines	***-1.35***	-0.10	***-3.21***	2.31E-03
479.30	4.87	$C_{23}H_{46}NO_7P$	PE(18:1)	7	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	***-1.57***	0.12	***-1.48***	2.93E-03
760.53	3.75	$C_{41}H_{77}O_{10}P$	PG(35:2)	5	Lipids: Glycerophospholipids	Glycerophosphoglycerols	-0.73	-0.12	***-1.39***	3.11E-03
763.54	3.74	$C_{40}H_{78}NO_{10}P$	PS(34:0)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	-0.35	0.02	-0.14	2.65E-02
246.05	12.26	$C_6H_{15}O_8P$	Glycerophosphoglycerol [PR]	7	Lipids: Glycerophospholipids	Undefined	***-1.14***	-0.46	***-1.10***	1.22E-02
546.46	3.77	$C_{35}H_{62}O_4$	bacteriohopane-32,33,34,35-tetrol	5	Lipids: Prenols	Hopanoids	0.85	-0.02	-0.63	7.79E-05
315.28	4.17	$C_{18}H_{37}NO_3$	Dehydrophytosphingosine	5	Lipids: Sphingolipids	Sphingoid bases	-0.14	-0.21	0.40	6.81E-03
155.99	8.59	$C_3H_8O_3S_2$	2-(Methylthio)ethanesulfonate	5	Metabolism of Cofactors and Vitamins	Folate biosynthesis	0.42	0.36	0.55	1.05E-03
267.10	9.01	$C_{10}H_{13}N_5O_4$	Adenosine	8	Nucleotide Metabolism	Purine metabolism	-0.11	0.18	***1.68***	1.44E-08
363.06	15.75	$C_{10}H_{14}N_5O_8P$	GMP	10	Nucleotide Metabolism	Purine metabolism; Glutamate metabolism	***-1.28***	-0.13	***-3.22***	8.22E-08

97.97	17.34	H_2O_4S	Sulfate	8	Nucleotide Metabolism	Purine metabolism; Cysteine metabolism; Sulfur metabolism	0.05	0.12	0.26	2.55E-06
283.09	12.38	$C_{10}H_{13}N_5O_5$	Guanosine	8	Nucleotide Metabolism	Purine metabolism	***-1.23***	-0.01	***-4.28***	4.90E-06
404.00	16.47	$C_9H_{14}N_2O_{12}P_2$	UDP	10	Nucleotide Metabolism	Pyrimidine metabolism; Peptidoglycan biosynthesis; Zeatin biosynthesis	-0.37	0.27	***-1.47***	2.08E-05
158.03	10.87	$C_5H_6N_2O_4$	(S)-Dihydroorotate	8	Nucleotide Metabolism	Pyrimidine metabolism	***-1.09***	-0.74	***-6.85***	2.08E-05
403.02	16.67	$C_9H_{15}N_3O_{11}P_2$	CDP	6	Nucleotide Metabolism	Pyrimidine metabolism	-0.18	0.18	***-1.57***	2.50E-05
324.04	14.13	$C_9H_{13}N_2O_9P$	UMP	10	Nucleotide Metabolism	Pyrimidine metabolism; Peptidoglycan biosynthesis	***-1.19***	-0.11	***-2.07***	3.72E-05
176.04	16.17	$C_5H_8N_2O_5$	*N*-Carbamoyl-L-aspartate	8	Nucleotide Metabolism	Pyrimidine metabolism; Alanine and aspartate metabolism	***-1.26***	-0.67	***-6.05***	8.31E-05
347.06	13.21	$C_{10}H_{14}N_5O_7P$	AMP	10	Nucleotide Metabolism	Purine metabolism; Zeatin biosynthesis	-0.92	-0.07	***-1.74***	1.06E-04
251.10	11.31	$C_{10}H_{13}N_5O_3$	5'-Deoxyadenosine	6	Nucleotide Metabolism	Undefined	-0.68	-0.04	***-1.44***	1.81E-04
268.08	10.73	$C_{10}H_{12}N_4O_5$	Inosine	8	Nucleotide Metabolism	Purine metabolism	***-1.49***	-0.08	***-4.76***	2.88E-04
347.06	12.50	$C_{10}H_{14}N_5O_7P$	3'-AMP	7	Nucleotide Metabolism	Purine metabolism	-0.82	0.02	***-1.64***	4.79E-04
114.04	10.64	$C_4H_6N_2O_2$	5,6-Dihydrouracil	8	Nucleotide Metabolism	Pyrimidine metabolism; beta-Alanine metabolism; Pantothenate and CoA biosynthesis	***-1.12***	-0.71	***-3.88***	1.00E-03
244.07	13.71	$C_9H_{12}N_2O_6$	Pseudouridine	6	Nucleotide Metabolism	Pyrimidine metabolism	-0.87	0.04	***-1.21***	1.59E-03
308.04	13.26	$C_9H_{13}N_2O_8P$	dUMP	8	Nucleotide Metabolism	Pyrimidine metabolism	0.60	0.09	0.87	1.68E-03
152.03	10.92	$C_5H_4N_4O_2$	Xanthine	10	Nucleotide Metabolism	Purine metabolism; Caffeine metabolism	-0.15	0.11	***-1.20***	1.68E-03
322.06	12.34	$C_{10}H_{15}N_2O_8P$	dTMP	10	Nucleotide Metabolism	Pyrimidine metabolism	***-1.21***	-0.09	0.95	2.22E-03
323.05	15.08	$C_9H_{14}N_3O_8P$	CMP	10	Nucleotide Metabolism	Pyrimidine metabolism	-0.92	-0.28	***-1.83***	6.05E-03
481.99	15.73	$C_{10}H_{17}N_2O_{14}P_3$	dTTP	6	Nucleotide Metabolism	Pyrimidine metabolism	-0.73	0.08	***-1.37***	6.51E-03
331.07	12.26	$C_{10}H_{14}N_5O_6P$	dAMP	8	Nucleotide Metabolism	Purine metabolism	-0.97	-0.05	-0.24	9.14E-03
243.09	11.73	$C_9H_{13}N_3O_5$	Cytidine	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.80	-0.09	***-1.29***	9.39E-03
244.07	9.77	$C_9H_{12}N_2O_6$	Uridine	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.49	0.37	-0.97	1.33E-02
136.04	10.01	$C_5H_4N_4O$	Hypoxanthine	10	Nucleotide Metabolism	Purine metabolism	-0.90	0.30	***-1.48***	1.98E-02
364.04	17.41	$C_{10}H_{13}N_4O_9P$	Xanthosine 5'-phosphate	8	Nucleotide Metabolism	Purine metabolism	-0.26	0.04	***-1.40***	2.36E-02
443.02	17.54	$C_{10}H_{15}N_5O_{11}P_2$	GDP	10	Nucleotide Metabolism	Purine metabolism	-0.20	0.19	-0.05	2.36E-02
112.03	9.75	$C_4H_4N_2O_2$	Orotate(Fragment)	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.42	0.19	-0.92	2.76E-02
402.02	14.31	$C_{10}H_{16}N_2O_{11}P_2$	dTDP	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.92	0.08	***-1.30***	3.25E-02

681.12	16.94	$C_{20}H_{33}N_3O_{19}P_2$	dihydro-UDP-*N*-acetylmuramate	5	Nucleotide Metabolism	Undefined	***-1.44***	0.03	***-4.97***	2.31E-10
151.05	16.21	$C_5H_5N_5O$	2-Hydroxyadenine	5	Nucleotide Metabolism	Undefined	***-1.18***	0.00	***-2.54***	2.70E-06
348.05	14.94	$C_{10}H_{13}N_4O_8P$	Inosine2'-phosphate	7	Nucleotide Metabolism	Undefined	-0.48	0.11	***-1.69***	7.14E-06
541.06	13.83	$C_{15}H_{21}N_5O_{13}P_2$	Cyclic ADP-ribose	5	Nucleotide Metabolism	Undefined	-0.98	0.00	***-1.95***	9.72E-06
122.05	13.90	$C_6H_6N_2O$	Isonicotineamide	5	Nucleotide Metabolism	Undefined	-0.81	-0.13	***-1.72***	1.20E-04
156.02	10.20	$C_5H_4N_2O_4$	Uracil 5-carboxylate	5	Nucleotide Metabolism	Undefined	-0.76	-0.69	***-4.77***	5.62E-04
265.12	11.26	$C_{11}H_{15}N_5O_3$	N6-Methyl-2'-deoxyadenosine	5	Nucleotide Metabolism	Undefined	-0.68	0.06	***-1.47***	7.00E-04
212.01	13.11	$C_5H_9O_7P$	P-DPD	6	Nucleotide Metabolism	Undefined	0.49	0.06	***-1.38***	8.46E-04
258.09	15.84	$C_{10}H_{14}N_2O_6$	Ribothymidine	5	Nucleotide Metabolism	Undefined	-0.60	-0.38	***-2.79***	1.59E-03
257.10	10.51	$C_{10}H_{15}N_3O_5$	5-Methylcytidine	5	Nucleotide Metabolism	Undefined	-0.83	0.02	***-1.12***	4.59E-03
230.09	11.00	$C_9H_{14}N_2O_5$	Aspartyl-L-proline	5	Peptide(di-)	Acidic peptide	-0.76	-0.03	***-1.17***	1.14E-03
214.13	8.23	$C_{10}H_{18}N_2O_3$	Val-Pro	7	Peptide(di-)	Hydrophobic peptide	0.18	0.26	0.32	2.36E-02
229.11	10.60	$C_9H_{15}N_3O_4$	Asn-Pro	5	Peptide(di-)	Polar peptide	-0.67	0.09	***-1.03***	4.75E-02
382.20	13.85	$C_{16}H_{26}N_6O_5$	Ala-Val-Gly-His	5	Peptide(tetra-)	Basic peptide	-0.77	0.15	***-1.28***	7.27E-04
340.17	12.43	$C_{15}H_{24}N_4O_5$	Ala-Gly-Pro-Pro	5	Peptide(tetra-)	Polar peptide	-0.68	0.22	0.51	3.59E-03
400.23	10.64	$C_{18}H_{32}N_4O_6$	Ala-Leu-Thr-Pro	5	Peptide(tetra-)	Hydrophobic peptide	0.06	0.31	0.59	1.01E-02
361.15	15.53	$C_{14}H_{23}N_3O_8$	Glu-Asp-Val	5	Peptide(tri-)	Hydrophobic peptide	***-1.76***	0.62	***-3.02***	5.30E-07
257.14	15.06	$C_{11}H_{19}N_3O_4$	Ala-Ala-Pro	7	Peptide(tri-)	Nonpolar peptide	-0.70	0.05	***-1.15***	1.28E-03
356.24	10.84	$C_{17}H_{32}N_4O_4$	Ile-Lys-Pro	5	Peptide(tri-)	Basic peptide	-0.44	0.13	-0.17	1.50E-03
313.16	11.71	$C_{14}H_{23}N_3O_5$	Thr-Pro-Pro	7	Peptide(tri-)	Polar peptide	***-1.28***	0.39	0.41	2.52E-03
343.17	11.73	$C_{15}H_{25}N_3O_6$	Ile-Asp-Pro	7	Peptide(tri-)	Hydrophobic peptide	-0.43	0.28	0.37	4.01E-03
370.23	11.76	$C_{16}H_{30}N_6O_4$	Val-Pro-Arg	5	Peptide(tri-)	Basic peptide	-0.74	-0.03	***-1.21***	4.65E-03
359.19	7.48	$C_{19}H_{25}N_3O_4$	Phe-Pro-Pro	5	Peptide(tri-)	Hydrophobic peptide	-0.19	0.04	0.12	1.62E-02

289.13	12.68	$C_{11}H_{19}N_3O_6$	Ophthalmicacid	7	Peptide(tri-)	Undefined	***-1.30***	0.03	***-3.31***	6.46E-06
208.06	9.95	$C_6H_{13}N_2O_4P$	*N*-acetyl-demethylphosphinothricin	7	Peptide(tri-)	Undefined	0.15	0.09	0.06	1.64E-03
283.13	14.02	$C_{11}H_{17}N_5O_4$	Gamma-glutamyl-Histidine	5	Peptides	Undefined	-0.72	0.10	***-1.12***	1.66E-03
246.12	12.41	$C_{10}H_{18}N_2O_5$	L-gamma-glutamyl-L-valine	5	Peptides	Undefined	-0.23	0.33	0.24	1.66E-03
204.07	15.59	$C_7H_{12}N_2O_5$	L-beta-aspartyl-L-alanine	5	Peptides	Undefined	-0.24	0.46	-0.22	1.16E-02
232.14	7.53	$C_{10}H_{20}N_2O_4$	Leucyl-Threonine	5	Peptides	Undefined	0.07	0.38	0.23	1.42E-02
230.16	5.19	$C_{11}H_{22}N_2O_3$	Valyl-Leucine	7	Peptides	Undefined	0.20	0.66	0.37	4.93E-02
158.07	8.80	$C_6H_{10}N_2O_3$	1-(Hydroxymethyl)-5,5-dimethyl-2,4-imidazolidinedione	7	Undefined	Undefined	-0.32	0.61	***-6.13***	1.98E-08
324.11	15.38	$C_{10}H_{28}Cl_2N_2OP_2$	Polixetonium chloride	5	Undefined	Undefined	***-1.67***	0.52	***-2.87***	1.31E-07
182.08	6.34	$C_{12}H_{10}N_2$	Harman	7	Undefined	Undefined	0.78	0.29	***1.77***	8.89E-06
326.19	3.78	$C_{18}H_{30}O_3S$	2-Dodecylbenzenesulfonic acid	5	Undefined	Undefined	0.17	0.08	0.88	1.06E-05
249.09	7.78	$C_{10}H_{19}NO_2S_2$	S-Acetyldihydrolipoamide	8	Undefined	Alanineandaspartatemetabolism	0.10	-0.16	0.69	2.53E-05
213.11	7.74	$C_9H_{15}N_3O_3$	Methyl 2-diazoacetamidohexonate	5	Undefined	Undefined	-0.50	0.28	***-1.80***	6.11E-05
141.99	11.58	$C_2H_7O_3PS$	dimethylthiophosphate	5	Undefined	methyl parathion degradation	0.08	0.02	0.33	7.13E-05
216.07	12.04	$C_8H_{12}N_2O_5$	N3-(4-methoxyfumaroyl)-L-2,3-diaminopropanoate	5	Undefined	Undefined	-0.68	0.19	***-1.14***	1.91E-04
302.22	7.35	$C_{20}H_{30}O_2$	Retinyl ester	5	Undefined	Undefined	-0.08	0.06	0.03	2.70E-04

312.18	3.80	$C_{17}H_{28}O_3S$	*N*-Undecylbenzenesulfonic acid	7	Undefined	Undefined	0.48	0.36	***1.10***	3.12E-04
266.10	10.15	$C_{11}H_{14}N_4O_4$	8-Oxodeoxycoformycin	5	Undefined	Undefined	-0.61	0.15	***-1.27***	3.32E-04
232.04	11.58	$C_5H_{13}O_8P$	D-arabitol 5-phosphate	5	Undefined	Undefined	0.03	0.12	0.12	4.10E-04
186.06	10.21	$C_7H_{10}N_2O_4$	(S)-AMPA	5	Undefined	Undefined	-0.70	0.07	***-1.26***	4.71E-04
230.09	9.53	$C_9H_{14}N_2O_5$	(1R,2S,3R)-2-Acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole	7	Undefined	Undefined	-0.76	0.09	***-1.10***	4.85E-04
254.06	7.51	$C_{15}H_{10}O_4$	Apigeninidin	5	Undefined	Undefined	0.95	0.12	0.68	4.85E-04
236.13	7.81	$C_{11}H_{16}N_4O_2$	CPX	5	Undefined	Undefined	-0.50	0.23	***-1.56***	6.34E-04
208.06	10.93	$C_6H_{13}N_2O_4P$	*N*-acetyl-demethylphosphinothricin	5	Undefined	Undefined	-0.11	-0.03	-0.13	6.34E-04
189.10	9.86	$C_8H_{15}NO_4$	(2S)-2-{[1-(R)-Carboxyethyl]amino}pentanoate	5	Undefined	Undefined	-0.13	-0.01	-0.21	6.46E-04
165.08	13.63	$C_9H_{11}NO_2$	3-Pyridinebutanoicacid	7	Undefined	Undefined	***-1.39***	-0.51	***-3.82***	6.60E-04
278.19	7.31	$C_{20}H_{24}N$	2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)	7	Undefined	Undefined	-0.02	0.03	0.05	6.96E-04
130.11	7.49	$C_6H_{14}N_2O$	*N*-Acetylputrescinium	5	Undefined	Undefined	0.41	0.37	0.37	7.16E-04
101.12	10.05	$C_6H_{15}N$	Hexylamine	5	Undefined	Undefined	-0.08	0.00	-0.11	9.05E-04
168.05	10.03	$C_7H_8N_2O_3$	2,3-Diaminosalicylic acid	5	Undefined	Undefined	-0.69	0.06	***-1.44***	1.23E-03
203.08	14.52	$C_8H_{13}NO_5$	2-acetamidoglucal	5	Undefined	Undefined	***-1.01***	-0.49	***-1.53***	1.26E-03
126.04	15.21	$C_5H_6N_2O_2$	Imidazol-4-ylacetate	5	Undefined	Undefined	-0.94	-0.33	***-2.66***	1.28E-03

160.11	7.47	$C_8H_{16}O_3$	Ethyl (R)-3-hydroxyhexanoate	7	Undefined	Undefined	-0.09	0.16	0.29	1.41E-03
233.18	7.17	$C_{15}H_{23}NO$	Deoxynuphharidine	5	Undefined	Undefined	-0.01	0.17	0.08	1.56E-03
129.15	8.17	$C_8H_{19}N$	Octylamine	5	Undefined	Undefined	-0.02	0.01	0.01	1.60E-03
921.38	13.86	$C_{37}H_{59}N_7O_{20}$	GlcNAc-1,6-anhMurNAc-L-Ala-gamma-D-Glu-DAP-D-Ala	7	Undefined	Undefined	***-2.29***	-0.07	***-3.17***	1.65E-03
125.05	15.81	$C_6H_7NO_2$	3-Hydroxyaminophenol	5	Undefined	Undefined	***-1.12***	-0.75	***-3.15***	1.73E-03
198.06	15.84	$C_8H_{10}N_2O_4$	(S)-2-Amino-3-(3-hydroxy-4-oxo-4H-pyridi*N*-1-yl)propanoate	5	Undefined	Undefined	-0.69	-0.30	***-2.26***	2.20E-03
193.11	11.62	$C_{11}H_{15}NO_2$	3,4-Methylenedioxymethamphetamine	7	Undefined	Undefined	-0.09	0.04	-0.13	2.23E-03
193.11	12.80	$C_{11}H_{15}NO_2$	Heliamine	7	Undefined	Undefined	-0.02	0.06	0.02	2.26E-03
172.05	10.82	$C_6H_8N_2O_4$	(R)-AMAA	5	Undefined	Undefined	-0.87	0.12	***-1.44***	2.52E-03
272.03	15.76	$C_7H_{13}O_9P$	alpha-(2,6-anhydro-3-deoxy-D-arabino-heptulopyranosid)onate 7-phosphate	5	Undefined	Undefined	***-1.17***	-0.85	***-3.01***	2.52E-03
250.10	8.57	$C_{12}H_{14}N_2O_4$	3-Oxohexobarbital	5	Undefined	Undefined	-0.34	0.28	***-1.02***	3.46E-03
163.08	10.89	$C_6H_{13}NO_4$	Bicine	5	Undefined	Undefined	-0.54	0.15	***-1.15***	3.54E-03
224.09	11.04	$C_9H_{12}N_4O_3$	Temurin	5	Undefined	Undefined	-0.57	0.13	***-1.40***	3.59E-03
171.06	10.68	$C_6H_9N_3O_3$	Metronidazole	5	Undefined	Undefined	-0.44	0.13	***-1.63***	3.99E-03
155.09	7.41	$C_8H_{13}NO_2$	Arecoline	5	Undefined	Undefined	0.05	0.01	0.12	4.84E-03
173.07	12.33	$C_7H_{11}NO_4$	5-Deoxy-5-aminoshikimic acid	5	Undefined	Undefined	-0.63	0.15	***-1.11***	5.84E-03

193.11	11.08	$C_{11}H_{15}NO_2$	Salsoline	7	Undefined	Undefined	-0.10	0.04	-0.24	7.00E-03
146.06	4.37	$C_6H_{10}O_4$	Dimethyl succinate	5	Undefined	Undefined	0.16	0.12	0.10	8.00E-03
238.13	7.48	$C_{12}H_{18}N_2O_3$	Secobarbital	5	Undefined	Undefined	-0.01	-0.08	-0.04	8.00E-03
182.09	4.41	$C_{10}H_{14}O_3$	5-Oxo-1,2-campholide	5	Undefined	D-camphor degradation	0.17	-0.10	0.19	8.74E-03
228.11	7.48	$C_{10}H_{16}N_2O_4$	(S)-ATPA	5	Undefined	Undefined	-0.76	0.05	-0.97	1.42E-02
359.19	5.25	$C_{21}H_{27}O_5$	19-oic-deoxycorticosterone	5	Undefined	Undefined	0.17	-0.21	0.54	1.81E-02
260.08	7.48	$C_{10}H_{16}N_2O_4S$	d-biotin d-sulfoxide	6	Undefined	Undefined	-0.76	0.11	***-1.02***	2.39E-02
228.11	5.42	$C_{10}H_{16}N_2O_4$	Tetraacetylethylenediamine	5	Undefined	Undefined	-0.59	0.07	***-1.04***	3.23E-02
1005.34	7.58	$C_{37}H_{66}N_7O_{17}P_3S$	4,8,12-Trimethyltridecanoyl-CoA	7	Undefined	Undefined	***-1.86***	-0.42	***-3.33***	4.04E-02

Table A1.2. Significant metabolites identified following exposure to polymyxin B and mitotane alone and in combination in *A. baumannii* ATCC 19606. Significant fold-changes are highlighted in bold and italic. One-way ANOVA for multiple comparison, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$.

MASS	RT	FORMULA	METABOLITE	CONFIDENCE	MAP	PATHWAY	Log_2-fold change			FDR
							Polmyxin B	Mitotane	Combination	
133.04	14.70	$C_4H_7NO_4$	L-Aspartate	10	Amino Acid Metabolism	Alanine and aspartate metabolism; Arginine and proline metabolism; Glycine, serine and threonine metabolism; Lysine biosynthesis; Arginine and proline metabolism; Histidine metabolism; beta-Alanine metabolism; Cyanoamino acid metabolism; Carbon fixation	-0.58	0.02	-0.40	3.79E-02
274.13	15.01	$C_{10}H_{18}N_4O_5$	N2-Succinyl-L-arginine	8	Amino Acid Metabolism	Arginine and proline metabolism	-0.51	-0.01	0.34	1.82E-02
115.06	12.73	$C_5H_9NO_2$	L-Proline	10	Amino Acid Metabolism	Arginine and proline metabolism; Novobiocin biosynthesis	0.35	***-1.41***	***-1.65***	2.89E-04
132.09	15.53	$C_5H_{12}N_2O_2$	D-Ornithine	7	Amino Acid Metabolism	D-Arginine and D-ornithine metabolism	-0.66	-0.03	-0.89	3.93E-02
103.06	13.72	$C_4H_9NO_2$	*N,N*-Dimethylglycine	7	Amino Acid Metabolism	Glycine, serine and threonine metabolism	0.07	-0.27	-0.69	1.03E-02
104.01	15.16	$C_3H_4O_4$	Hydroxypyruvate	6	Amino Acid Metabolism	Glycine, serine and threonine metabolism; Glyoxylate and dicarboxylate metabolism	0.13	-0.03	-0.19	3.79E-02
258.09	13.56	$C_{10}H_{14}N_2O_6$	(1-Ribosylimidazole)-4-acetate	5	Amino Acid Metabolism	Histidine metabolism	-0.90	-0.31	-0.79	2.09E-02
174.06	14.21	$C_6H_{10}N_2O_4$	*N*-Formimino-L-glutamate	7	Amino Acid Metabolism	Histidine metabolism	-0.66	-0.57	***-1.56***	8.60E-03
138.04	10.37	$C_6H_6N_2O_2$	Urocanate	7	Amino Acid Metabolism	Histidine metabolism	0.11	0.30	0.71	6.01E-03
161.11	13.16	$C_7H_{15}NO_3$	L-Carnitine	10	Amino Acid Metabolism	Lysine degradation	***-1.19***	***-1.25***	***-2.49***	1.72E-04
103.06	11.30	$C_4H_9NO_2$	(R)-3-Amino-2-methylpropanoate	5	Amino Acid Metabolism	Undefined	-0.02	-0.25	0.11	3.85E-02
174.10	13.55	$C_7H_{14}N_2O_3$	N5-Ethyl-L-glutamine	5	Amino Acid Metabolism	Undefined	-0.91	-0.18	***-1.03***	2.20E-02
188.08	10.28	$C_7H_{12}N_2O_4$	*N*-Acetylglutamine	9	Amino Acid Metabolism	Undefined	-0.86	-0.29	***-1.10***	4.34E-02
416.11	7.52	$C_{21}H_{20}O_9$	Daidzin	5	Biosynthesis of Secondary Metabolites	Isoflavonoid biosynthesis	0.95	-0.07	0.77	2.39E-02
136.04	11.88	$C_4H_8O_5$	[FA trihydroxy(4:0)] 2,3,4-trihydroxy-butanoic acid	5	Carbohydrate Metabolism	Ascorbate and aldarate metabolism	0.81	0.58	0.59	7.06E-03

180.06	14.68	$C_6H_{12}O_6$	D-Mannose	8	Carbohydrate Metabolism	Fructose and mannose metabolism; Galactose metabolism	-0.01	-0.97	-0.13	4.40E-02
342.12	15.77	$C_{12}H_{22}O_{11}$	Lactose	10	Carbohydrate Metabolism	Galactose metabolism	***-1.55***	***-1.32***	***-3.29***	2.88E-03
422.08	17.19	$C_{12}H_{23}O_{14}P$	alpha,alpha'-Trehalose 6-phosphate	8	Carbohydrate Metabolism	Starch and sucrose metabolism	***-1.78***	-0.21	***-2.51***	4.60E-03
545.20	16.94	$C_{20}H_{35}NO_{16}$	alpha-D-Galactosyl-*N*-acetyllactosamine	5	Carbohydrate Metabolism	Undefined	-0.84	***-1.45***	***-1.99***	1.82E-02
503.18	16.96	$C_{18}H_{33}NO_{15}$	beta-D-Galactopyranosyl-(1->4)-2-amino-2-deoxy-beta-D-glucopyranosyl-(1->6)-D-mannose	7	Carbohydrate Metabolism	Undefined	-0.77	***-1.85***	***-2.73***	3.24E-02
663.11	13.82	$C_{21}H_{27}N_7O_{14}P_2$	NAD^+	10	Energy Metabolism	Oxidative phosphorylation; Glutamate metabolism; Nicotinate and nicotinamide metabolism	-0.82	0.03	-0.57	7.06E-03
515.68	5.20	$C_{39}H_{68}N_7O_{17}P_3S$	Oleoyl-CoA	5	Lipid Metabolism	Biosynthesis of unsaturated fatty acids	***-1.37***	-0.45	-0.79	4.29E-02
145.11	28.45	$C_7H_{15}NO_2$	Acetylcholine	7	Lipid Metabolism	Glycerophospholipid metabolism	0.14	0.06	0.12	1.69E-02
334.07	15.65	$C_9H_{19}O_{11}P$	*sn*-glycero-3-Phospho-1-inositol	5	Lipid Metabolism	Undefined	0.23	-0.61	-0.05	1.17E-02
118.06	7.48	$C_5H_{10}O_3$	5-Hydroxypentanoate	7	Lipids: Fatty Acyls	Fatty Acids and Conjugates	-0.32	-0.64	***-1.46***	1.28E-02
548.48	3.76	$C_{35}H_{64}O_4$	Cohibin A	5	Lipids: Fatty Acyls	Undefined	0.86	-0.35	-0.32	9.45E-04
602.53	3.73	$C_{39}H_{70}O_4$	1-(14-methyl-pentadecanoyl)-2-(8-[3]-ladderane-octanyl)-*sn*-glycerol	5	Lipids: Glycerolipids	Diradylglycerols	-0.66	-0.04	-0.15	3.26E-02
410.24	7.50	$C_{19}H_{39}O_7P$	PA(16:0)	7	Lipids: Glycerophospholipids	Glycerophosphates	0.95	0.18	0.85	1.17E-02
495.33	5.03	$C_{24}H_{50}NO_7P$	PC(16:0)	7	Lipids: Glycerophospholipids	Glycerophosphocholines	0.57	0.22	0.03	4.40E-02
689.50	4.24	$C_{37}H_{72}NO_8P$	PE(32:1)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	***1.18***	-0.34	0.11	2.92E-04
687.48	4.23	$C_{37}H_{70}NO_8P$	PE(32:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	0.67	-0.42	-0.07	2.28E-03
715.52	4.18	$C_{39}H_{74}NO_8P$	PE(34:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	-0.83	-0.14	-0.26	2.39E-02

743.55	4.12	$C_{41}H_{78}NO_8P$	PE(36:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	***-1.12***	-0.11	-0.16	2.88E-03
720.49	3.76	$C_{38}H_{73}O_{10}P$	PG(32:1)	5	Lipids: Glycerophospholipids	Glycerophosphoglycerols	0.81	-0.43	-0.55	3.48E-04
760.53	3.75	$C_{41}H_{77}O_{10}P$	PG(35:2)	5	Lipids: Glycerophospholipids	Glycerophosphoglycerols	-0.66	-0.18	-0.11	6.41E-03
572.30	4.50	$C_{25}H_{49}O_{12}P$	PI(16:0)	5	Lipids: Glycerophospholipids	Glycerophosphoinositols	0.50	0.28	0.27	3.85E-02
735.50	3.76	$C_{38}H_{74}NO_{10}P$	PS(32:0)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	0.60	-0.41	-0.12	3.14E-03
733.52	4.28	$C_{39}H_{76}NO_9P$	PS(O-33:1)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	***1.17***	-0.26	0.46	3.48E-04
546.46	3.77	$C_{35}H_{62}O_4$	[PR] bacteriohopane-32,33,34,35-tetrol	5	Lipids: Prenols	Hopanoids	0.51	-0.39	-0.09	3.14E-03
155.99	8.59	$C_3H_8O_3S_2$	2-(Methylthio)ethanesulfonate	5	Metabolism of Cofactors and Vitamins	Folate biosynthesis	0.07	-0.63	-0.30	2.55E-02
267.10	9.01	$C_{10}H_{13}N_5O_4$	Adenosine	8	Nucleotide Metabolism	Purine metabolism	-0.09	0.25	0.32	1.69E-02
112.03	9.75	$C_4H_4N_2O_2$	Orotate(Fragment)	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.16	-0.46	-0.36	1.69E-02
244.07	13.71	$C_9H_{12}N_2O_6$	Pseudouridine	6	Nucleotide Metabolism	Pyrimidine metabolism	-0.82	-0.24	-0.64	1.28E-02
244.07	9.77	$C_9H_{12}N_2O_6$	Uridine	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.07	-0.50	-0.39	3.85E-02
310.51	16.31	$C_{15}H_{22}N_5O_{16}P_3$	ADP ribose 1'',2''-phosphate	5	Nucleotide Metabolism	Undefined	-0.69	0.12	-0.63	2.02E-02
541.06	13.83	$C_{15}H_{21}N_5O_{13}P_2$	Cyclic ADP-ribose	5	Nucleotide Metabolism	Undefined	-0.77	0.06	-0.66	1.69E-02
212.01	13.11	$C_5H_9O_7P$	P-DPD	6	Nucleotide Metabolism	Undefined	0.15	-0.35	0.15	3.93E-02
156.02	10.20	$C_5H_4N_2O_4$	Uracil 5-carboxylate	5	Nucleotide Metabolism	Undefined	***-1.31***	0.04	0.08	2.88E-03
400.23	10.64	$C_{18}H_{32}N_4O_6$	Ala-Leu-Thr-Pro	5	Peptide(tetra-)	Hydrophobic peptide	-0.15	-0.46	-0.20	3.85E-02
361.15	15.53	$C_{14}H_{23}N_3O_8$	Glu-Asp-Val	5	Peptide(tri-)	Hydrophobic peptide	***-1.35***	***-1.08***	***-2.23***	6.12E-03
193.11	11.08	$C_{11}H_{15}NO_2$	Salsoline	7	Undefined	Undefined	0.02	0.14	0.02	4.43E-02
230.09	9.53	$C_9H_{14}N_2O_5$	(1R,2S,3R)-2-Acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)imidazole	7	Undefined	Undefined	-0.58	-0.25	-0.35	2.39E-02
359.19	5.25	$C_{21}H_{27}O_5$	19-oic-deoxycorticosterone	5	Undefined	Undefined	0.11	-0.46	0.07	4.07E-02

326.19	3.78	$C_{18}H_{30}O_3S$	2-Dodecylbenzenesulfonic acid	5	Undefined	Undefined	0.25	0.05	0.37	9.67E-03
165.08	13.63	$C_9H_{11}NO_2$	3-Pyridinebutanoica cid	7	Undefined	Undefined	-0.92	-0.91	***-1.73***	3.93E-02
266.10	11.08	$C_{11}H_{14}N_4O_4$	8-Oxodeoxycoformycin	5	Undefined	Undefined	-0.45	0.19	-0.07	3.88E-02
254.06	7.51	$C_{15}H_{10}O_4$	Apigeninidin	5	Undefined	Undefined	0.94	-0.16	0.67	2.02E-02
232.04	11.58	$C_5H_{13}O_8P$	D-arabitol 5-phosphate	5	Undefined	Undefined	0.37	0.14	0.47	5.31E-03
101.12	10.05	$C_6H_{15}N$	Hexylamine	5	Undefined	Undefined	-0.09	-0.06	0.16	2.87E-02
320.05	15.86	$C_8H_{17}O_{11}P$	octulose 8-phosphate	5	Undefined	Undefined	***-1.02***	0.10	-0.26	3.30E-02
324.11	15.38	$C_{10}H_{28}Cl_2N_2OP_2$	Polixetonium chloride	5	Undefined	Undefined	***-1.38***	***-1.05***	***-2.06***	2.20E-02

Table A1.3. Significant metabolites identified following exposure to polymyxin B and mitotane alone and in combination in polymyxin-resistant *A. baumannii* FADDI-AB225. Significant fold-changes are highlighted in bold and italic. One-way ANOVA for multiple comparison, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$.

MASS	RT	FORMULA	METABOLITE	CONFIDENCE	MAP	PATHWAY	Log_2-fold change			FDR
							Polmyxin B	Mitotane	Combination	
301.06	14.31	$C_8H_{16}NO_9P$	*N*-Acetyl-D-glucosamine 6-phosphate	8	Amino Acid Metabolism	Glutamate metabolism; Aminosugars metabolism	-0.67	-0.76	***-1.08***	4.49E-02
160.08	11.13	$C_6H_{12}N_2O_3$	D-Alanyl-D-alanine	6	Amino Acid Metabolism	D-Alanine metabolism; Peptidoglycan biosynthesis	-0.97	0.63	0.37	4.82E-02
103.06	11.30	$C_4H_9NO_2$	(R)-3-Amino-2-methylpropanoate	5	Amino Acid Metabolism	Undefined	0.14	-0.15	-0.03	3.12E-02
202.06	11.99	$C_7H_{10}N_2O_5$	N3-fumaramoyl-L-2,3-diaminopropanoate	5	Amino Acid Metabolism	Undefined	-0.80	-0.02	-0.21	4.49E-02
255.71	5.19	$C_{21}H_{36}N_7O_{16}P_3S$	CoA	8	Carbohydrate Metabolism	Citrate cycle (TCA cycle); Fatty acid metabolism; Pantothenate and CoA biosynthesis	***-1.12***	***-1.05***	***-1.61***	6.70E-03
196.06	13.48	$C_6H_{12}O_7$	D-Gluconic acid	10	Carbohydrate Metabolism	Pentose phosphate pathway	***-1.21***	***-1.01***	-0.47	4.49E-02
166.05	12.88	$C_5H_{10}O_6$	D-Arabinonate	7	Carbohydrate Metabolism	D-arabinose degradation III	-0.15	-0.17	-0.31	4.82E-02
504.17	16.32	$C_{18}H_{32}O_{16}$	Maltotriose	10	Carbohydrate Metabolism	glycogen degradation I	-0.45	0.05	0.12	4.82E-02
97.98	12.44	H_3O_4P	Orthophosphate	9	Energy Metabolism	Oxidative phosphorylation; Photosynthesis; Peptidoglycan biosynthesis	0.14	0.05	-0.02	4.82E-02
515.68	5.20	$C_{39}H_{68}N_7O_{17}P_3S$	Oleoyl-CoA	5	Lipid Metabolism	Biosynthesis of unsaturated fatty acids	-0.80	***-1.05***	***-1.54***	4.49E-02
785.16	11.04	$C_{27}H_{33}N_9O_{15}P_2$	FAD	10	Metabolism of Cofactors and Vitamins	Riboflavin metabolism	-0.35	-0.53	-0.50	4.49E-02
97.97	17.34	H_2O_4S	Sulfate	8	Nucleotide Metabolism	Purine metabolism; Cysteine metabolism; Sulfur metabolism	-0.12	-0.27	-0.13	4.82E-02
921.38	13.86	$C_{37}H_{59}N_7O_{20}$	GlcNAc-1,6-anhMurNAc-L-Ala-gamma-D-Glu-DAP-D-Ala	7	Undefined	Undefined	***-3.01***	0.26	-0.64	4.49E-02
1005.34	7.58	$C_{37}H_{66}N_7O_{17}P_3S$	4,8,12-Trimethyltridecanoyl-CoA	7	Undefined	Undefined	***-1.34***	***-1.29***	***-2.00***	4.49E-02
186.06	10.21	$C_7H_{10}N_2O_4$	(S)-AMPA	5	Undefined	Undefined	-0.67	-0.06	-0.15	4.49E-02

Table A1.4. Significant metabolites identified following exposure to polymyxin B and mitotane alone and in combination in polymyxin-resistant *A. baumannii* FADDI-AB065. Significant fold-changes are highlighted in bold and italic. One-way ANOVA for multiple comparison, FDR ≤ 0.05; Fisher's LSD, $p \leq 0.05$.

MASS	RT	FORMULA	METABOLITE	CONFIDENCE	MAP	PATHWAY	Log_2-fold change			FDR
							Polmyxin B	Mitotane	Combination	
117.08	11.18	$C_5H_{11}NO_2$	Betaine	10	Amino Acid Metabolism	Glycine, serine and threonine metabolism	-0.45	***-4.56***	***-2.20***	3.15E-06
102.03	7.47	$C_4H_6O_3$	Succinate semialdehyde	6	Amino Acid Metabolism	Glutamate metabolism; Tyrosine metabolism; Butanoate metabolism; Vitamin B6 metabolism	-0.06	0.42	-0.26	8.42E-05
132.09	15.53	$C_5H_{12}N_2O_2$	D-Ornithine	7	Amino Acid Metabolism	D-Arginine and D-ornithine metabolism	0.30	0.85	***2.29***	2.54E-04
679.10	15.27	$C_{20}H_{31}N_3O_{19}P_2$	UDP-*N*-acetylmuramate	8	Amino Acid Metabolism	D-Glutamine and D-glutamate metabolism; Aminosugars metabolism; Peptidoglycan biosynthesis	0.18	-0.62	***1.75***	4.86E-04
160.08	11.13	$C_6H_{12}N_2O_3$	D-Alanyl-D-alanine	6	Amino Acid Metabolism	D-Alanine metabolism; Peptidoglycan biosynthesis	0.01	***-1.73***	0.13	4.86E-04
161.07	14.36	$C_6H_{11}NO_4$	L-2-Aminoadipate	9	Amino Acid Metabolism	Lysine biosynthesis; Lysine degradation; Penicillin and cephalosporin biosynthesis	0.97	0.21	***2.13***	8.27E-04
174.06	14.21	$C_6H_{10}N_2O_4$	*N*-Formimino-L-glutamate	7	Amino Acid Metabolism	Histidine metabolism	-0.11	***-1.75***	-0.29	8.27E-04
232.11	13.18	$C_9H_{16}N_2O_5$	gamma-Glutamyl-gamma-aminobutyrate	6	Amino Acid Metabolism	Arginine and proline metabolism	0.06	***-1.35***	-0.67	1.63E-03
139.99	16.02	$C_2H_5O_5P$	Acetyl phosphate	8	Amino Acid Metabolism	Taurine and hypotaurine metabolism; Pyruvate metabolism	0.29	***-2.79***	0.27	2.75E-03
188.12	8.30	$C_8H_{16}N_2O_3$	N6-Acetyl-L-lysine	5	Amino Acid Metabolism	Lysine degradation	0.05	-0.48	-0.50	2.88E-03
230.11	9.33	$C_{13}H_{14}N_2O_2$	(1xi,3xi)-1,2,3,4-Tetrahydro-1-methyl-beta-carboline-3-carboxylic acid	7	Amino Acid Metabolism	Undefined	0.09	-0.27	0.70	2.98E-03
301.06	14.31	$C_8H_{16}NO_9P$	*N*-Acetyl-D-glucosamine 6-phosphate	8	Amino Acid Metabolism	Glutamate metabolism; Aminosugars metabolism	-0.19	***1.34***	***1.18***	2.98E-03

190.10	23.12	$C_7H_{14}N_2O_4$	LL-2,6-Diaminoheptanedioate	6	Amino Acid Metabolism	Lysine biosynthesis	0.09	***-1.05***	-0.19	5.14E-03
165.05	13.41	$C_5H_{11}NO_3S$	L-Methionine S-oxide	6	Amino Acid Metabolism	Methionine metabolism	-0.02	-0.87	-0.23	5.32E-03
175.10	15.88	$C_6H_{13}N_3O_3$	L-Citrulline	8	Amino Acid Metabolism	Arginine and proline metabolism	-0.10	-0.60	***1.14***	5.36E-03
103.06	13.72	$C_4H_9NO_2$	*N,N*-Dimethylglycine	7	Amino Acid Metabolism	Glycine, serine and threonine metabolism	0.11	0.04	0.13	6.00E-03
138.04	10.37	$C_6H_6N_2O_2$	Urocanate	7	Amino Acid Metabolism	Histidine metabolism	0.10	***-1.13***	0.28	7.85E-03
226.11	15.51	$C_9H_{14}N_4O_3$	Carnosine	5	Amino Acid Metabolism	Alanine and aspartate metabolism; Histidine metabolism; beta-Alanine metabolism	0.14	-0.98	0.06	8.23E-03
104.01	15.16	$C_3H_4O_4$	Hydroxypyruvate	6	Amino Acid Metabolism	Glycine, serine and threonine metabolism; Glyoxylate and dicarboxylate metabolism	-0.56	***-1.45***	***-1.07***	9.00E-03
129.04	14.61	$C_5H_7NO_3$	5-Oxoproline	7	Amino Acid Metabolism	Glutathione metabolism	0.50	***-1.08***	-0.70	1.23E-02
130.06	4.93	$C_6H_{10}O_3$	4-Methyl-2-oxopentanoate	10	Amino Acid Metabolism	Valine, leucine and isoleucine degradation; Valine, leucine and isoleucine biosynthesis	0.04	-0.70	-0.31	1.59E-02
129.08	7.55	$C_6H_{11}NO_2$	L-Pipecolate	5	Amino Acid Metabolism	Lysine degradation; Alkaloid biosynthesis II	-0.16	-0.10	-0.12	2.86E-02
137.08	28.46	$C_8H_{11}NO$	Tyramine	7	Amino Acid Metabolism	Tyrosine metabolism; Alkaloid biosynthesis I	0.02	-0.08	-0.13	3.02E-02
116.05	7.45	$C_5H_8O_3$	5-Oxopentanoate	5	Amino Acid Metabolism	Lysine degradation	-0.10	-0.18	-0.01	3.16E-02
102.03	7.11	$C_4H_6O_3$	2-Methyl-3-oxopropanoate	7	Amino Acid Metabolism	Valine-LeucineandIsoleucineDegradation	0.03	-0.06	0.06	4.41E-02
188.12	14.86	$C_8H_{16}N_2O_3$	N2-Acetyl-L-lysine	7	Amino Acid Metabolism	Lysine biosynthesis	-0.02	-0.49	***1.32***	4.66E-02
103.06	11.30	$C_4H_9NO_2$	(R)-3-Amino-2-methylpropanoate	5	Amino Acid Metabolism	Undefined	0.02	-0.04	0.04	3.26E-03
188.08	10.28	$C_7H_{12}N_2O_4$	*N*-Acetylglutamine	9	Amino Acid Metabolism	Undefined	0.20	***-2.64***	-0.26	7.59E-03
416.11	7.52	$C_{21}H_{20}O_9$	Daidzin	5	Biosynthesis of Secondary Metabolites	Isoflavonoid biosynthesis	0.22	0.77	***1.23***	2.54E-04
270.05	7.50	$C_{15}H_{10}O_5$	Apigenin	7	Biosynthesis of Secondary Metabolites	Flavonoid biosynthesis; Isoflavonoid biosynthesis	0.24	***1.05***	***1.30***	3.28E-04
290.04	15.77	$C_7H_{15}O_{10}P$	D-Sedoheptulose 7-phosphate	6	Carbohydrate Metabolism	Pentose phosphate pathway; Carbon fixation	-0.06	***-3.45***	0.56	4.55E-04

185.99	16.45	$C_3H_7O_7P$	3-Phospho-D-glycerate	10	Carbohydrate Metabolism	Glycolysis / Gluconeogenesis; Glycine, serine and threonine metabolism; Glycerolipid metabolism; Glyoxylate and dicarboxylate metabolism; Carbon fixation	0.17	***-1.93***	0.10	4.55E-04
200.01	15.77	$C_4H_9O_7P$	D-Erythrose 4-phosphate	8	Carbohydrate Metabolism	Pentose phosphate pathway; Phenylalanine, tyrosine and tryptophan biosynthesis; Carbon fixation; Vitamin B6 metabolism	0.22	***-2.74***	0.58	4.88E-04
230.02	15.29	$C_5H_{11}O_8P$	D-Ribose 5-phosphate	8	Carbohydrate Metabolism	Pentose phosphate pathway; Purine metabolism; Carbon fixation	0.14	-0.94	0.50	8.26E-04
134.02	15.46	$C_4H_6O_5$	(S)-Malate	10	Carbohydrate Metabolism	Citrate cycle (TCA cycle); Glutamate metabolism; Alanine and aspartate metabolism; Pyruvate metabolism; Glyoxylate and dicarboxylate metabolism; Carbon fixation; Reductive carboxylate cycle (CO2 fixation)	0.82	-0.85	-0.82	9.69E-04
130.03	14.09	$C_5H_6O_4$	2,5-Dioxopentanoate	7	Carbohydrate Metabolism	Ascorbate and aldarate metabolism; Arginine and proline metabolism	-0.41	-0.37	-0.35	1.31E-03
179.08	13.28	$C_6H_{13}NO_5$	D-Galactosamine	7	Carbohydrate Metabolism	Galactose metabolism	0.22	-0.61	0.80	1.57E-03
196.06	13.48	$C_6H_{12}O_7$	D-Gluconic acid	10	Carbohydrate Metabolism	Pentose phosphate pathway	-0.32	-0.36	-0.64	3.27E-03
607.08	14.65	$C_{17}H_{27}N_3O_{17}P_2$	UDP-*N*-acetyl-D-glucosamine	10	Carbohydrate Metabolism	Aminosugars metabolism; Lipopolysaccharide biosynthesis; Peptidoglycan biosynthesis	-0.01	***-1.09***	0.76	4.19E-03
342.12	15.77	$C_{12}H_{22}O_{11}$	Lactose	10	Carbohydrate Metabolism	Galactose metabolism	0.20	-0.17	***1.52***	6.64E-03
566.05	15.85	$C_{15}H_{24}N_2O_{17}P_2$	UDP-glucose	10	Carbohydrate Metabolism	Pentose and glucuronate interconversions; Galactose metabolism; Ascorbate and aldarate metabolism; Pyrimidine metabolism; Starch and sucrose metabolism; Nucleotide sugars metabolism; Glycerolipid metabolism; Zeatin biosynthesis; Biosynthesis of ansamycins	0.06	-0.75	***1.57***	7.15E-03
259.05	15.95	$C_6H_{14}NO_8P$	alpha-D-Glucosamine 1-phosphate	8	Carbohydrate Metabolism	Aminosugars metabolism	0.12	***-1.43***	0.52	7.85E-03
106.03	16.37	$C_3H_6O_4$	D-Glycerate	6	Carbohydrate Metabolism	Glycine, serine and threonine metabolism; Glycerolipid metabolism; Glyoxylate and dicarboxylate metabolism	0.19	***-1.22***	-0.08	8.69E-03
422.08	17.19	$C_{12}H_{23}O_{14}P$	alpha,alpha'-Trehalose 6-phosphate	8	Carbohydrate Metabolism	Starch and sucrose metabolism	-0.06	-0.49	***1.52***	9.54E-03
621.06	17.20	$C_{17}H_{25}N_3O_{18}P_2$	UDP-*N*-acetyl-D-mannosaminouronate	8	Carbohydrate Metabolism	Aminosugars metabolism	0.00	***-2.51***	0.68	1.96E-02

84.02	13.28	$C_4H_4O_2$	3-Butynoate	5	Carbohydrate Metabolism	Butanoate metabolism	0.11	-0.61	0.72	2.05E-02
260.03	15.64	$C_6H_{13}O_9P$	D-Glucose 6-phosphate	10	Carbohydrate Metabolism	Starch and sucrose metabolism; Streptomycin biosynthesis; Inositol phosphate metabolism	-0.33	***-1.55***	0.02	2.18E-02
255.71	11.83	$C_{21}H_{36}N_7O_{16}P_3S$	CoA	8	Carbohydrate Metabolism	Citrate cycle (TCA cycle); Fatty acid metabolism; Pantothenate and CoA biosynthesis	0.62	***-1.27***	***1.91***	2.57E-02
166.05	12.43	$C_5H_{10}O_6$	D-Xylonate	5	Carbohydrate Metabolism	Pentose and glucuronate interconversions	0.02	-0.10	0.13	4.85E-02
130.03	11.31	$C_5H_6O_4$	Mesaconate	7	Carbohydrate Metabolism	C5-Branched dibasic acid metabolism	0.18	-0.82	-0.42	4.98E-02
662.09	15.84	$C_{19}H_{28}N_4O_{18}P_2$	UDP-2,3-diacetamido-2,3-dideoxy-alpha-D-glucuronate	5	Carbohydrate Metabolism	Undefined	-0.74	-0.87	***1.34***	9.33E-03
189.10	12.26	$C_8H_{15}NO_4$	Castanospermine	5	Carbohydrate Metabolism	Undefined	-0.07	-0.11	0.16	4.32E-02
663.11	13.82	$C_{21}H_{27}N_7O_{14}P_2$	NAD^+	10	Energy Metabolism	Oxidative phosphorylation; Glutamate metabolism; Nicotinate and nicotinamide metabolism	0.34	-0.70	***1.15***	4.55E-04
97.98	12.44	H_3O_4P	Orthophosphate	9	Energy Metabolism	Oxidative phosphorylation; Photosynthesis; Peptidoglycan biosynthesis	-0.05	-0.28	-0.36	4.55E-04
210.07	13.72	$C_7H_{14}O_7$	Sedoheptulose	7	Energy Metabolism	Carbon fixation	0.43	***-1.82***	***1.21***	1.98E-03
743.08	16.31	$C_{21}H_{28}N_7O_{17}P_3$	$NADP^+$	10	Energy Metabolism	Photosynthesis; Glutathione metabolism; Nicotinate and nicotinamide metabolism	0.22	-0.59	***1.32***	3.53E-03
238.07	13.23	$C_8H_{14}O_8$	3-Deoxy-D-manno-octulosonate	8	Glycan Biosynthesis and Metabolism	Lipopolysaccharide biosynthesis	0.29	0.73	***2.65***	8.42E-05
172.01	14.45	$C_3H_9O_6P$	*sn*-Glycerol 3-phosphate	10	Lipid Metabolism	Glycerolipid metabolism; Glycerophospholipid metabolism	-0.05	-0.21	***1.55***	3.06E-03
215.06	15.43	$C_5H_{14}NO_6P$	*sn*-glycero-3-Phosphoethanolamine	7	Lipid Metabolism	Glycerophospholipid metabolism; Ether lipid metabolism	-0.69	***-1.71***	***-1.62***	4.41E-03
145.11	28.45	$C_7H_{15}NO_2$	Acetylcholine	7	Lipid Metabolism	Glycerophospholipid metabolism	0.13	-0.04	-0.03	2.30E-02
102.03	13.33	$C_4H_6O_3$	Acetoacetate	8	Lipid Metabolism	Synthesis and degradation of ketone bodies; Valine, leucine and isoleucine degradation; Tyrosine metabolism; Propanoate metabolism; Styrene degradation; Butanoate metabolism	-0.07	-0.16	0.15	2.60E-02
257.10	14.36	$C_8H_{20}NO_6P$	*sn*-glycero-3-Phosphocholine	10	Lipid Metabolism	Glycerophospholipid metabolism; Ether lipid metabolism	-0.09	-0.76	-0.25	3.28E-02
334.07	15.65	$C_9H_{19}O_{11}P$	sn-glycero-3-Phospho-1-inositol	5	Lipid metabolism	Undefined	-0.18	***-1.50***	-0.39	5.69E-03

300.27	3.98	$C_{18}H_{36}O_3$	FA hydroxy(18:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	-0.12	***-1.14***	***-1.94***	6.05E-05
312.27	7.51	$C_{19}H_{36}O_3$	FA oxo(19:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.05	***1.41***	***1.45***	2.54E-04
576.51	3.73	$C_{37}H_{68}O_4$	Cohibin C	5	Lipids: Fatty Acyls	Undefined	0.05	0.04	0.18	2.98E-03
548.48	3.76	$C_{35}H_{64}O_4$	Cohibin A	5	Lipids: Fatty Acyls	Undefined	-0.13	-0.21	-0.06	5.69E-03
268.24	3.92	$C_{17}H_{32}O_2$	FA methyl(16:1)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	-0.36	-0.44	-0.98	2.40E-02
103.06	11.79	$C_4H_9NO_2$	FA amino(4:0)	5	Lipids: Fatty Acyls	Amino Fatty Acids	0.07	-0.01	0.04	3.23E-02
574.50	3.74	$C_{37}H_{66}O_4$	Montecristin	5	Lipids: Fatty Acyls	Undefined	-0.02	-0.53	0.04	3.81E-02
242.22	3.98	$C_{15}H_{30}O_2$	FA methyl(14:0)	5	Lipids: Fatty Acyls	Fatty Acids and Conjugates	0.08	-0.23	-0.32	4.62E-02
467.30	4.92	$C_{22}H_{46}NO_7P$	PC(14:0)	5	Lipids: Glycerophospholipids	Glycerophosphocholines	0.00	***5.58***	***4.75***	8.69E-11
733.52	4.28	$C_{39}H_{76}NO_9P$	PS(O-33:1)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	-0.18	0.67	0.39	3.41E-05
743.55	4.12	$C_{41}H_{78}NO_8P$	PE(36:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	0.16	0.62	0.70	9.46E-05
479.30	7.51	$C_{23}H_{46}NO_7P$	PE(18:1)	7	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	-0.15	-0.75	-0.75	1.27E-03
715.52	4.18	$C_{39}H_{74}NO_8P$	PE(34:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	0.12	-0.65	-0.33	1.61E-03
495.33	5.03	$C_{24}H_{50}NO_7P$	PC(16:0)	7	Lipids: Glycerophospholipids	Glycerophosphocholines	-0.08	***-1.52***	-0.26	1.75E-03
572.30	4.50	$C_{25}H_{49}O_{12}P$	PI(16:0)	5	Lipids: Glycerophospholipids	Glycerophosphoinositols	-0.22	***-1.42***	-0.29	2.98E-03
410.24	7.50	$C_{19}H_{39}O_7P$	PA(16:0)	7	Lipids: Glycerophospholipids	Glycerophosphates	-0.12	-0.22	***1.52***	3.53E-03
689.50	4.24	$C_{37}H_{72}NO_8P$	PE(32:1)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	-0.19	-0.13	-0.01	3.53E-03
521.35	4.92	$C_{26}H_{52}NO_7P$	PC(18:1)	7	Lipids: Glycerophospholipids	Glycerophosphocholines	-0.13	***-1.46***	-0.34	5.00E-03
687.48	4.23	$C_{37}H_{70}NO_8P$	PE(32:2)	5	Lipids: Glycerophospholipids	Glycerophosphoethanolamines	-0.16	-0.90	-0.42	1.33E-02
735.50	3.76	$C_{38}H_{74}NO_{10}P$	PS(32:0)	5	Lipids: Glycerophospholipids	Glycerophosphoserines	-0.08	-0.70	-0.04	3.16E-02
488.76	3.85	$C_{46}H_{81}N_3O_{15}P_2$	CDP-DG(16:0/18:2(9Z,12Z))	5	Lipids: Glycerophospholipids	Undefined	0.36	0.28	***1.01***	4.81E-02
523.36	4.88	$C_{26}H_{54}NO_7P$	PC(18:0)	5	Lipids: Glycerophospholipids	Glycerophosphocholines	-0.26	-0.85	-0.43	4.85E-02
246.05	12.26	$C_6H_{15}O_8P$	Glycerophosphoglycerol	7	Lipids: Glycerophospholipids	Undefined	-0.81	***-1.47***	***-1.40***	7.15E-03

546.46	3.77	$C_{35}H_{62}O_4$	[PR] bacteriohopane-32,33,34,35-tetrol	5	Lipids: Prenols	Hopanoids	-0.14	-0.71	-0.17	1.96E-02
562.39	4.20	$C_{33}H_{54}O_7$	Cholesterolglucuronide	5	Lipids: Sterol lipids	Pentose and glucuronate interconversions; Starch and sucrose metabolism	-0.22	-0.99	-0.21	1.65E-02
195.08	10.39	$C_7H_9N_5O_2$	2-Amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine	6	Metabolism of Cofactors and Vitamins	Folate biosynthesis	0.25	***-2.92***	***1.14***	6.17E-03
155.99	8.59	$C_3H_8O_3S_2$	2-(Methylthio)ethanesulfonate	5	Metabolism of Cofactors and Vitamins	Folate biosynthesis	-0.09	-0.69	-0.57	6.55E-03
112.03	8.57	$C_4H_4N_2O_2$	Uracil	10	Nucleotide Metabolism	Pyrimidine metabolism; beta-Alanine metabolism; Pantothenate and CoA biosynthesis	-0.17	-0.73	-0.73	4.55E-04
322.06	12.34	$C_{10}H_{15}N_2O_8P$	dTMP	10	Nucleotide Metabolism	Pyrimidine metabolism	-0.11	***1.04***	***3.01***	4.77E-04
111.04	10.57	$C_4H_5N_3O$	Cytosine	10	Nucleotide Metabolism	Pyrimidine metabolism	-0.28	-0.73	***1.06***	4.86E-04
331.07	12.26	$C_{10}H_{14}N_5O_6P$	dAMP	8	Nucleotide Metabolism	Purine metabolism	-0.44	-0.31	***1.77***	4.86E-04
347.06	13.21	$C_{10}H_{14}N_5O_7P$	AMP	10	Nucleotide Metabolism	Purine metabolism; Zeatin biosynthesis	0.09	0.34	***1.11***	4.86E-04
243.09	11.73	$C_9H_{13}N_3O_5$	Cytidine	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.33	-0.74	***1.13***	7.54E-04
323.05	15.08	$C_9H_{14}N_3O_8P$	CMP	10	Nucleotide Metabolism	Pyrimidine metabolism	-0.28	-0.40	***1.70***	9.23E-04
324.04	14.13	$C_9H_{13}N_2O_9P$	UMP	10	Nucleotide Metabolism	Pyrimidine metabolism; Peptidoglycan biosynthesis	-0.14	-0.32	***1.74***	1.31E-03
364.04	17.41	$C_{10}H_{13}N_4O_9P$	Xanthosine 5'-phosphate	8	Nucleotide Metabolism	Purine metabolism	0.05	***-2.70***	***1.10***	1.40E-03
308.04	13.26	$C_9H_{13}N_2O_8P$	dUMP	8	Nucleotide Metabolism	Pyrimidine metabolism	-0.56	***-3.08***	-0.08	2.63E-03
152.03	10.92	$C_5H_4N_4O_2$	Xanthine	10	Nucleotide Metabolism	Purine metabolism; Caffeine metabolism	0.18	-0.65	1.00	2.98E-03
404.00	16.06	$C_9H_{14}N_2O_{12}P_2$	UDP	10	Nucleotide Metabolism	Pyrimidine metabolism; Peptidoglycan biosynthesis; Zeatin biosynthesis	-0.04	-0.76	0.87	5.02E-03
227.09	10.34	$C_9H_{13}N_3O_4$	Deoxycytidine	10	Nucleotide Metabolism	Pyrimidine metabolism	-0.19	***-1.66***	***3.16***	7.96E-03
403.02	16.67	$C_9H_{15}N_3O_{11}P_2$	CDP	6	Nucleotide Metabolism	Pyrimidine metabolism	-0.12	-0.31	***1.33***	9.50E-03
411.03	14.03	$C_{10}H_{15}N_5O_9P_2$	dADP	5	Nucleotide Metabolism	Purine metabolism	-0.29	0.09	***1.21***	1.33E-02

402.02	14.31	$C_{10}H_{16}N_2O_{11}P_2$	dTDP	8	Nucleotide Metabolism	Pyrimidine metabolism	0.03	-0.49	***1.35***	2.05E-02
251.10	11.31	$C_{10}H_{13}N_5O_3$	5'-Deoxyadenosine	6	Nucleotide Metabolism	Undefined	0.08	-0.82	0.02	3.32E-02
267.10	9.01	$C_{10}H_{13}N_5O_4$	Adenosine	8	Nucleotide Metabolism	Purine metabolism	-0.77	-0.12	***-1.06***	3.41E-02
347.06	12.50	$C_{10}H_{14}N_5O_7P$	3'-AMP	7	Nucleotide Metabolism	Purine metabolism	-0.08	-0.22	0.72	3.66E-02
383.11	13.97	$C_{14}H_{17}N_5O_8$	Succinyladenosine	7	Nucleotide Metabolism	Undefined	0.94	***1.91***	***2.71***	8.72E-04
541.06	13.83	$C_{15}H_{21}N_5O_{13}P_2$	Cyclic ADP-ribose	5	Nucleotide Metabolism	Undefined	0.31	-0.66	***1.19***	2.03E-03
310.51	16.31	$C_{15}H_{22}N_5O_{16}P_3$	ADP ribose 1,2-phosphate	5	Nucleotide Metabolism	Undefined	0.36	-0.19	***1.57***	3.88E-03
122.05	13.90	$C_6H_6N_2O$	Isonicotineamide	5	Nucleotide Metabolism	Undefined	0.31	-0.24	0.86	3.44E-02
348.05	14.94	$C_{10}H_{13}N_4O_8P$	Inosine 2'-phosphate	7	Nucleotide Metabolism	Undefined	-0.53	***-1.37***	0.23	4.54E-02
258.09	16.23	$C_{10}H_{14}N_2O_6$	Ribothymidine	7	Nucleotide Metabolism	Undefined	***-1.04***	0.03	***1.25***	4.66E-02
216.15	7.48	$C_{10}H_{20}N_2O_3$	Val-Val	5	Peptide(di-)	Hydrophobic peptide	-0.19	-0.30	-0.47	3.24E-03
186.10	11.16	$C_8H_{14}N_2O_3$	Ala-Pro	5	Peptide(di-)	Nonpolar peptide	0.02	-0.79	-0.61	3.53E-03
291.12	9.22	$C_{14}H_{17}N_3O_4$	Trp-Ser	5	Peptide(di-)	Hydrophobic peptide	-0.35	***-3.49***	0.99	5.69E-03
248.12	7.47	$C_{10}H_{20}N_2O_3S$	Met-Val	5	Peptide(di-)	Hydrophobic peptide	-0.21	-0.95	-0.87	1.61E-02
172.08	12.13	$C_7H_{12}N_2O_3$	Glycylproline	5	Peptide(di-)	Polar peptide	-0.09	-0.69	-0.43	2.40E-02
174.10	9.56	$C_7H_{14}N_2O_3$	Val-Gly	5	Peptide(di-)	Hydrophobic peptide	0.07	-0.84	-0.70	3.10E-02
340.17	12.43	$C_{15}H_{24}N_4O_5$	Ala-Gly-Pro-Pro	5	Peptide(tetra-)	Polar peptide	0.36	-0.74	0.64	3.27E-03
313.16	11.71	$C_{14}H_{23}N_3O_5$	Thr-Pro-Pro	7	Peptide(tri-)	Polar peptide	0.10	***-3.31***	0.14	2.66E-03
361.15	15.53	$C_{14}H_{23}N_3O_8$	Glu-Asp-Val	5	Peptide(tri-)	Hydrophobic peptide	-0.02	***-1.59***	***1.02***	1.39E-02
343.17	11.73	$C_{15}H_{25}N_3O_6$	Ile-Asp-Pro	7	Peptide(tri-)	Hydrophobic peptide	0.28	0.06	0.90	4.41E-02
289.13	12.68	$C_{11}H_{19}N_3O_6$	Ophthalmicacid	7	Peptide(tri-)	Undefined	0.70	***-2.00***	***1.29***	1.73E-02
218.07	8.45	$C_8H_{14}N_2O_3S$	Cysteinyl-Proline	5	Peptides	Undefined	-0.55	***-1.69***	-0.99	4.28E-04
244.18	5.02	$C_{12}H_{24}N_2O_3$	Isoleucyl-Leucine	7	Peptides	Undefined	-0.03	-0.74	***-1.61***	4.19E-03
202.13	6.31	$C_9H_{18}N_2O_3$	Alanyl-Leucine	7	Peptides	Undefined	-0.09	***-1.13***	-0.92	6.55E-03

232.14	6.06	$C_{10}H_{20}N_2O_4$	Threoninyl-Leucine	7	Peptides	Undefined	-0.04	-0.67	-0.41	1.34E-02
260.14	11.22	$C_{11}H_{20}N_2O_5$	L-gamma-glutamyl-L-isoleucine	5	Peptides	Undefined	-0.09	-0.54	-0.53	1.73E-02
921.38	13.86	$C_{37}H_{59}N_7O_{20}$	GlcNAc-1,6-anhMurNAc-L-Ala-gamma-D-Glu-DAP-D-Ala	7	Undefined	Undefined	0.59	***-1.88***	-0.66	2.54E-04
254.06	7.51	$C_{15}H_{10}O_4$	Apigeninidin	5	Undefined	Undefined	0.23	0.88	***1.19***	4.55E-04
193.11	11.08	$C_{11}H_{15}NO_2$	Salsoline	7	Undefined	Undefined	-0.05	-0.11	0.00	4.55E-04
112.02	12.75	$C_5H_4O_3$	2-Furoate	7	Undefined	Undefined	0.54	0.07	***2.80***	4.55E-04
272.03	15.76	$C_7H_{13}O_9P$	alpha-(2,6-anhydro-3-deoxy-D-arabino-heptulopyranosid)onate 7-phosphate	5	Undefined	Undefined	-0.07	***-1.70***	0.58	9.96E-04
193.11	12.80	$C_{11}H_{15}NO_2$	Heliamine	7	Undefined	Undefined	0.01	-0.07	0.01	1.12E-03
237.09	12.53	$C_8H_{15}NO_7$	*N*-Acetyl-D-glucosaminate	5	Undefined	Undefined	0.24	-0.28	***2.29***	1.57E-03
490.27	3.85	$C_{51}H_{80}O_{18}$	Ziziphin	5	Undefined	Undefined	0.16	0.83	0.79	1.57E-03
129.15	8.17	$C_8H_{19}N$	Octylamine	5	Undefined	Undefined	-0.01	-0.04	0.01	1.63E-03
220.06	12.63	$C_8H_{12}O_7$	dihomocitrate	5	Undefined	Undefined	***1.87***	***1.28***	***4.50***	1.89E-03
128.06	7.49	$C_5H_8N_2O_2$	L-Cyclo(alanylglycyl)	5	Undefined	Undefined	-0.01	-0.03	0.14	2.98E-03
101.12	10.05	$C_6H_{15}N$	Hexylamine	5	Undefined	Undefined	0.00	-0.03	0.02	3.01E-03
188.12	7.90	$C_8H_{16}N_2O_3$	6-Acetamido-3-aminohexanoate	5	Undefined	Undefined	-0.21	-0.92	-0.84	3.06E-03
126.04	7.52	$C_5H_6N_2O_2$	Imidazol-4-ylacetate	7	Undefined	Undefined	0.17	-0.38	***1.59***	3.47E-03
193.11	11.62	$C_{11}H_{15}NO_2$	3,4-Methylenedioxymethamphetamine	7	Undefined	Undefined	-0.04	-0.11	0.01	5.02E-03

320.05	15.86	$C_8H_{17}O_{11}P$	octulose 8-phosphate	5	Undefined	Undefined	0.61	-0.56	***2.63***	5.80E-03
103.10	14.30	$C_5H_{13}NO$	Neurine	5	Undefined	Undefined	0.01	0.03	0.04	7.85E-03
249.09	7.78	$C_{10}H_{19}NO_2S_2$	S-Acetyldihydrolipoamide	8	Undefined	Alanineandaspartatemetabolism	0.13	***1.07***	***-1.40***	1.39E-02
114.03	15.76	$C_5H_6O_3$	L-erythro-ascorbate	7	Undefined	Undefined	-0.06	-0.12	-0.01	1.96E-02
278.19	7.31	$C_{20}H_{24}N$	2-ethyl-1,5-dimethyl-3,3-diphenylpyrrolinium (EDDP)	7	Undefined	Undefined	0.00	-0.11	-0.04	2.04E-02
232.04	11.58	$C_5H_{13}O_8P$	D-arabitol 5-phosphate	5	Undefined	Undefined	0.08	0.07	0.17	2.04E-02
312.18	7.28	$C_{17}H_{28}O_3S$	*N*-Undecylbenzenesulfonic acid	5	Undefined	Undefined	0.10	0.05	0.19	2.33E-02
141.99	11.58	$C_2H_7O_3PS$	dimethylthiophosphate	5	Undefined	methyl parathion degradation	0.04	0.02	-0.01	2.89E-02
324.11	15.38	$C_{10}H_{28}Cl_2N_2OP_2$	Polixetonium chloride	5	Undefined	Undefined	-0.08	-0.36	0.58	2.99E-02
113.94	20.95	$H_2O_3S_2$	$H_2S_2O_3$	8	Undefined	sulfur disproportionation II (aerobic) ; superpathway of sulfur oxidation (Acidianus ambivalens) ; superpathway of tetrathionate reduction (Salmonella typhimurium) ; tetrathionate reduction I (to thiosulfate) ; sulfate reduction IV (dissimilatory)	0.17	-0.20	-0.43	4.39E-02
175.08	12.09	$C_7H_{13}NO_4$	Calystegin B2	7	Undefined	Undefined	0.05	0.03	0.13	4.66E-02

APPENDIX 2: ADDITIONAL SUPPORTING DATA FOR CHAPTER 5

Table A2.1. Differentially expressed genes (log_2 fold-change ≥ |1|, FDR ≤ 0.05) in A549 cells infected with *A. baumannii* ATCC 19606. Statistical significance was calculated using F-statistic with Benjamini Hochberg adjustment to control the FDR. Unannotated products are labelled as 0.

Systematic Name	Product	Log_2 fold-change	Relative expression /intensity	FDR
NM_004591	CCL20	6.67	9.52	1.59E-06
NM_000584	CXCL8	5.58	8.39	5.02E-07
NM_000758	CSF2	4.99	8.66	8.18E-06
NM_022377	ICAM4	4.97	8.04	9.01E-05
NM_002090	CXCL3	4.85	11.12	1.87E-06
NR_015361	LOC440896	4.84	8.03	4.30E-05
NM_000594	TNF	4.73	9.04	6.73E-07
NM_002089	CXCL2	4.61	13.18	6.06E-07
NM_000201	ICAM1	4.54	9.40	2.00E-05
NM_001511	CXCL1	4.36	12.98	5.12E-06
NR_036513	LINC01405	4.01	7.66	1.14E-03
ENST00000554254	0	3.94	11.26	8.65E-05
NM_001964	EGR1	3.91	10.86	4.42E-05
NM_006290	TNFAIP3	3.90	10.05	1.54E-05
NM_002852	PTX3	3.80	8.14	6.68E-06
NM_030979	PABPC3	3.76	11.28	1.18E-07
NM_000575	IL1A	3.57	7.15	7.55E-05
NM_139239	NFKBID	3.49	8.98	1.35E-04
NM_002341	LTB	3.46	8.68	2.31E-05
ENST00000424827	0	3.44	11.18	8.07E-08
ENST00000469078	ZNF90	3.35	11.13	3.50E-08
ENST00000419060	0	3.34	8.75	2.15E-05
NM_000600	IL6	3.31	8.02	9.17E-06
NM_003714	STC2	3.30	9.30	1.18E-07
ENST00000465511	0	3.19	9.84	1.91E-07
NM_019058	DDIT4	3.04	15.02	3.48E-06
NM_031419	NFKBIZ	3.03	8.75	6.68E-06
NM_001145033	C11orf96	3.02	7.78	9.74E-05
NM_139314	ANGPTL4	3.02	14.39	1.37E-05
ENST00000453464	RNF223	3.01	9.62	2.24E-07
NR_038262	MIR210HG	3.00	7.69	1.08E-04
NM_181726	ANKRD37	2.98	11.28	1.39E-07
NR_002182	NACAP1	2.98	8.82	1.01E-04
A_33_P3414017	0	2.97	11.00	3.56E-04
lnc-RP11-422N16.3.1-1:1	lnc-RP11-422N16.3.1-1	2.93	9.35	1.79E-05
ENST00000590085	0	2.92	7.84	2.15E-05
NM_006732	FOSB	2.91	10.48	3.20E-04
NM_001965	EGR4	2.91	6.85	1.08E-04

lnc-RHPN1-2:1	lnc-RHPN1-2	2.91	7.61	3.87E-03
A_21_P0014808	0	2.83	9.87	1.09E-06
NM_014330	PPP1R15A	2.79	12.15	6.05E-05
ENST00000462503	0	2.76	9.82	7.45E-07
lnc-KRT83-1:1	lnc-KRT83-1	2.75	9.38	4.06E-06
NM_000576	IL1B	2.75	7.84	3.75E-03
ENST00000497298	0	2.70	10.40	3.53E-06
lnc-TSC22D1-1:4	lnc-TSC22D1-1	2.68	9.71	8.31E-03
A_33_P3333523	0	2.66	12.96	8.48E-07
NM_004428	EFNA1	2.65	12.61	1.39E-07
ENST00000443413	0	2.65	10.99	1.09E-06
BU535024	0	2.64	8.82	1.30E-04
NM_173200	NR4A3	2.59	11.10	2.43E-04
ENST00000498256	0	2.58	9.82	1.58E-06
TCONS_l2_00014098	XLOC_l2_007656	2.56	9.74	1.30E-05
ENST00000511464	0	2.55	9.46	2.86E-06
NM_001285486	NEURL3	2.55	10.46	1.64E-05
NM_002192	INHBA	2.52	7.00	3.58E-05
lnc-KIF25-2:2	lnc-KIF25-2	2.51	11.72	2.36E-04
NM_001674	ATF3	2.46	10.17	2.71E-04
ENST00000426283	0	2.45	6.98	1.22E-05
NM_003992	CLK3	2.44	9.85	1.87E-06
NM_001040619	ATF3	2.44	10.28	7.21E-06
THC2544321	0	2.43	7.02	1.55E-03
ENST00000497246	0	2.40	7.11	2.52E-04
ENST00000415103	0	2.40	8.63	5.22E-04
NM_173502	PRSS36	2.40	8.88	1.04E-06
NM_005252	FOS	2.38	9.75	2.83E-05
NM_005204	MAP3K8	2.37	10.95	7.55E-05
NM_002198	IRF1	2.37	10.23	6.68E-06
NM_001025366	VEGFA	2.37	9.05	1.21E-04
A_33_P3279456	0	2.35	13.02	1.18E-07
ENST00000615248	0	2.34	7.78	4.48E-04
NM_001008540	CXCR4	2.34	10.74	2.35E-03
NM_005658	TRAF1	2.33	9.22	9.03E-05
NM_004233	CD83	2.31	9.99	1.54E-05
NM_002985	CCL5	2.30	8.14	4.56E-03
TCONS_l2_00022742	XLOC_l2_011908	2.29	6.20	1.69E-05
ENST00000517927	MIR146A	2.28	6.40	4.56E-03
ENST00000467688	0	2.28	11.22	1.80E-07
NM_203411	TMEM88	2.28	9.32	4.12E-05
NM_003670	BHLHE40	2.27	12.87	5.29E-06
ENST00000447193	0	2.27	10.54	1.39E-07
NM_002228	JUN	2.27	12.99	5.59E-05
ENST00000513465	0	2.26	10.28	1.09E-06
NM_020529	NFKBIA	2.25	15.31	8.18E-06

NM_001080424	KDM6B	2.24	8.04	1.10E-03
NM_001124	ADM	2.22	16.01	1.37E-05
A_19_P00808923	0	2.22	14.12	1.18E-07
NM_172109	KCNQ2	2.21	10.90	1.05E-05
A_33_P3329991	0	2.21	10.65	2.77E-07
THC2753069	0	2.17	12.28	5.97E-06
lnc-CCDC51-1:1	lnc-CCDC51-1	2.17	7.93	1.25E-04
NM_001300918	HMGA2	2.17	9.93	6.00E-05
lnc-AC011239.1.1-1:3	lnc-AC011239.1.1-1	2.17	5.67	2.94E-02
NM_002701	POU5F1	2.16	9.18	5.12E-06
lnc-HDDC3-1:2	lnc-HDDC3-1	2.16	10.69	2.53E-05
lnc-IRS2-1:1	lnc-IRS2-1	2.15	5.48	1.43E-02
lnc-RP11-723O4.6.1-1:1	lnc-RP11-723O4.6.1-1	2.15	7.27	3.11E-04
NM_001002021	PFKL	2.14	10.47	1.37E-05
NM_000963	PTGS2	2.13	11.65	2.27E-03
NM_001002914	KCTD11	2.12	12.16	9.25E-06
TCONS_l2_00010603	XLOC_l2_005692	2.12	9.45	8.82E-05
AF395440	DNAJA1P5	2.12	11.09	1.16E-06
ENST00000417077	0	2.11	10.09	3.48E-06
NM_181611	KRTAP19-5	2.11	7.89	4.12E-05
ENST00000451170	0	2.11	9.10	8.12E-06
ENST00000493052	0	2.10	12.49	6.11E-07
NM_000450	SELE	2.09	6.13	8.21E-04
NM_001956	EDN2	2.09	11.15	1.01E-03
ENST00000439303	0	2.08	9.16	3.43E-05
lnc-DOLPP1-1:1	lnc-DOLPP1-1	2.08	11.97	3.05E-05
NM_004419	DUSP5	2.07	11.47	1.80E-05
NM_002610	PDK1	2.07	9.96	1.35E-05
ENST00000549896	0	2.07	5.60	4.60E-02
NM_020801	ARRDC3	2.07	9.02	7.23E-05
NR_040024	LOC100131655	2.06	9.82	3.48E-06
lnc-DLL1-4:1	lnc-DLL1-4	2.05	6.78	4.14E-03
A_33_P3365963	0	2.05	11.61	3.50E-05
ENST00000444823	0	2.05	7.25	4.59E-04
NM_001258038	SPRY1	2.04	9.70	6.52E-06
NM_000399	EGR2	2.03	6.74	1.23E-04
NM_006186	NR4A2	2.03	8.87	9.19E-04
TCONS_l2_00001619	XLOC_l2_001192	2.03	12.54	7.45E-07
lnc-RP11-17A1.2.1-2:1	lnc-RP11-17A1.2.1-2	2.03	5.57	4.35E-02
NM_005398	PPP1R3C	2.03	13.23	2.88E-04
lnc-CCHCR1-1:1	lnc-CCHCR1-1	2.03	6.89	7.76E-05
ENST00000482002	0	2.02	11.86	6.06E-07
ENST00000453717	0	2.02	12.15	3.48E-06
NM_012405	ICMT	2.02	7.52	1.24E-03
NM_005384	NFIL3	2.01	12.43	3.67E-07

NM_145203	CSNK1A1L	2.01	6.95	3.35E-04
NM_025079	ZC3H12A	2.01	12.01	2.93E-05
ENST00000414733	0	2.01	11.55	2.17E-06
NM_002616	PER1	2.01	10.39	4.49E-05
NM_004210	NEURL1	2.01	10.56	1.90E-05
lnc-TMCO3-1:1	lnc-TMCO3-1	2.00	9.40	8.04E-04
NM_198541	IGFL1	2.00	10.06	4.71E-03
NM_005655	KLF10	2.00	12.19	7.12E-05
ENST00000424673	0	2.00	7.53	6.78E-05
ENST00000508108	0	1.99	7.44	1.05E-05
ENST00000507681	0	1.99	7.87	4.14E-02
ENST00000442125	0	1.96	11.08	1.95E-06
ENST00000558517	0	1.96	6.46	5.19E-06
NM_014470	RND1	1.95	10.39	3.05E-05
NM_004083	DDIT3	1.95	11.21	1.01E-03
NM_001161528	LRRD1	1.94	6.74	1.37E-05
ENST00000463344	0	1.94	14.29	1.38E-06
NM_001300	KLF6	1.92	14.11	1.22E-05
lnc-CDK5R1-2:1	lnc-CDK5R1-2	1.92	9.50	6.72E-05
lnc-OR4M2-7:1	lnc-OR4M2-7	1.91	8.93	5.98E-04
NM_022073	EGLN3	1.91	8.78	3.09E-04
lnc-ANKRD11-2:1	lnc-ANKRD11-2	1.91	8.32	1.43E-03
ENST00000474162	0	1.90	8.97	1.59E-05
ENST00000447135	0	1.90	8.48	4.95E-04
lnc-PLOD2-1:1	lnc-PLOD2-1	1.88	8.36	2.29E-05
NM_012323	MAFF	1.87	11.42	4.18E-05
NM_032330	CAPNS2	1.86	8.50	1.56E-04
NM_000189	HK2	1.86	6.65	8.40E-05
ENST00000434894	0	1.86	7.89	4.96E-03
NM_001278720	RHBDL1	1.86	11.18	9.97E-05
lnc-C16orf42-2:1	lnc-C16orf42-2	1.85	12.88	8.47E-05
ENST00000425480	0	1.85	7.16	4.49E-05
NM_006813	PNRC1	1.85	11.95	1.09E-06
NR_004389	SNORA16B	1.85	7.77	6.32E-03
NR_033244	LOC729080	1.85	7.72	3.93E-04
NM_030762	BHLHE41	1.85	8.21	2.52E-05
NM_014417	BBC3	1.84	12.64	1.09E-05
NM_018433	KDM3A	1.84	9.03	2.17E-06
ENST00000561802	0	1.84	7.55	1.37E-05
NM_078476	BTN2A1	1.84	6.91	8.18E-06
NM_001167676	FAM229A	1.84	13.85	5.44E-05
ENST00000488396	0	1.84	12.88	1.66E-06
NM_004864	GDF15	1.84	16.15	3.47E-06
NM_002648	PIM1	1.83	12.43	8.48E-07
ENST00000480085	0	1.82	12.41	1.66E-06
NM_016951	CKLF	1.82	6.46	5.97E-04

ENST00000443168	0	1.82	7.56	1.80E-03
ENST00000414870	0	1.82	12.68	1.16E-06
NM_013332	HILPDA	1.81	15.05	4.02E-05
TCONS_l2_00014417	XLOC_l2_007884	1.81	5.16	8.95E-03
NR_001446	ANXA2P3	1.81	9.28	9.19E-06
NM_001145115	PPP1R3G	1.81	8.56	1.66E-05
ENST00000482292	0	1.80	9.29	1.00E-02
ENST00000567054	0	1.80	9.08	9.03E-05
NM_006734	HIVEP2	1.80	10.19	4.68E-04
ENST00000563903	0	1.79	8.87	6.93E-06
NM_001080476	GRXCR1	1.79	5.25	1.66E-02
NM_003201	TFAM	1.79	6.87	4.49E-05
NM_017592	MED29	1.79	6.72	5.72E-06
NM_001013631	HNRNPCL1	1.79	6.85	4.23E-05
ENST00000484315	0	1.78	7.77	1.05E-05
ENST00000413691	0	1.77	6.55	4.69E-04
NM_001165	BIRC3	1.77	13.60	4.79E-05
ENST00000561636	0	1.76	8.68	7.83E-05
A_33_P3277805	0	1.75	11.44	3.18E-05
lnc-MME-4:1	lnc-MME-4	1.75	12.40	8.71E-05
NM_000710	BDKRB1	1.73	9.35	1.23E-02
ENST00000488538	0	1.73	8.33	6.52E-04
NR_026667	RPS10P7	1.73	13.62	1.54E-05
ENST00000523572	0	1.73	8.03	2.14E-03
ENST00000580924	0	1.72	11.46	1.04E-06
ENST00000458044	LOC101927851	1.72	10.02	3.50E-05
NM_001085	SERPINA3	1.71	10.34	2.20E-02
NM_001570	IRAK2	1.71	12.33	1.19E-04
XR_427815	LOC101927675	1.71	8.70	4.38E-05
NM_001001343	FNDC9	1.71	7.01	4.16E-05
NM_002982	CCL2	1.70	15.05	4.05E-04
NM_000759	CSF3	1.70	5.68	5.50E-03
NM_005967	NAB2	1.70	13.72	3.68E-04
NM_004417	DUSP1	1.70	13.81	4.07E-04
lnc-ERLEC1-1:1	lnc-ERLEC1-1	1.69	8.62	2.28E-05
NR_033769	ASB9P1	1.68	8.37	4.93E-04
NM_001002857	ANXA2	1.68	13.46	6.68E-06
NM_002136	HNRNPA1	1.68	9.17	3.03E-05
lnc-BDKRB1-1:1	lnc-BDKRB1-1	1.67	8.93	1.69E-02
NM_001190706	MTRNR2L9	1.67	11.55	4.06E-06
NM_020648	TWSG1	1.67	7.45	4.36E-04
ENST00000421078	0	1.65	8.47	1.15E-02
NM_207468	FAM177B	1.65	8.15	3.63E-02
NM_012403	ANP32C	1.65	6.99	2.26E-03
NM_004232	SOCS6	1.65	7.98	9.22E-03
ENST00000511127	0	1.64	5.89	7.36E-04

NM_003897	IER3	1.64	15.03	1.34E-05
ENST00000567299	0	1.64	8.00	4.05E-04
ENST00000489168	0	1.63	8.29	6.16E-04
lnc-CNBD1-4:6	lnc-CNBD1-4	1.63	5.39	1.24E-02
ENST00000475592	0	1.62	9.40	8.18E-06
ENST00000433260	0	1.61	5.68	1.08E-03
ENST00000446719	0	1.61	7.20	1.30E-05
ENST00000513279	0	1.61	10.45	6.93E-06
NM_199327	SPRY1	1.61	8.20	4.34E-03
NM_001201329	PPP1R3B	1.61	11.44	3.37E-05
ENST00000402485	0	1.61	11.47	1.05E-05
ENST00000477300	0	1.61	9.09	2.43E-05
NM_014228	SLC6A7	1.60	5.54	1.08E-04
NR_002768	HYMAI	1.60	8.32	7.34E-03
NR_021487	PSMG3-AS1	1.60	13.16	2.93E-05
NM_001013638	PRR25	1.60	11.43	2.73E-05
NM_018293	ZNF654	1.60	8.65	1.05E-04
ENST00000431058	0	1.60	13.98	2.55E-06
THC2546670	0	1.59	7.92	3.38E-03
ENST00000567888	MIR940	1.59	9.57	1.84E-03
lnc-KLHL25-11:1	lnc-KLHL25-11	1.59	5.41	9.91E-03
ENST00000520239	0	1.59	8.64	6.08E-04
ENST00000455793	0	1.59	10.78	1.35E-06
NM_080606	BHLHE23	1.59	12.09	1.34E-03
XR_432412	LOC102725053	1.59	7.21	1.69E-03
lnc-RTL1-2:2	lnc-RTL1-2	1.58	10.22	4.46E-05
NM_005257	GATA6	1.58	10.61	2.88E-04
NM_016084	RASD1	1.58	9.49	5.97E-04
TCONS_l2_00030232	XLOC_l2_015542	1.57	8.26	3.06E-04
ENST00000376463	TLE1	1.57	8.33	1.08E-03
NM_003407	ZFP36	1.57	13.73	1.39E-05
NR_027686	LINC00176	1.57	9.94	5.96E-05
ENST00000520558	0	1.56	8.75	6.68E-06
ENST00000505048	0	1.56	12.18	3.29E-04
NM_018948	ERRFI1	1.56	14.20	6.96E-05
NM_003155	STC1	1.56	7.03	3.32E-03
NM_022162	NOD2	1.56	5.74	4.70E-03
lnc-GJA10-5:1	lnc-GJA10-5	1.56	5.09	4.52E-02
ENST00000526810	0	1.56	8.46	3.20E-04
ENST00000449255	0	1.55	6.34	3.56E-04
NM_030647	KDM7A	1.55	7.86	7.35E-04
NM_025047	ARL14	1.55	8.95	1.30E-03
NM_015508	TIPARP	1.54	14.26	1.81E-04
A_33_P3411384	0	1.54	7.46	6.39E-04
ENST00000518355	0	1.54	5.43	4.00E-03
NR_003242	PFN1P2	1.53	12.44	8.78E-06

NM_006096	NDRG1	1.53	14.35	6.68E-06
NM_001102609	C5orf58	1.53	8.70	4.49E-05
NM_001134771	SLC12A5	1.53	11.54	3.03E-04
ENST00000397595	0	1.53	8.86	1.18E-05
lnc-SERPINC1-1:9	lnc-SERPINC1-1	1.53	10.25	6.68E-06
NR_119384	ZNF295-AS1	1.53	6.76	1.41E-02
NM_001043351	TPM3	1.52	7.02	1.98E-03
NM_003152	STAT5A	1.52	9.19	1.24E-03
lnc-ATG7-2:1	lnc-ATG7-2	1.51	9.37	1.28E-04
ENST00000417412	0	1.51	10.31	4.61E-05
THC2507863	0	1.51	9.64	1.20E-03
NM_006018	HCAR3	1.51	6.99	4.20E-03
A_33_P3276927	0	1.51	7.32	1.97E-04
ENST00000446953	0	1.51	10.66	1.05E-05
NM_020130	C8orf4	1.50	12.40	3.43E-04
ENST00000423476	0	1.50	6.85	2.68E-03
ENST00000604654	0	1.50	7.80	7.41E-05
lnc-CTC-236F12.4.1-3:2	lnc-CTC-236F12.4.1-3	1.50	5.31	2.87E-02
NM_015675	GADD45B	1.50	14.48	1.64E-03
NM_181608	KRTAP19-2	1.49	8.70	7.87E-05
lnc-FKBP3-3:1	lnc-FKBP3-3	1.49	11.08	1.99E-05
A_33_P3370217	0	1.49	5.25	2.63E-02
NM_002360	MAFK	1.48	12.60	2.15E-04
lnc-SIX6-1:1	lnc-SIX6-1	1.48	5.94	1.09E-03
NM_001025390	AMPD3	1.48	8.45	1.32E-03
NM_173728	ARHGEF15	1.48	8.94	7.86E-06
A_33_P3407049	0	1.47	7.49	4.42E-02
ENST00000430550	0	1.47	6.71	3.18E-05
NM_175924	ILDR1	1.47	13.27	9.39E-05
NM_052952	DIRC1	1.47	9.46	1.68E-05
NR_120513	LOC101928861	1.47	6.05	1.52E-02
lnc-FBXO25-3:1	lnc-FBXO25-3	1.47	9.47	9.19E-05
ENST00000423967	0	1.47	5.26	2.37E-02
ENST00000453852	0	1.47	9.47	4.18E-05
ENST00000483219	0	1.46	12.89	1.34E-05
ENST00000394891	0	1.46	6.56	1.01E-03
TCONS_l2_00007037	XLOC_l2_003877	1.46	5.25	2.53E-02
NM_002908	REL	1.46	9.90	3.26E-05
NM_172387	NFATC1	1.46	8.61	9.12E-03
NM_052880	PIK3IP1	1.45	6.59	1.50E-02
NM_016449	DRICH1	1.45	9.56	1.33E-03
NR_033826	SNRPD2P2	1.45	7.88	2.28E-03
ENST00000565674	0	1.45	8.74	1.38E-05
ENST00000496795	0	1.45	8.70	1.12E-04
NM_020429	SMURF1	1.44	7.70	1.80E-03
NM_004430	EGR3	1.44	5.35	3.54E-04

ENST00000473357	SLC2A11	1.44	15.04	3.50E-05
NM_001202234	NR4A1	1.44	5.35	1.20E-04
NM_001781	CD69	1.44	5.51	8.11E-04
NM_012118	CCRN4L	1.43	7.82	1.80E-03
ENST00000373544	RABEPK	1.43	9.65	3.48E-05
ENST00000577279	LOC102724532	1.43	9.24	1.87E-02
lnc-SEZ6L2-1:1	lnc-SEZ6L2-1	1.43	10.36	6.93E-06
NM_004952	EFNA3	1.42	7.05	8.24E-04
TCONS_l2_00030495	XLOC_l2_015738	1.42	5.95	4.95E-04
XR_242926	LOC101928554	1.42	5.63	3.70E-02
A_33_P3407235	0	1.41	5.93	5.13E-04
NM_001190438	NCOR1	1.41	7.57	5.41E-03
TCONS_l2_00024768	XLOC_l2_013000	1.41	5.71	1.25E-02
NR_028496	OSTCP1	1.40	8.32	4.36E-04
A_33_P3280355	0	1.40	14.47	6.68E-06
ENST00000429953	0	1.40	6.16	4.81E-02
NM_003456	ZNF205	1.40	9.37	2.14E-03
ENST00000521326	0	1.40	7.28	9.07E-04
NP1243929	0	1.40	9.81	4.12E-05
ENST00000423733	PLCG1	1.40	9.60	1.54E-05
NM_004331	BNIP3L	1.40	11.41	6.72E-05
NM_021724	NR1D1	1.39	8.67	3.53E-03
NM_000499	CYP1A1	1.39	7.62	3.83E-03
ENST00000609439	0	1.39	8.44	2.40E-03
NM_014931	PPP6R1	1.38	7.89	2.27E-02
NM_002658	PLAU	1.37	15.70	7.25E-06
NM_001216	CA9	1.37	7.45	1.71E-02
ENST00000606622	0	1.37	5.19	3.87E-03
NR_120643	TMEM26-AS1	1.37	9.40	9.03E-05
lnc-SNX20-5:1	lnc-SNX20-5	1.37	10.70	6.68E-06
ENST00000441295	0	1.37	6.73	1.66E-03
ENST00000398190	0	1.37	12.90	1.18E-05
ENST00000461109	0	1.36	9.10	4.38E-05
NM_001243042	HLA-C	1.36	12.55	2.15E-05
NM_001123068	PPIAL4G	1.36	15.26	1.37E-05
NM_144492	CLDN14	1.36	6.48	1.05E-02
NM_001134438	PHLDB2	1.36	6.14	2.01E-02
NM_002203	ITGA2	1.36	6.39	3.97E-04
ENST00000377951	0	1.35	13.58	3.10E-05
NM_152899	IL4I1	1.35	8.30	4.12E-04
lnc-AIG1-1:1	lnc-AIG1-1	1.35	5.60	2.11E-02
ENST00000495104	0	1.35	15.05	6.78E-05
lnc-C11orf36-2:1	lnc-C11orf36-2	1.35	6.02	5.29E-03
NM_001142459	ASB10	1.34	10.46	5.63E-04
THC2691933	0	1.34	7.13	1.32E-05
NM_001145031	PLAU	1.34	10.87	5.70E-05

lnc-NUFIP1-1:1	lnc-NUFIP1-1	1.34	5.33	3.25E-02
NM_020959	ANO8	1.34	6.41	4.45E-03
lnc-RTN2-1:1	lnc-RTN2-1	1.34	6.40	1.61E-02
NM_001031737	CCDC78	1.33	12.04	3.20E-04
NM_003259	ICAM5	1.33	7.07	1.75E-02
NR_003326	SNORD116-11	1.33	6.33	3.03E-04
NM_006133	DAGLA	1.33	6.25	1.08E-03
ENST00000412800	0	1.32	10.74	1.05E-05
ENST00000525280	0	1.32	9.20	4.79E-05
lnc-ARSD-1:1	lnc-ARSD-1	1.32	9.29	3.18E-05
NM_032833	PPP1R15B	1.32	13.61	2.15E-05
NM_138439	FLYWCH2	1.31	5.84	6.61E-05
ENST00000567765	0	1.31	6.03	3.84E-03
lnc-PCYOX1-1:1	lnc-PCYOX1-1	1.31	9.03	5.59E-03
NM_152523	CCNYL1	1.31	7.48	1.81E-02
lnc-FAM20C-4:3	lnc-FAM20C-4	1.31	5.44	2.22E-02
lnc-CCDC90A-5:1	lnc-CCDC90A-5	1.31	10.50	3.76E-03
NM_001101337	C3orf79	1.31	5.27	7.47E-03
NM_021960	MCL1	1.30	11.15	5.24E-03
A_33_P3241596	0	1.30	12.26	2.34E-04
AF416714	0	1.30	13.02	1.27E-04
ENST00000487308	0	1.30	7.01	6.82E-04
ENST00000429552	0	1.30	15.13	3.48E-06
NM_000623	BDKRB2	1.30	10.49	1.38E-02
NM_002006	FGF2	1.30	8.60	2.31E-04
NM_031894	FTHL17	1.30	14.48	4.49E-05
NM_002999	SDC4	1.30	12.35	5.44E-05
A_33_P3346526	0	1.30	8.47	1.71E-04
lnc-OST4-2:3	lnc-OST4-2	1.30	12.54	8.36E-05
ENST00000603072	0	1.29	8.23	4.16E-04
ENST00000508126	SPSB4	1.29	11.00	1.47E-05
A_33_P3293698	0	1.29	5.47	3.95E-02
lnc-MTHFD2L-1:1	lnc-MTHFD2L-1	1.29	6.07	5.97E-04
XR_426334	NCRNA00249	1.29	5.51	3.69E-02
ENST00000447259	0	1.29	13.54	8.78E-06
lnc-PLTP-1:1	lnc-PLTP-1	1.28	8.59	3.25E-04
NM_001105576	SOWAHD	1.28	7.95	4.81E-03
NM_014755	SERTAD2	1.28	11.68	1.78E-05
ENST00000366413	LOC646513	1.28	11.16	3.60E-05
NM_001142595	P4HA1	1.28	10.65	2.15E-05
NM_015626	WSB1	1.28	12.97	5.54E-04
NR_073397	ERICH1-AS1	1.27	7.91	8.04E-04
A_33_P3519223	0	1.27	6.64	3.69E-03
XR_424592	LOC102724362	1.27	10.85	3.75E-05
A_33_P3235831	0	1.27	6.76	9.11E-04
NM_001124758	SPNS2	1.27	10.87	2.07E-04

NM_020439	CAMK1G	1.27	6.86	1.04E-02
NM_013271	PCSK1N	1.26	12.23	2.93E-04
NR_037629	LOC728739	1.26	8.90	5.53E-05
ENST00000423237	0	1.26	8.82	5.35E-06
lnc-AC005493.1-1:10	lnc-AC005493.1-1	1.26	5.19	6.01E-03
ENST00000440938	0	1.26	14.55	6.68E-06
NR_024438	ACTG1P4	1.26	12.18	3.10E-05
ENST00000475432	HIF3A	1.26	11.27	6.68E-06
lnc-MYEF2-5:1	lnc-MYEF2-5	1.25	5.06	3.46E-03
NM_001286233	SLC2A14	1.25	10.89	2.49E-02
NM_005438	FOSL1	1.25	11.82	3.76E-04
NM_003315	DNAJC7	1.25	12.07	1.32E-02
lnc-MBP-1:2	lnc-MBP-1	1.25	9.42	4.49E-05
lnc-TRA2A-1:1	lnc-TRA2A-1	1.25	13.70	1.89E-04
ENST00000464444	0	1.25	14.88	4.09E-06
ENST00000407656	0	1.24	8.41	2.45E-03
ENST00000534728	0	1.24	7.98	1.73E-03
ENST00000565297	0	1.24	5.21	7.67E-03
ENST00000458667	LOC102725353	1.24	11.46	3.06E-04
ENST00000483240	0	1.24	7.24	3.06E-02
TCONS_l2_00001954	XLOC_l2_000018	1.24	8.56	2.49E-03
NR_027053	LOC646214	1.24	8.05	6.94E-03
ENST00000416004	0	1.24	5.29	3.40E-02
ENST00000412562	0	1.24	9.57	1.78E-05
AK026323	ALPK1	1.23	14.65	5.03E-05
NM_031955	SPATA16	1.23	4.90	1.99E-02
AK130932	0	1.23	11.43	1.13E-04
NM_001924	GADD45A	1.23	13.06	2.45E-03
NM_030643	APOL4	1.23	5.61	2.19E-02
NR_046420	UPK1A-AS1	1.23	6.31	1.49E-03
ENST00000427868	LINC00665	1.23	7.89	2.09E-02
NR_126369	GACAT1	1.23	7.88	5.29E-05
CU677518	0	1.23	8.36	2.96E-03
NR_026816	PSORS1C3	1.23	6.87	2.75E-04
NM_002991	CCL24	1.22	10.81	4.09E-06
NM_178815	ARL5B	1.22	9.23	8.71E-05
ENST00000489727	0	1.22	7.23	3.57E-04
NM_001160223	RNF170	1.22	15.18	7.87E-05
ENST00000407780	0	1.22	9.76	2.11E-03
NM_005100	AKAP12	1.22	12.63	7.03E-05
NM_000675	ADORA2A	1.22	5.92	2.09E-03
TCONS_l2_00026769	XLOC_l2_013931	1.22	7.24	6.94E-06
ENST00000419201	0	1.21	8.41	8.26E-06
BM918074	SNORA71A	1.21	9.80	1.11E-03
A_33_P3337742	0	1.21	9.19	2.27E-05
NM_004556	NFKBIE	1.21	12.82	5.03E-05

ENST00000441188	0	1.21	6.10	2.48E-03
NR_121635	FOXD3-AS1	1.21	14.30	2.36E-04
ENST00000505488	0	1.21	5.34	3.38E-02
NR_024356	FBLL1	1.21	7.94	2.99E-02
ENST00000454614	0	1.21	10.87	3.20E-04
NM_016584	IL23A	1.21	9.25	1.07E-04
ENST00000454986	0	1.21	6.07	3.35E-03
NM_006979	SLC39A7	1.20	11.06	9.33E-03
NM_001561	TNFRSF9	1.20	7.41	6.32E-03
NM_004155	SERPINB9	1.20	10.99	8.41E-04
lnc-LRGUK-1:1	lnc-LRGUK-1	1.20	6.86	9.24E-04
NM_033027	CSRNP1	1.20	10.13	4.39E-04
lnc-PPP2R2A-1:1	lnc-PPP2R2A-1	1.19	5.95	7.53E-03
A_33_P3389578	0	1.19	6.71	6.14E-03
A_33_P3422712	0	1.19	9.37	4.63E-04
NM_001288631	FAM222B	1.19	9.79	3.67E-03
ENST00000622038	0	1.19	5.23	1.08E-02
BC137009	0	1.19	10.42	7.87E-05
NM_134268	CYGB	1.19	12.47	1.35E-05
NM_012421	RLF	1.19	11.12	1.04E-05
NM_004433	ELF3	1.19	13.48	1.10E-04
NM_019066	MAGEL2	1.19	5.98	7.01E-04
lnc-LDHC-1:1	lnc-LDHC-1	1.19	7.68	3.37E-02
ENST00000596769	0	1.18	11.55	1.60E-04
ENST00000396994	0	1.18	11.19	3.37E-05
NM_014260	PFDN6	1.18	12.13	9.97E-03
NM_001234	CAV3	1.18	6.65	9.92E-03
ENST00000524369	0	1.18	9.12	5.44E-05
ENST00000426697	0	1.18	5.39	4.07E-02
NM_001242758	HLA-A	1.18	15.46	9.80E-05
ENST00000618028	0	1.18	5.24	3.15E-02
ENST00000312710	0	1.18	5.92	6.00E-05
lnc-PCF11-1:12	lnc-PCF11-1	1.17	7.99	1.18E-02
A_21_P0014324	0	1.17	6.88	1.10E-02
NM_005261	GEM	1.17	9.93	1.08E-02
ENST00000392885	0	1.17	6.06	1.94E-02
ENST00000444388	0	1.17	9.00	8.60E-05
A_33_P3224971	0	1.17	11.06	2.81E-04
lnc-RIT2-1:1	lnc-RIT2-1	1.17	9.06	1.21E-04
lnc-ARRDC3-1:6	lnc-ARRDC3-1	1.16	10.45	4.62E-03
NM_032521	PARD6B	1.16	10.34	3.48E-05
NM_024943	TMEM156	1.16	9.02	1.13E-02
NM_153607	CREBRF	1.16	9.10	6.39E-04
NM_015714	G0S2	1.16	10.36	2.11E-03
NM_032413	C15orf48	1.16	12.30	7.80E-03
NM_001190702	MTRNR2L8	1.16	13.42	1.37E-05

NR_126381	LINC01162	1.15	5.22	1.19E-02
lnc-LYRM2-1:1	lnc-LYRM2-1	1.15	5.26	4.54E-02
NM_203356	CTAGE5	1.15	8.21	5.75E-03
ENST00000423841	0	1.15	14.72	6.68E-06
AK128371	PTK2B	1.15	9.68	3.18E-04
A_33_P3316379	0	1.14	13.31	1.35E-05
ENST00000575402	0	1.14	10.38	2.93E-05
NM_002543	OLR1	1.14	6.27	3.74E-02
lnc-AL360004.1-2:1	lnc-AL360004.1-2	1.14	5.38	3.44E-03
NM_000602	SERPINE1	1.14	11.66	1.81E-03
NM_001001852	PIM3	1.14	13.90	3.10E-05
lnc-MINA-3:1	lnc-MINA-3	1.14	11.27	3.50E-05
NM_001286462	C21orf58	1.14	8.79	1.28E-04
NR_110219	LOC101927285	1.14	9.32	1.21E-04
ENST00000585776	0	1.13	7.58	1.23E-02
ENST00000623879	0	1.13	12.69	3.56E-04
ENST00000434541	0	1.13	7.76	1.12E-03
ENST00000396131	0	1.13	13.72	1.37E-05
NM_001272068	SHISA5	1.13	12.36	2.93E-05
NM_024017	HOXB9	1.13	11.78	8.47E-04
ENST00000416191	0	1.13	6.93	5.00E-03
NM_001206	KLF9	1.12	10.14	7.38E-03
ENST00000420237	LOC101929648	1.12	5.16	3.02E-02
A_33_P3322430	0	1.12	11.47	2.31E-04
lnc-C22orf26-2:10	lnc-C22orf26-2	1.12	5.23	1.62E-02
ENST00000584867	0	1.12	11.70	7.32E-04
A_33_P3349025	0	1.12	5.22	4.42E-02
NM_003044	SLC6A12	1.12	6.67	1.77E-02
lnc-SCRG1-1:4	lnc-SCRG1-1	1.11	6.90	2.53E-03
ENST00000338548	PSMD10	1.11	9.69	4.07E-04
NM_001001955	OR4C13	1.11	5.08	9.71E-03
NM_004767	GPR37L1	1.11	8.00	4.36E-02
XR_429801	LOC100133182	1.11	13.33	9.81E-06
TCONS_l2_00014564	XLOC_l2_008009	1.11	5.80	3.28E-02
NM_144697	CIART	1.11	7.03	2.09E-02
ENST00000342691	0	1.11	6.92	1.06E-02
A_33_P3327140	0	1.11	8.91	2.40E-04
NM_014883	FAM13A	1.10	8.78	2.26E-02
NM_005178	BCL3	1.10	14.97	8.17E-03
NR_034167	LINC00851	1.10	5.46	4.78E-02
TCONS_l2_00027342	XLOC_l2_013808	1.10	7.24	1.33E-05
NR_110303	LOC101929295	1.10	6.31	1.99E-02
lnc-STXBP6-1:2	lnc-STXBP6-1	1.10	5.05	2.76E-02
ENST00000447729	0	1.10	6.78	3.24E-03
NM_001122	PLIN2	1.09	10.26	4.49E-05
NM_005238	ETS1	1.09	7.62	1.98E-02

NR_044995	GAS6-AS1	1.09	9.97	2.51E-02
AW303581	0	1.09	12.83	2.89E-05
XR_245260	LOC101928760	1.09	11.67	1.59E-04
TCONS_l2_00013854	XLOC_l2_007456	1.08	12.14	8.27E-05
NM_001198	PRDM1	1.08	9.65	8.15E-03
ENST00000459875	0	1.08	12.57	7.67E-05
NM_001164469	TMED7-TICAM2	1.08	8.72	1.94E-05
lnc-CTBP1-1:1	lnc-CTBP1-1	1.08	14.43	1.03E-03
NM_001037442	RUFY3	1.08	5.57	4.06E-02
lnc-ARRDC3-1:1	lnc-ARRDC3-1	1.08	10.15	3.52E-04
NM_001164404	GOLGA6C	1.08	6.25	1.30E-03
NM_001202439	NCR3LG1	1.08	9.40	4.01E-03
NM_031412	GABARAPL1	1.08	11.18	2.36E-04
ENST00000449715	0	1.08	7.10	4.52E-05
A_33_P3336038	0	1.08	15.75	2.35E-05
ENST00000400023	C22orf34	1.07	5.19	3.25E-02
AK126671	FLJ44715	1.07	6.48	6.83E-03
NM_001265594	PLEKHG5	1.07	10.60	2.52E-04
NM_000104	CYP1B1	1.07	13.02	2.20E-03
XR_426298	LOC101929622	1.07	13.10	4.02E-05
lnc-THNSL1-2:1	lnc-THNSL1-2	1.07	6.46	2.07E-02
AF086546	0	1.07	6.67	2.30E-02
ENST00000442006	0	1.07	6.01	4.62E-03
ENST00000570531	0	1.07	5.89	4.81E-03
NR_125821	LOC102723701	1.07	14.12	3.69E-05
lnc-ARRDC3-1:13	lnc-ARRDC3-1	1.07	7.38	1.99E-02
lnc-WARS2-2:1	lnc-WARS2-2	1.07	5.58	3.59E-03
NM_001012631	IL32	1.07	10.04	1.14E-03
NM_003998	NFKB1	1.06	12.19	3.32E-05
ENST00000510120	0	1.06	6.36	4.00E-03
NM_173484	KLF17	1.06	12.32	9.63E-04
NM_014950	ZBTB1	1.06	8.16	2.60E-03
NM_001242829	IP6K1	1.06	14.64	3.43E-05
lnc-ZKSCAN1-1:4	lnc-ZKSCAN1-1	1.06	9.84	2.89E-05
NR_026790	HCG11	1.06	9.98	1.06E-04
NR_126385	TXNDC12-AS1	1.06	5.40	4.06E-02
lnc-BBC3-1:1	lnc-BBC3-1	1.06	7.79	1.46E-02
NR_028326	LINC01001	1.05	14.12	3.99E-04
NM_005542	INSIG1	1.05	11.31	4.13E-03
TCONS_l2_00025857	XLOC_l2_013383	1.05	12.32	9.06E-05
ENST00000374439	MUSK	1.05	8.87	1.09E-03
lnc-ZKSCAN1-1:5	lnc-ZKSCAN1-1	1.05	10.46	2.89E-05
ENST00000428814	0	1.05	9.17	8.65E-04
ENST00000513626	LUCAT1	1.05	8.62	2.06E-03
NM_144975	SLFN5	1.05	12.12	6.08E-04
TCONS_l2_00030893	XLOC_l2_015885	1.05	16.98	2.37E-05

NM_006601	PTGES3	1.04	10.63	1.19E-04
lnc-CBLB-1:1	lnc-CBLB-1	1.04	5.53	4.68E-02
ENST00000524858	0	1.04	9.97	1.48E-04
ENST00000517910	0	1.04	11.98	3.18E-04
NR_045484	LOC646626	1.04	8.63	1.50E-03
NR_110811	LINC01363	1.04	5.27	4.33E-03
NM_001193621	PINLYP	1.04	7.05	5.93E-04
NM_003955	SOCS3	1.04	11.31	2.01E-02
TCONS_l2_00025976	XLOC_l2_013460	1.04	10.67	2.86E-05
AK057884	0	1.04	12.98	2.38E-03
lnc-CSRP2BP-1:1	lnc-CSRP2BP-1	1.04	6.67	2.79E-02
NR_024451	JHDM1D-AS1	1.03	7.40	9.80E-05
ENST00000556713	0	1.03	9.96	4.36E-04
NM_004525	LRP2	1.03	8.67	3.09E-03
NM_004660	DDX3Y	1.03	10.41	4.06E-05
NM_004621	TRPC6	1.03	9.58	2.36E-04
ENST00000420303	0	1.03	6.78	5.04E-03
BC040156	LOC284570	1.03	6.71	1.08E-02
NM_004567	PFKFB4	1.03	10.17	1.02E-02
NM_018126	TMEM33	1.03	7.38	4.75E-03
lnc-IFRD2-2:1	lnc-IFRD2-2	1.03	8.87	9.08E-04
ENST00000566307	0	1.02	7.89	2.37E-02
XR_247116	FLJ39095	1.02	5.25	4.44E-02
NM_001012979	TCEAL5	1.02	10.70	1.04E-03
NM_001098402	ZBTB21	1.02	11.03	5.80E-05
ENST00000529743	0	1.02	8.16	2.35E-02
lnc-WDR7-4:1	lnc-WDR7-4	1.02	5.10	1.23E-02
NM_001172630	ARHGAP33	1.02	13.84	4.16E-04
lnc-DNASE1L3-1:1	lnc-DNASE1L3-1	1.02	11.71	1.75E-04
ENST00000419275	PPIAL4G	1.02	7.27	7.11E-03
lnc-RP11-167N24.6.1-1:1	lnc-RP11-167N24.6.1-1	1.02	5.09	1.58E-02
NM_001422	ELF5	1.02	5.05	2.86E-03
NM_015288	JADE2	1.02	8.60	4.96E-03
ENST00000591045	0	1.02	8.35	4.38E-03
NM_000596	IGFBP1	1.01	13.02	2.35E-02
lnc-PLEKHG6-2:1	lnc-PLEKHG6-2	1.01	5.18	2.21E-02
lnc-DENND5A-1:1	lnc-DENND5A-1	1.01	8.41	2.36E-04
ENST00000523812	GLI4	1.01	14.81	5.21E-05
NM_181531	BTN2A2	1.01	8.52	6.75E-03
XR_246125	LOC101929021	1.01	8.96	7.89E-04
NM_177458	LYNX1	1.01	8.62	1.98E-03
ENST00000436474	0	1.01	10.49	3.28E-05
NM_001166663	CD244	1.01	6.79	1.59E-02
NM_001006641	SLC25A25	1.01	11.30	4.85E-04
NM_001242901	DPP9-AS1	1.01	8.00	2.07E-04

ENST00000457863	0	1.00	6.24	2.28E-02
NM_015021	ZNF292	1.00	8.15	1.75E-02
NM_003446	ZNF157	1.00	8.97	7.46E-04
NM_001427	EN2	1.00	13.86	4.63E-04
NM_006136	CAPZA2	1.00	9.42	2.44E-03
lnc-SRGAP3-1:17	lnc-SRGAP3-1	1.00	7.58	3.18E-02
NM_001128636	ELFN1	1.00	9.19	1.33E-03
NR_028502	MIR22HG	-1.00	11.01	2.85E-03
NM_005982	SIX1	-1.00	10.77	2.81E-04
NM_001202	BMP4	-1.01	9.55	1.12E-02
ENST00000452760	0	-1.01	6.64	9.24E-03
NR_002742	SNORD52	-1.02	9.44	1.17E-03
NM_000435	NOTCH3	-1.03	11.39	6.01E-03
NM_001040167	LFNG	-1.03	10.45	8.44E-03
NM_173717	ELAC2	-1.03	9.29	1.41E-03
NM_018011	ARGLU1	-1.04	10.98	3.86E-02
lnc-ANKRD13B-1:1	lnc-ANKRD13B-1	-1.04	8.25	1.24E-03
ENST00000570151	0	-1.05	9.03	2.88E-04
NM_001134364	MAP4	-1.06	8.78	4.56E-03
NM_001131015	CIZ1	-1.06	8.57	2.65E-03
lnc-SGSH-1:3	lnc-SGSH-1	-1.06	8.54	2.52E-04
NM_001010938	TNK2	-1.08	6.23	1.53E-02
NM_001077183	TSC2	-1.09	7.23	1.47E-02
NM_001170406	CDK1	-1.10	6.12	2.95E-02
NM_001159746	ABR	-1.10	8.64	2.74E-03
NM_004655	AXIN2	-1.12	7.50	8.52E-03
NM_021979	HSPA2	-1.15	12.83	1.17E-04
NM_020119	ZC3HAV1	-1.15	12.89	4.57E-04
A_33_P3389668	0	-1.15	5.54	6.06E-03
NM_001202522	DDR1	-1.16	6.88	8.78E-03
NR_033186	C1orf220	-1.16	7.02	2.65E-03
NM_001286476	C21orf58	-1.19	8.68	3.53E-05
NM_024508	ZBED2	-1.21	9.18	6.72E-05
NR_003672	SNHG7	-1.21	9.19	8.67E-04
ENST00000527983	0	-1.21	6.51	1.09E-02
NM_032637	SKP2	-1.27	8.59	1.06E-03
NR_024046	NRADDP	-1.27	6.26	1.53E-02
NM_001080461	UNCX	-1.27	14.63	1.21E-04
ENST00000568686	0	-1.28	10.16	7.03E-05
NM_001271938	MEGF8	-1.32	11.75	3.35E-06
NM_014562	OTX1	-1.45	9.18	6.61E-05
NM_199461	NANOS1	-1.46	9.59	2.52E-05
NM_001135654	PABPC4	-1.47	6.67	2.45E-02
NM_005529	HSPG2	-1.53	7.10	6.82E-03
NM_015672	RIMBP3	-1.62	10.01	4.63E-04
NM_153201	HSPA8	-1.63	15.09	5.10E-04

NM_053001	OSR2	-1.74	8.56	3.01E-04
ENST00000532091	0	-1.79	7.33	9.48E-04
NM_001456	FLNA	-2.27	11.28	5.08E-03
NM_005345	HSPA1A	-3.25	12.11	3.02E-05
NM_005346	HSPA1B	-3.34	10.99	3.43E-05

Table A2.2. Differentially expressed genes (log_2 fold-change ≥ |1|, FDR ≤ 0.05) in A549 cells infected with *A. baumannii* ATCC 19606 and treated with polymyxin B. Statistical significance was calculated using F-statistic with Benjamini Hochberg adjustment to control the FDR. Unannotated products are labelled as 0.

Systematic Name	Product	Log_2 fold-change	Relative expression/intensity	FDR
NM_004591	CCL20	6.63	9.52	1.61E-06
NM_000584	CXCL8	5.78	8.39	3.51E-07
NM_000758	CSF2	5.08	8.66	7.95E-06
NM_022377	ICAM4	5.00	8.04	9.79E-05
NM_000594	TNF	4.75	9.04	6.42E-07
NM_002089	CXCL2	4.62	13.18	5.67E-07
NR_015361	LOC440896	4.61	8.03	6.60E-05
NM_000201	ICAM1	4.54	9.40	2.18E-05
NM_001511	CXCL1	4.45	12.98	4.53E-06
NM_002090	CXCL3	4.27	11.12	4.67E-06
NM_006290	TNFAIP3	4.08	10.05	1.20E-05
ENST00000554254	0	3.86	11.26	1.09E-04
NM_030979	PABPC3	3.84	11.28	9.72E-08
NM_002852	PTX3	3.71	8.14	7.94E-06
NM_001964	EGR1	3.57	10.86	9.13E-05
NM_000575	IL1A	3.49	7.15	9.75E-05
ENST00000424827	0	3.49	11.18	6.96E-08
ENST00000469078	ZNF90	3.33	11.13	3.65E-08
ENST00000465511	0	3.30	9.84	1.64E-07
NM_003714	STC2	3.24	9.30	9.72E-08
NM_139239	NFKBID	3.17	8.98	2.97E-04
NM_002341	LTB	3.14	8.68	4.93E-05
NM_181726	ANKRD37	3.13	11.28	9.72E-08
NM_139314	ANGPTL4	3.07	14.39	1.26E-05
NM_000576	IL1B	3.03	7.84	2.36E-03
NR_002182	NACAP1	3.02	8.82	1.03E-04
NM_031419	NFKBIZ	3.01	8.75	6.68E-06
NM_001145033	C11orf96	2.95	7.78	1.29E-04
A_21_P0014808	0	2.93	9.87	7.95E-07
NM_001965	EGR4	2.93	6.85	1.18E-04
NM_000600	IL6	2.92	8.02	2.19E-05
ENST00000453464	RNF223	2.91	9.62	2.71E-07
ENST00000497298	0	2.80	10.40	2.95E-06
A_33_P3333523	0	2.75	12.96	6.71E-07
ENST00000443413	0	2.73	10.99	7.95E-07
NM_001674	ATF3	2.72	10.17	1.45E-04
ENST00000462503	0	2.71	9.82	7.58E-07

A_33_P3414017	0	2.70	11.00	7.60E-04
NM_006732	FOSB	2.69	10.48	6.09E-04
BU535024	0	2.68	8.82	1.29E-04
THC2544321	0	2.68	7.02	9.60E-04
ENST00000498256	0	2.68	9.82	1.11E-06
NR_038262	MIR210HG	2.67	7.69	2.71E-04
NM_173200	NR4A3	2.67	11.10	2.11E-04
ENST00000590085	0	2.65	7.84	4.62E-05
lnc-KRT83-1:1	lnc-KRT83-1	2.63	9.38	5.36E-06
NM_019058	DDIT4	2.60	15.02	9.23E-06
ENST00000419060	0	2.58	8.75	1.28E-04
ENST00000497246	0	2.54	7.11	1.82E-04
ENST00000426283	0	2.52	6.98	1.04E-05
ENST00000415103	0	2.50	8.63	4.54E-04
A_33_P3279456	0	2.48	13.02	9.72E-08
NM_004428	EFNA1	2.47	12.61	1.98E-07
lnc-RP11-422N16.3.1-1:1	lnc-RP11-422N16.3.1-1	2.42	9.35	6.90E-05
NM_001008540	CXCR4	2.41	10.74	2.27E-03
NM_003992	CLK3	2.41	9.85	2.03E-06
NM_005204	MAP3K8	2.38	10.95	8.21E-05
ENST00000513465	0	2.38	10.28	7.30E-07
ENST00000447193	0	2.38	10.54	9.72E-08
NM_001025366	VEGFA	2.37	9.05	1.34E-04
lnc-RHPN1-2:1	lnc-RHPN1-2	2.36	7.61	1.34E-02
NM_002985	CCL5	2.36	8.14	4.44E-03
NR_126020	LOC285766	2.34	5.51	4.02E-02
ENST00000517927	MIR146A	2.34	6.40	4.42E-03
A_33_P3329991	0	2.33	10.65	1.93E-07
TCONS_l2_00014098	XLOC_l2_007656	2.33	9.74	2.49E-05
ENST00000511464	0	2.31	9.46	5.46E-06
ENST00000467688	0	2.30	11.22	1.66E-07
A_19_P00808923	0	2.25	14.12	9.72E-08
NM_002136	HNRNPA1	2.24	9.17	5.20E-06
lnc-KIF25-2:2	lnc-KIF25-2	2.23	11.72	5.77E-04
lnc-TSC22D1-1:4	lnc-TSC22D1-1	2.23	9.71	2.38E-02
NM_001124	ADM	2.22	16.01	1.43E-05
NM_001285486	NEURL3	2.22	10.46	4.67E-05
ENST00000414733	0	2.22	11.55	1.10E-06
ENST00000451170	0	2.21	9.10	6.33E-06
NM_001040619	ATF3	2.20	10.28	1.37E-05
ENST00000493052	0	2.19	12.49	4.15E-07
NM_020529	NFKBIA	2.19	15.31	9.94E-06
NM_002192	INHBA	2.18	7.00	1.03E-04
ENST00000434894	0	2.18	7.89	2.25E-03
NM_014330	PPP1R15A	2.16	12.15	3.96E-04

ENST00000444823	0	2.14	7.25	3.78E-04
NM_000963	PTGS2	2.14	11.65	2.52E-03
NM_004233	CD83	2.13	9.99	2.80E-05
TCONS_l2_00022742	XLOC_l2_011908	2.13	6.20	2.96E-05
NM_173502	PRSS36	2.13	8.88	2.07E-06
ENST00000482002	0	2.12	11.86	3.90E-07
NM_002198	IRF1	2.12	10.23	1.32E-05
AF395440	DNAJA1P5	2.12	11.09	1.11E-06
ENST00000424673	0	2.12	7.53	5.21E-05
NM_003670	BHLHE40	2.11	12.87	8.34E-06
NM_172109	KCNQ2	2.10	10.90	1.44E-05
NM_012405	ICMT	2.10	7.52	1.11E-03
ENST00000558517	0	2.10	6.46	3.30E-06
NM_005384	NFIL3	2.10	12.43	2.61E-07
ENST00000439303	0	2.09	9.16	3.77E-05
NM_001002914	KCTD11	2.08	12.16	1.06E-05
ENST00000463344	0	2.08	14.29	7.95E-07
NM_005658	TRAF1	2.08	9.22	2.25E-04
NM_014470	RND1	2.08	10.39	2.19E-05
NM_001080424	KDM6B	2.06	8.04	2.14E-03
ENST00000508108	0	2.06	7.44	8.78E-06
NR_004389	SNORA16B	2.05	7.77	4.00E-03
NR_036513	LINC01405	2.05	7.66	4.31E-02
NM_005398	PPP1R3C	2.05	13.23	2.87E-04
NM_022073	EGLN3	2.05	8.78	2.07E-04
TCONS_l2_00010603	XLOC_l2_005692	2.05	9.45	1.26E-04
NM_006186	NR4A2	2.04	8.87	1.01E-03
lnc-CDK5R1-2:1	lnc-CDK5R1-2	2.03	9.50	5.21E-05
ENST00000453717	0	2.03	12.15	3.57E-06
TCONS_l2_00001619	XLOC_l2_001192	2.02	12.54	7.30E-07
NM_145203	CSNK1A1L	2.02	6.95	3.65E-04
ENST00000442125	0	2.02	11.08	1.61E-06
NM_002228	JUN	2.02	12.99	1.38E-04
NM_002610	PDK1	2.00	9.96	1.77E-05
ENST00000417077	0	1.99	10.09	5.20E-06
NM_198541	IGFL1	1.98	10.06	5.54E-03
NM_000450	SELE	1.98	6.13	1.30E-03
NM_016951	CKLF	1.98	6.46	3.73E-04
lnc-HDDC3-1:2	lnc-HDDC3-1	1.98	10.69	4.94E-05
ENST00000474162	0	1.97	8.97	1.31E-05
NM_001258038	SPRY1	1.96	9.70	8.03E-06
NR_040024	LOC100131655	1.96	9.82	5.04E-06
NM_001013631	HNRNPCL1	1.96	6.85	2.55E-05
ENST00000523572	0	1.95	8.03	1.12E-03
THC2753069	0	1.95	12.28	1.10E-05
ENST00000480085	0	1.93	12.41	1.10E-06

NM_078476	BTN2A1	1.93	6.91	6.52E-06
NM_004419	DUSP5	1.92	11.47	3.39E-05
NM_001300918	HMGA2	1.92	9.93	1.56E-04
NM_001145115	PPP1R3G	1.91	8.56	1.20E-05
ENST00000615248	0	1.90	7.78	2.00E-03
NM_001956	EDN2	1.90	11.15	2.08E-03
NM_203411	TMEM88	1.90	9.32	1.55E-04
lnc-ANKRD11-2:1	lnc-ANKRD11-2	1.90	8.32	1.73E-03
A_33_P3365963	0	1.89	11.61	6.60E-05
lnc-DOLPP1-1:1	lnc-DOLPP1-1	1.89	11.97	6.38E-05
NM_000189	HK2	1.88	6.65	8.77E-05
NM_025079	ZC3H12A	1.87	12.01	5.04E-05
ENST00000425480	0	1.87	7.16	4.93E-05
NM_005252	FOS	1.87	9.75	1.52E-04
ENST00000561636	0	1.86	8.68	6.29E-05
lnc-MTOR-1:1	lnc-MTOR-1	1.86	5.55	2.99E-02
lnc-PLOD2-1:1	lnc-PLOD2-1	1.86	8.36	2.62E-05
NR_033244	LOC729080	1.86	7.72	4.23E-04
ENST00000421078	0	1.85	8.47	7.52E-03
NR_001446	ANXA2P3	1.85	9.28	8.48E-06
lnc-OR4M2-7:1	lnc-OR4M2-7	1.85	8.93	8.70E-04
ENST00000447135	0	1.84	8.48	6.83E-04
ENST00000443168	0	1.84	7.56	1.92E-03
lnc-TGFBRAP1-6:1	lnc-TGFBRAP1-6	1.84	5.50	4.95E-02
NM_003201	TFAM	1.83	6.87	4.58E-05
NM_012323	MAFF	1.83	11.42	5.35E-05
ENST00000580924	0	1.83	11.46	6.71E-07
NM_001165	BIRC3	1.82	13.60	4.69E-05
ENST00000477300	0	1.82	9.09	1.14E-05
lnc-CCDC51-1:1	lnc-CCDC51-1	1.82	7.93	4.85E-04
NM_006813	PNRC1	1.82	11.95	1.12E-06
NM_013332	HILPDA	1.81	15.05	4.62E-05
NM_005967	NAB2	1.81	13.72	2.63E-04
NR_033769	ASB9P1	1.81	8.37	3.39E-04
ENST00000488396	0	1.80	12.88	1.96E-06
NM_001085	SERPINA3	1.80	10.34	2.04E-02
ENST00000567054	0	1.80	9.08	1.01E-04
NM_030762	BHLHE41	1.80	8.21	3.27E-05
ENST00000484315	0	1.80	7.77	9.94E-06
ENST00000413691	0	1.79	6.55	5.02E-04
NM_002648	PIM1	1.78	12.43	8.33E-07
ENST00000567299	0	1.78	8.00	2.50E-04
NM_004864	GDF15	1.77	16.15	4.42E-06
NM_018433	KDM3A	1.77	9.03	3.06E-06
NM_001144933	EFCAB3	1.76	8.71	9.67E-03
NM_004210	NEURL1	1.76	10.56	5.02E-05

ENST00000414870	0	1.76	12.68	1.45E-06
NM_002616	PER1	1.75	10.39	1.29E-04
NR_003242	PFN1P2	1.75	12.44	4.18E-06
ENST00000563903	0	1.74	8.87	8.72E-06
A_33_P3277805	0	1.74	11.44	3.74E-05
ENST00000455793	0	1.74	10.78	7.30E-07
NM_001216	CA9	1.74	7.45	6.08E-03
NM_002982	CCL2	1.74	15.05	3.93E-04
ENST00000458044	LOC101927851	1.73	10.02	3.74E-05
ENST00000446719	0	1.73	7.20	8.48E-06
A_33_P3251408	0	1.73	5.69	1.99E-02
ENST00000489168	0	1.72	8.29	4.77E-04
THC2546670	0	1.72	7.92	2.38E-03
ENST00000561802	0	1.72	7.55	2.38E-05
NM_001002021	PFKL	1.71	10.47	6.60E-05
NM_001190706	MTRNR2L9	1.71	11.55	3.57E-06
TCONS_l2_00022686	XLOC_l2_011885	1.71	5.44	6.74E-03
NM_000399	EGR2	1.71	6.74	4.67E-04
NR_026667	RPS10P7	1.71	13.62	1.80E-05
NM_000759	CSF3	1.70	5.68	6.36E-03
lnc-C16orf42-2:1	lnc-C16orf42-2	1.70	12.88	1.68E-04
NM_001002857	ANXA2	1.70	13.46	6.61E-06
NM_017592	MED29	1.69	6.72	8.03E-06
lnc-RP11-723O4.6.1-1:1	lnc-RP11-723O4.6.1-1	1.69	7.27	1.70E-03
ENST00000475592	0	1.69	9.40	6.78E-06
ENST00000520558	0	1.68	8.75	4.48E-06
NM_020648	TWSG1	1.68	7.45	4.63E-04
ENST00000402485	0	1.68	11.47	8.48E-06
ENST00000513279	0	1.68	10.45	5.81E-06
NM_004083	DDIT3	1.68	11.21	2.96E-03
NM_001167676	FAM229A	1.68	13.85	1.14E-04
NM_020801	ARRDC3	1.67	9.02	3.57E-04
ENST00000446953	0	1.66	10.66	6.12E-06
ENST00000394891	0	1.66	6.56	4.75E-04
ENST00000505048	0	1.66	12.18	2.38E-04
lnc-BDKRB1-1:1	lnc-BDKRB1-1	1.65	8.93	2.08E-02
XR_432412	LOC102725053	1.65	7.21	1.47E-03
ENST00000488538	0	1.65	8.33	1.01E-03
ENST00000431058	0	1.65	13.98	2.01E-06
ENST00000376463	TLE1	1.65	8.33	8.89E-04
NM_005655	KLF10	1.65	12.19	2.99E-04
NM_000710	BDKRB1	1.64	9.35	1.87E-02
TCONS_l2_00030232	XLOC_l2_015542	1.63	8.26	2.51E-04
ENST00000453852	0	1.63	9.47	2.31E-05
ENST00000482292	0	1.61	9.29	2.03E-02

NM_001570	IRAK2	1.61	12.33	2.01E-04
NM_199327	SPRY1	1.61	8.20	4.87E-03
NM_001201329	PPP1R3B	1.61	11.44	3.78E-05
NM_006734	HIVEP2	1.60	10.19	1.13E-03
ENST00000511127	0	1.60	5.89	1.00E-03
NM_001300	KLF6	1.59	14.11	4.58E-05
NM_032330	CAPNS2	1.59	8.50	5.17E-04
lnc-RTL1-2:2	lnc-RTL1-2	1.58	10.22	5.06E-05
NM_005257	GATA6	1.58	10.61	3.13E-04
TCONS_l2_00009674	XLOC_l2_005179	1.58	5.89	4.62E-02
lnc-TMCO3-1:1	lnc-TMCO3-1	1.57	9.40	4.05E-03
NM_001278720	RHBDL1	1.56	11.18	3.76E-04
ENST00000397595	0	1.56	8.86	1.04E-05
ENST00000423733	PLCG1	1.56	9.60	8.48E-06
NM_016084	RASD1	1.55	9.49	7.55E-04
ENST00000565674	0	1.55	8.74	9.76E-06
NM_018948	ERRFI1	1.55	14.20	8.22E-05
NM_006096	NDRG1	1.55	14.35	6.52E-06
NM_020959	ANO8	1.54	6.41	2.18E-03
A_33_P3276927	0	1.54	7.32	1.82E-04
NM_004232	SOCS6	1.54	7.98	1.52E-02
NM_018293	ZNF654	1.53	8.65	1.57E-04
NM_002701	POU5F1	1.53	9.18	4.76E-05
lnc-MME-4:1	lnc-MME-4	1.52	12.40	2.58E-04
NR_119384	ZNF295-AS1	1.52	6.76	1.69E-02
NM_001145031	PLAU	1.52	10.87	2.80E-05
NM_001161528	LRRD1	1.52	6.74	7.60E-05
NR_028496	OSTCP1	1.52	8.32	2.90E-04
NM_025047	ARL14	1.51	8.95	1.71E-03
ENST00000423476	0	1.51	6.85	2.91E-03
NM_003155	STC1	1.51	7.03	4.45E-03
ENST00000449255	0	1.50	6.34	4.86E-04
lnc-ERLEC1-1:1	lnc-ERLEC1-1	1.50	8.62	5.37E-05
NM_001013638	PRR25	1.50	11.43	4.58E-05
A_33_P3407049	0	1.50	7.49	4.62E-02
NR_033826	SNRPD2P2	1.50	7.88	2.17E-03
NM_003407	ZFP36	1.49	13.73	2.18E-05
NM_004331	BNIP3L	1.48	11.41	5.21E-05
NM_001102609	C5orf58	1.47	8.70	6.60E-05
ENST00000483219	0	1.47	12.89	1.30E-05
NM_001001343	FNDC9	1.46	7.01	1.28E-04
XR_425856	LOC102723839	1.46	4.97	1.71E-02
NM_015626	WSB1	1.46	12.97	2.42E-04
NM_080606	BHLHE23	1.46	12.09	2.59E-03
NM_173728	ARHGEF15	1.46	8.94	8.69E-06
ENST00000496795	0	1.45	8.70	1.22E-04

lnc-FKBP3-3:1	lnc-FKBP3-3	1.45	11.08	2.55E-05
NM_001134771	SLC12A5	1.45	11.54	4.67E-04
NM_001043351	TPM3	1.45	7.02	3.06E-03
lnc-CCHCR1-1:1	lnc-CCHCR1-1	1.45	6.89	8.79E-04
lnc-SERPINC1-1:9	lnc-SERPINC1-1	1.45	10.25	8.78E-06
ENST00000604654	0	1.44	7.80	1.06E-04
ENST00000461109	0	1.44	9.10	3.47E-05
NM_001202234	NR4A1	1.44	5.35	1.32E-04
ENST00000377951	0	1.44	13.58	2.24E-05
lnc-FBXO25-3:1	lnc-FBXO25-3	1.43	9.47	1.23E-04
NR_021487	PSMG3-AS1	1.43	13.16	6.59E-05
ENST00000373544	RABEPK	1.43	9.65	3.95E-05
lnc-SNX20-5:1	lnc-SNX20-5	1.43	10.70	5.07E-06
NM_004430	EGR3	1.43	5.35	4.10E-04
XR_427815	LOC101927675	1.43	8.70	1.64E-04
THC2507863	0	1.42	9.64	2.06E-03
NM_001025390	AMPD3	1.42	8.45	1.95E-03
ENST00000417412	0	1.42	10.31	8.11E-05
NM_006018	HCAR3	1.42	6.99	6.74E-03
NM_001190438	NCOR1	1.42	7.57	6.11E-03
A_33_P3280355	0	1.42	14.47	6.61E-06
ENST00000433260	0	1.41	5.68	2.82E-03
NM_016449	DRICH1	1.41	9.56	1.82E-03
NM_020429	SMURF1	1.40	7.70	2.44E-03
ENST00000424886	0	1.40	9.06	1.52E-05
NM_001105576	SOWAHD	1.40	7.95	3.28E-03
NM_004417	DUSP1	1.40	13.81	1.65E-03
NM_001123068	PPIAL4G	1.40	15.26	1.17E-05
NM_001142595	P4HA1	1.40	10.65	1.20E-05
ENST00000430550	0	1.40	6.71	5.02E-05
NM_138439	FLYWCH2	1.39	5.84	5.02E-05
lnc-SCRG1-1:4	lnc-SCRG1-1	1.39	6.90	7.01E-04
NM_001286233	SLC2A14	1.39	10.89	1.83E-02
NM_003152	STAT5A	1.39	9.19	2.57E-03
NR_037629	LOC728739	1.39	8.90	3.43E-05
TCONS_l2_00030495	XLOC_l2_015738	1.39	5.95	6.61E-04
lnc-CTBP1-1:1	lnc-CTBP1-1	1.39	14.43	2.09E-04
ENST00000429552	0	1.39	15.13	2.16E-06
NM_022162	NOD2	1.38	5.74	1.02E-02
lnc-MTHFD2L-1:1	lnc-MTHFD2L-1	1.38	6.07	4.11E-04
NM_181611	KRTAP19-5	1.38	7.89	8.19E-04
NM_003259	ICAM5	1.37	7.07	1.79E-02
ENST00000398190	0	1.37	12.90	1.15E-05
THC2691933	0	1.37	7.13	1.15E-05
A_33_P3411384	0	1.37	7.46	1.54E-03
lnc-SEZ6L2-1:1	lnc-SEZ6L2-1	1.37	10.36	9.23E-06

NM_033027	CSRNP1	1.37	10.13	1.95E-04
ENST00000609439	0	1.36	8.44	3.02E-03
NR_024438	ACTG1P4	1.36	12.18	1.94E-05
A_33_P3519223	0	1.36	6.64	2.77E-03
NM_004952	EFNA3	1.36	7.05	1.27E-03
ENST00000495104	0	1.36	15.05	7.52E-05
ENST00000489727	0	1.36	7.23	1.90E-04
ENST00000412800	0	1.35	10.74	9.08E-06
NM_032833	PPP1R15B	1.35	13.61	1.94E-05
NM_012118	CCRN4L	1.35	7.82	2.94E-03
NM_005438	FOSL1	1.35	11.82	2.49E-04
ENST00000526810	0	1.35	8.46	9.41E-04
ENST00000525280	0	1.35	9.20	4.90E-05
ENST00000603072	0	1.34	8.23	3.66E-04
NR_002768	HYMAI	1.34	8.32	2.06E-02
NM_015508	TIPARP	1.34	14.26	5.37E-04
NM_172387	NFATC1	1.34	8.61	1.65E-02
NM_030647	KDM7A	1.34	7.86	2.18E-03
A_33_P3346526	0	1.33	8.47	1.56E-04
NM_002006	FGF2	1.33	8.60	2.10E-04
NM_152899	IL4I1	1.33	8.30	5.29E-04
NM_001243042	HLA-C	1.33	12.55	2.80E-05
NM_001234	CAV3	1.32	6.65	6.42E-03
ENST00000412562	0	1.32	9.57	1.20E-05
ENST00000440938	0	1.32	14.55	5.20E-06
NM_003446	ZNF157	1.32	8.97	1.26E-04
BC137009	0	1.32	10.42	4.62E-05
NM_014755	SERTAD2	1.32	11.68	1.51E-05
NM_152523	CCNYL1	1.32	7.48	2.07E-02
NM_001025370	VEGFA	1.32	10.56	7.85E-04
NM_001781	CD69	1.32	5.51	1.62E-03
NM_002203	ITGA2	1.31	6.39	5.48E-04
A_33_P3337742	0	1.31	9.19	1.36E-05
NR_027686	LINC00176	1.31	9.94	2.31E-04
ENST00000392885	0	1.31	6.06	1.32E-02
NM_001242758	HLA-A	1.31	15.46	5.61E-05
NM_052880	PIK3IP1	1.30	6.59	2.84E-02
ENST00000464444	0	1.30	14.88	3.30E-06
ENST00000423967	0	1.30	5.26	4.49E-02
ENST00000591045	0	1.30	8.35	1.12E-03
ENST00000567765	0	1.29	6.03	4.59E-03
ENST00000534728	0	1.29	7.98	1.58E-03
NM_002908	REL	1.29	9.90	8.08E-05
NP1243929	0	1.28	9.81	8.26E-05
NM_002658	PLAU	1.28	15.70	1.15E-05
NR_024356	FBLL1	1.28	7.94	2.70E-02

NR_026790	HCG11	1.28	9.98	3.47E-05
ENST00000565297	0	1.27	5.21	7.93E-03
ENST00000396994	0	1.27	11.19	2.25E-05
NM_001286462	C21orf58	1.27	8.79	6.82E-05
NM_006133	DAGLA	1.27	6.25	1.67E-03
ENST00000564363	0	1.27	7.00	3.46E-02
ENST00000342691	0	1.26	6.92	6.42E-03
lnc-LDHC-1:1	lnc-LDHC-1	1.26	7.68	3.03E-02
NM_002991	CCL24	1.25	10.81	3.65E-06
ENST00000529743	0	1.25	8.16	1.05E-02
ENST00000407656	0	1.25	8.41	2.73E-03
ENST00000366413	LOC646513	1.25	11.16	4.80E-05
A_33_P3316379	0	1.25	13.31	8.48E-06
NM_001142459	ASB10	1.24	10.46	1.07E-03
NR_003326	SNORD116-11	1.24	6.33	5.23E-04
NM_014228	SLC6A7	1.24	5.54	7.24E-04
TCONS_l2_00019332	XLOC_l2_010225	1.24	4.94	2.14E-02
NM_003897	IER3	1.24	15.03	8.91E-05
ENST00000423237	0	1.24	8.82	6.04E-06
NM_002543	OLR1	1.24	6.27	3.03E-02
ENST00000427868	LINC00665	1.24	7.89	2.36E-02
NR_104143	LINC00682	1.24	5.40	3.97E-02
NM_001124758	SPNS2	1.23	10.87	2.71E-04
ENST00000407780	0	1.23	9.76	2.28E-03
NM_014417	BBC3	1.23	12.64	1.68E-04
TCONS_l2_00027342	XLOC_l2_013808	1.23	7.24	6.68E-06
NR_027053	LOC646214	1.23	8.05	8.65E-03
NM_031894	FTHL17	1.22	14.48	7.56E-05
NM_181608	KRTAP19-2	1.22	8.70	3.55E-04
NM_000623	BDKRB2	1.22	10.49	2.15E-02
NM_004767	GPR37L1	1.22	8.00	3.36E-02
NM_144492	CLDN14	1.22	6.48	2.07E-02
ENST00000508126	SPSB4	1.22	11.00	2.38E-05
NM_014883	FAM13A	1.21	8.78	1.72E-02
lnc-MINA-3:1	lnc-MINA-3	1.21	11.27	2.55E-05
ENST00000487308	0	1.20	7.01	1.28E-03
NM_021724	NR1D1	1.20	8.67	8.97E-03
A_33_P3235831	0	1.20	6.76	1.42E-03
ENST00000447259	0	1.20	13.54	1.36E-05
lnc-PCF11-1:12	lnc-PCF11-1	1.20	7.99	1.25E-02
lnc-ARRDC3-1:1	lnc-ARRDC3-1	1.20	10.15	1.89E-04
lnc-WARS2-2:1	lnc-WARS2-2	1.19	5.58	2.07E-03
lnc-MBP-1:2	lnc-MBP-1	1.19	9.42	7.40E-05
AK126671	FLJ44715	1.18	6.48	4.56E-03
NM_203356	CTAGE5	1.18	8.21	5.85E-03

lnc-RP11-582J16.5.1-3:1	lnc-RP11-582J16.5.1-3	1.18	8.10	1.66E-03
A_21_P0014324	0	1.18	6.88	1.27E-02
NM_058237	PPP4R4	1.17	5.42	4.10E-02
ENST00000396131	0	1.17	13.72	1.15E-05
NM_001104	ACTN3	1.17	6.88	5.32E-03
NM_012403	ANP32C	1.17	6.99	1.65E-02
ENST00000441295	0	1.17	6.73	4.72E-03
XR_424592	LOC102724362	1.17	10.85	7.23E-05
NM_032413	C15orf48	1.17	12.30	8.65E-03
NM_207322	C2CD4A	1.17	5.42	1.33E-02
lnc-ARSD-1:1	lnc-ARSD-1	1.16	9.29	7.83E-05
ENST00000454614	0	1.16	10.87	4.60E-04
NM_015021	ZNF292	1.16	8.15	1.02E-02
NM_005224	ARID3A	1.16	9.91	1.46E-03
ENST00000441188	0	1.15	6.10	3.73E-03
NM_015714	G0S2	1.15	10.36	2.45E-03
NM_024943	TMEM156	1.15	9.02	1.37E-02
TCONS_l2_00026769	XLOC_l2_013931	1.15	7.24	1.01E-05
TCONS_l2_00013854	XLOC_l2_007456	1.15	12.14	6.33E-05
NM_020439	CAMK1G	1.15	6.86	1.97E-02
ENST00000558641	0	1.15	9.19	3.65E-04
NR_045484	LOC646626	1.15	8.63	9.24E-04
ENST00000473357	SLC2A11	1.14	15.04	1.82E-04
ENST00000379253	SAT1	1.14	8.76	8.03E-04
ENST00000457863	0	1.14	6.24	1.49E-02
NM_004566	PFKFB3	1.14	11.73	4.60E-04
NM_001190702	MTRNR2L8	1.14	13.42	1.52E-05
TCONS_l2_00014564	XLOC_l2_008009	1.14	5.80	3.41E-02
ENST00000475062	0	1.13	8.62	4.90E-05
NM_020130	C8orf4	1.13	12.40	2.45E-03
NM_052952	DIRC1	1.13	9.46	1.04E-04
ENST00000420303	0	1.13	6.78	3.38E-03
NM_001164469	TMED7-TICAM2	1.13	8.72	1.52E-05
NM_002234	KCNA5	1.13	5.07	4.22E-02
NM_001206	KLF9	1.13	10.14	8.56E-03
ENST00000511219	0	1.13	5.57	2.13E-02
ENST00000570531	0	1.12	5.89	4.03E-03
lnc-TRA2A-1:1	lnc-TRA2A-1	1.12	13.70	4.29E-04
NM_005067	SIAH2	1.12	13.32	1.09E-04
lnc-TPM4-2:2	lnc-TPM4-2	1.12	5.03	1.33E-02
NM_000757	CSF1	1.12	7.38	3.46E-02
lnc-SESN3-1:1	lnc-SESN3-1	1.12	5.40	2.52E-02
lnc-OST4-2:3	lnc-OST4-2	1.11	12.54	2.71E-04
ENST00000524369	0	1.11	9.12	9.23E-05
lnc-PPP2R2A-1:1	lnc-PPP2R2A-1	1.11	5.95	1.30E-02

NR_120643	TMEM26-AS1	1.10	9.40	4.54E-04
NM_003456	ZNF205	1.10	9.37	9.83E-03
NM_001122	PLIN2	1.10	10.26	5.01E-05
NM_001013398	IGFBP3	1.10	13.00	4.04E-02
NM_005238	ETS1	1.10	7.62	2.19E-02
NM_175924	ILDR1	1.10	13.27	8.08E-04
ENST00000513626	LUCAT1	1.10	8.62	1.76E-03
ENST00000434541	0	1.10	7.76	1.57E-03
NM_001198	PRDM1	1.10	9.65	9.08E-03
THC2573499	0	1.09	5.61	5.78E-03
NM_001164404	GOLGA6C	1.09	6.25	1.37E-03
ENST00000436474	0	1.09	10.49	2.15E-05
NR_003682	MGC70870	1.09	10.35	1.26E-04
NR_073397	ERICH1-AS1	1.09	7.91	2.45E-03
lnc-LRGUK-1:1	lnc-LRGUK-1	1.09	6.86	1.95E-03
ENST00000584867	0	1.09	11.70	9.98E-04
NM_198893	ZNF160	1.08	8.69	1.68E-04
NM_000596	IGFBP1	1.08	13.02	2.09E-02
NM_004556	NFKBIE	1.08	12.82	1.26E-04
A_33_P3336038	0	1.07	15.75	2.55E-05
NM_002999	SDC4	1.07	12.35	2.21E-04
NM_017964	SLC30A6	1.07	6.75	2.11E-02
NR_046420	UPK1A-AS1	1.07	6.31	3.91E-03
ENST00000459875	0	1.07	12.57	9.15E-05
ENST00000423841	0	1.07	14.72	1.05E-05
NM_134268	CYGB	1.07	12.47	2.83E-05
ENST00000458667	LOC102725353	1.07	11.46	9.21E-04
NR_121635	FOXD3-AS1	1.07	14.30	5.90E-04
NM_001265594	PLEKHG5	1.07	10.60	2.74E-04
NM_015675	GADD45B	1.07	14.48	1.28E-02
NM_012421	RLF	1.06	11.12	2.19E-05
NM_006931	SLC2A3	1.06	7.00	2.61E-02
NM_002641	PIGA	1.06	11.17	4.58E-05
NM_001101337	C3orf79	1.06	5.27	2.41E-02
ENST00000475432	HIF3A	1.06	11.27	1.94E-05
ENST00000444388	0	1.06	9.00	1.89E-04
NM_001165877	ATP5L2	1.06	13.46	4.67E-05
NM_004567	PFKFB4	1.06	10.17	1.06E-02
NM_178815	ARL5B	1.05	9.23	2.71E-04
ENST00000447729	0	1.05	6.78	4.49E-03
lnc-RIT2-1:1	lnc-RIT2-1	1.05	9.06	2.73E-04
NM_153607	CREBRF	1.05	9.10	1.35E-03
lnc-METAP1-2:1	lnc-METAP1-2	1.05	10.41	2.97E-03
ENST00000559752	0	1.05	8.49	2.07E-02
NM_032649	CNDP1	1.05	5.12	2.30E-02
TCONS_l2_00001954	XLOC_l2_000018	1.05	8.56	7.52E-03

NM_001005466	OR10G2	1.05	6.30	3.51E-03
lnc-NFYB-1:1	lnc-NFYB-1	1.05	10.35	2.10E-04
lnc-DNASE1L3-1:1	lnc-DNASE1L3-1	1.04	11.71	1.63E-04
NM_004621	TRPC6	1.04	9.58	2.31E-04
ENST00000428814	0	1.04	9.17	9.97E-04
lnc-ZKSCAN1-1:4	lnc-ZKSCAN1-1	1.04	9.84	3.47E-05
lnc-LECT2-1:3	lnc-LECT2-1	1.04	4.99	4.98E-02
A_33_P3213134	0	1.04	9.50	8.73E-05
NM_000499	CYP1A1	1.04	7.62	1.97E-02
NM_014950	ZBTB1	1.04	8.16	3.36E-03
ENST00000419201	0	1.04	8.41	2.47E-05
ENST00000567888	MIR940	1.04	9.57	2.14E-02
NM_001098402	ZBTB21	1.04	11.03	6.19E-05
lnc-PLTP-1:1	lnc-PLTP-1	1.04	8.59	1.52E-03
NM_001272068	SHISA5	1.03	12.36	5.51E-05
NM_005542	INSIG1	1.03	11.31	5.07E-03
AF086546	0	1.03	6.67	3.09E-02
NM_001145139	CXorf49B	1.03	6.74	2.41E-03
NM_015288	JADE2	1.03	8.60	5.38E-03
lnc-C15orf57-1:1	lnc-C15orf57-1	1.03	10.36	1.92E-04
NM_001128636	ELFN1	1.03	9.19	1.31E-03
lnc-NAA35-1:2	lnc-NAA35-1	1.02	9.46	3.26E-04
NM_003044	SLC6A12	1.02	6.67	2.99E-02
A_33_P3407235	0	1.02	5.93	4.44E-03
NM_032271	TRAF7	1.02	11.04	5.51E-05
lnc-ARRDC3-1:13	lnc-ARRDC3-1	1.02	7.38	2.78E-02
ENST00000521326	0	1.02	7.28	7.05E-03
ENST00000375322	MAP3K8	1.02	11.68	5.15E-04
NM_001031737	CCDC78	1.02	12.04	2.18E-03
NM_144972	LDHAL6A	1.02	10.65	5.04E-05
NR_003680	RPL13AP17	1.01	8.18	1.18E-02
A_32_P113154	0	1.01	15.27	1.17E-05
ENST00000420492	0	1.01	7.85	9.40E-03
NM_018366	BLOC1S4	1.01	9.26	1.97E-03
ENST00000473094	0	1.01	13.47	2.55E-05
A_33_P3224971	0	1.01	11.06	8.13E-04
NM_006136	CAPZA2	1.01	9.42	2.67E-03
ENST00000510120	0	1.01	6.36	6.16E-03
NR_110219	LOC101927285	1.01	9.32	3.13E-04
BM918074	SNORA71A	1.01	9.80	3.99E-03
lnc-IFRD2-2:1	lnc-IFRD2-2	1.01	8.87	1.17E-03
NR_103761	CYP4F62P	1.01	8.60	2.37E-04
NM_004433	ELF3	1.00	13.48	4.02E-04
ENST00000415184	0	1.00	11.10	1.52E-05
NM_006516	SLC2A1	1.00	14.09	3.13E-04
lnc-SIX6-1:1	lnc-SIX6-1	1.00	5.94	1.21E-02

AK057884	0	1.00	12.98	3.34E-03
lnc-AC016722.2.1-2:1	lnc-AC016722.2.1-2	-1.03	7.63	2.01E-03
NR_002739	SNORD56	-1.03	9.18	2.58E-03
NM_004441	EPHB1	-1.04	5.12	1.73E-02
A_33_P3304369	0	-1.04	8.34	8.30E-05
A_33_P3404221	0	-1.05	6.78	3.24E-02
NM_001202	BMP4	-1.07	9.55	1.02E-02
NM_024508	ZBED2	-1.07	9.18	1.74E-04
NM_021979	HSPA2	-1.10	12.83	1.76E-04
NM_032637	SKP2	-1.12	8.59	2.67E-03
NR_002742	SNORD52	-1.12	9.44	7.69E-04
NM_014562	OTX1	-1.15	9.18	3.69E-04
NM_001040152	PEG10	-1.15	11.30	1.35E-02
A_33_P3389668	0	-1.17	5.54	6.54E-03
NM_005322	HIST1H1B	-1.21	9.87	2.16E-03
NM_153201	HSPA8	-1.21	15.09	3.79E-03
ENST00000568686	0	-1.26	10.16	9.05E-05
ENST00000532091	0	-1.26	7.33	8.87E-03
NM_001271938	MEGF8	-1.28	11.75	4.00E-06
NM_001080461	UNCX	-1.29	14.63	1.26E-04
NM_199461	NANOS1	-1.29	9.59	6.15E-05
NM_053001	OSR2	-1.36	8.56	1.71E-03
NR_003672	SNHG7	-1.37	9.19	4.37E-04
NR_024046	NRADDP	-1.37	6.26	1.25E-02
NM_015672	RIMBP3	-1.42	10.01	1.26E-03
NM_005345	HSPA1A	-3.27	12.11	3.18E-05
NM_005346	HSPA1B	-3.41	10.99	3.38E-05

Table A2.3. List of enriched GO terms, KEGG pathways and their relative genes identified using all the significantly up-regulated genes in A549 cells infected with *A. baumannii* ATCC19606.

GO term/KEGG pathway	Gene symbol	Fold Enrichment	FDR
GO biological process			
GO:0006954~inflammatory response	NFKBIZ, IRAK2, TNFAIP3, REL, CXCL2, CXCR4, BDKRB1, CCL20, CCL24, SERPINA3, PTX3, NFKB1, CXCL3, CXCL1, IL1B, OLR1, IL6, IL23A, CCL5, TMED7-TICAM2, CCL2, SELE, NFKBID, IL1A, TNF, ADORA2A, TNFRSF9, FOS, ELF3, BDKRB2, ZC3H12A, PTGS2, CXCL8	5.34	3.59E-11
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	NOD2, NR4A1, MAFK, RLF, 0, NR4A3, KDM6B, INHBA, IRF1, NFKB1, BHLHE23, POU5F1, ELF5, HOXB9, IL6, FOSL1, ATF3, NFATC1, CSF289, IL23A, HMGA2, KDM3A, CSRNP1, TFAM, FGF2, FOS, GATA6, SERPINE1, NR4A2, HIF3A, VEGFA, EGR2, REL, CSF3, JUN, KLF6, NFKBIA, IL1B, ZNF292, PER1, MAFF, IL1A, TNF, FOSB, ETS1, ELF3, BCL3, ZC3H12A, DDIT3, EGR1	3.13	2.24E-09
GO:0071222~cellular response to lipopolysaccharide	ZFP36, IL6, CSF2, TMED7-TICAM2, CCL2, TNFAIP3, CSF3, TNF, PLAU, CCL20, NR1D1, SERPINE1, NFKB1, ICAM1, ZC3H12A, CXCL8	7.74	3.80E-06
GO:0045766~positive regulation of angiogenesis	PTK2B, CCL5, VEGFA, IL1A, CCL24, FGF2, ADM, ETS1, GATA6, ANGPTL4, SERPINE1, ZC3H12A, IL1B, CXCL8, CYP1B1	8.01	8.93E-06
GO:0001666~response to hypoxia	PTK2B, ITGA2, CCL2, VEGFA, KDM3A, PLAU, CYP1A1, CXCR4, EGLN3, DDIT4, SOCS3, CYGB, ADM, ETS1, ANGPTL4, NR4A2, IL1B, EGR1	5.72	3.03E-05
GO:0006366~transcription from RNA polymerase II promoter	NCOR1, EGR2, MAFK, REL, JUN, CCRN4L, IRF1, NFKB1, HIVEP2, POU5F1, ELF5, ZNF292, FOSL1, ATF3, NFATC1, 0, MAFF, HMGA2, NFIL3, CSRNP1, TFAM, FOSB, ETS1, FOS, ELF3, GATA6, HIF3A, EGR1	3.35	1.37E-04
GO:0006915~apoptotic process	NR4A1, TNFAIP3, PPP1R15A, MAP3K8, CXCR4, EGLN3, BIRC3, C8orf4, IRF1, NFKB1, NFKBIA, SERPINB9, TRAF1, BBC3, IL1B, PTK2B, GADD45B, IL1A, PIM1, IER3, CSRNP1, GADD45A, ADORA2A, TNFRSF9, HIF3A, PIM3, ZC3H12A, DDIT3	3.05	8.93E-04
GO:0008285~negative regulation of cell proliferation	IL6, KLF10, PTK2B, FOSL1, 0, NEURL1, PPP1R15A, IL1A, JUN, SPRY1, INHBA, FGF2, IRF1, ADM, ETS1, TNFRSF9, NDRG1, CXCL1, BDKRB2, IL1B, PTGS2, CXCL8, CYP1B1	3.48	1.42E-03
GO:0071456~cellular response to hypoxia	VEGFA, GATA6, NDRG1, STC2, TRPC6, STC1, ICAM1, BDKRB2, BBC3, BNIP3L, PLAU, PTGS2	6.95	2.07E-03

GO:0006955~immune response	IL6, CSF2, CCL5, CCL2, NFIL3, IL1A, CSF3, CXCL2, TNF, CCL20, CCL24, HLA-C, LTB, ETS1, TNFRSF9, CXCL3, CXCL1, HLA-A, GEM, SERPINB9, IL1B, CXCL8, IL32	3.39	2.20E-03
GO:0070098~chemokine-mediated signaling pathway	CCL20, PTK2B, CCL24, CCL5, CCL2, CXCL3, CXCL1, CXCL2, CXCL8, CXCR4	8.72	3.38E-03
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	NCOR1, KLF10, VEGFA, BHLHE40, REL, ZBTB1, 0, KLF17, NR4A3, ZNF157, NFKB1, CREBRF, PRDM1, POU5F1, IL1B, ZFP36, ATF3, 0, BHLHE41, PER1, ZNF205, HMGA2, NFIL3, TNF, NR1D1, FOSB, GATA6, NR4A2, EFNA1, DDIT3, EGR1	2.62	4.41E-03
GO:0045893~positive regulation of transcription, DNA-templated	EGR2, RLF, JUN, KDM7A, KLF6, INHBA, IRF1, NFKB1, IL1B, IL6, SERTAD2, NFATC1, HMGA2, KDM3A, TNF, NR1D1, TFAM, FGF2, ETS1, FOS, ELF3, GATA6, BCL3, DDIT3, EGR1	3.00	5.77E-03
GO:0006935~chemotaxis	CCL20, CCL24, FOSL1, CCL5, FGF2, CCL2, CKLF, CXCL1, CXCL2, PLAU, CXCL8, CXCR4	6.04	8.22E-03
GO:0007623~circadian rhythm	EGR3, NR1D1, NCOR1, KLF10, PER1, SERPINE1, NFIL3, KLF9, JUN, CCRN4L	7.65	1.03E-02
GO:0071407~cellular response to organic cyclic compound	NOD2, CCL5, TIPARP, CCL2, NFKBIA, TNF, CYP1A1, IL1B, CYP1B1	8.71	1.41E-02
GO:0030593~neutrophil chemotaxis	CCL20, CCL24, CCL5, CCL2, CXCL3, CKLF, EDN2, IL1B, CXCL8	8.45	1.78E-02
GO:0042493~response to drug	IL6, PTK2B, FOSL1, CA9, ITGA2, CCL5, JUN, PLIN2, CYP1A1, FOSB, INHBA, SOCS3, FOS, GATA6, SLC12A5, ICAM1, BDKRB2, PTGS2, DDIT3	3.34	2.88E-02
GO:0007568~aging	IL6, CCL5, CCL2, PPP1R15A, JUN, CYP1A1, SOCS3, FGF2, ADM, FOS, TRPC6, IGFBP1, IL1B, DDIT3	4.25	4.66E-02
GO molecular function			
GO:0003700~transcription factor activity, sequence-specific DNA binding	KLF10, EGR2, MAFK, BHLHE40, REL, JUN, CCRN4L, KLF6, 0, KLF17, ZNF157, IRF1, ZNF90, NFKB1, CREBRF, HIVEP2, PRDM1, POU5F1, EGR4, EGR3, FOSL1, ATF3, NFATC1, 0, MAFF, ZNF205, NFIL3, KDM3A, CSRNP1, NR1D1, FOSB, TFAM, FOS, ETS1, ELF3, GATA6, KLF9, STAT5A, BCL3, EGR1, DDIT3	2.65	3.60E-05
GO:0043565~sequence-specific DNA binding	FOSL1, ATF3, SPNS2, CSF289, MAFF, NR4A1, ZNF205, MAFK, NFIL3, JUN, CSRNP1, FOSB, NR4A3, KDM6B, IRF1, FOS, ETS1, ELF3, CREBRF, NR4A2, HIVEP2, POU5F1, ELF5, HOXB9, DDIT3, EGR1	3.12	1.38E-03
GO:0008009~chemokine activity	CCL20, CCL24, CCL5, CCL2, CXCL3, CKLF, CXCL1, CXCL2, CXCL8	11.44	1.39E-03
GO:0005125~cytokine activity	IL6, CSF2, IL23A, VEGFA, IL1A, CSF3, TNF, LTB, INHBA, FGF2, GDF15, CKLF, IL1B, IL32	4.95	7.39E-03
GO:0001077~transcriptional activator activity, RNA polymerase II core promoter	FOSL1, EGR2, NR4A1, HMGA2, JUN, NR4A3, TFAM, FOSB, IRF1, FOS, ELF3, NR4A2, ZNF292, DDIT3, EGR1	4.01	3.41E-02

proximal region sequence-specific binding			
GO:0000982~transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	ATF3, FOSL1, FOSB, ETS1, FOS, JUN	15.57	4.72E-02
GO cellular component			
GO:0005615~extracellular space	VEGFA, APOL4, ICAM4, CSF3, STC1, CXCL2, PLAU, CCL20, CCL24, PTX3, SERPINA3, GDF15, CXCL3, CXCL1, IGFBP1, TWSG1, PCSK1N, ANXA2, SERPINB9, IGFL1, IL1B, IL32, IL6, CSF2, CCL5, CCL2, SELE, IL1A, HILPDA, TNF, LTB, ADM, FGF2, TNFRSF9, CKLF, ANGPTL4, SERPINE1, STC2, EDN2, lnc-KRT83-1, ICAM1, CXCL8	2.05	1.38E-02
KEGG pathway			
hsa04668:TNF signaling pathway	IL6, NOD2, CSF2, CCL5, CCL2, SELE, TNFAIP3, JUN, CXCL2, TNF, MAP3K8, BIRC3, CCL20, SOCS3, FOS, NFKB1, NFKBIA, CXCL3, CXCL1, ICAM1, BCL3, TRAF1, PTGS2, IL1B	12.32	4.79E-16
hsa05323:Rheumatoid arthritis	IL6, CSF2, IL23A, CCL5, CCL2, VEGFA, IL1A, JUN, TNF, CCL20, LTB, FOS, ICAM1, IL1B, CXCL8	9.27	5.46E-07
hsa04621:NOD-like receptor signaling pathway	IL6, NOD2, CCL5, CCL2, NFKB1, NFKBIA, TNFAIP3, TNF, IL1B, CXCL8, BIRC3	10.88	5.08E-05
hsa04064:NF-kappa B signaling pathway	TMED7-TICAM2, TNFAIP3, TNF, PLAU, BIRC3, LTB, NFKBIA, NFKB1, ICAM1, TRAF1, IL1B, PTGS2, CXCL8	8.13	5.45E-05
hsa05133:Pertussis	IL6, IL23A, TMED7-TICAM2, IRF1, FOS, NFKB1, IL1A, JUN, TNF, IL1B, CXCL8	7.98	1.06E-03
hsa05132:Salmonella infection	IL6, CSF2, FOS, NFKB1, CXCL3, CXCL1, IL1A, JUN, CXCL2, IL1B, CXCL8	7.21	2.75E-03
hsa05142:Chagas disease (American trypanosomiasis)	IL6, CCL5, CCL2, FOS, NFKB1, NFKBIA, SERPINE1, JUN, BDKRB2, TNF, IL1B, CXCL8	6.28	3.11E-03
hsa05166:HTLV-I infection	ZFP36, IL6, ATF3, CSF2, FOSL1, NFATC1, EGR2, JUN, TNF, HLA-C, FOS, ETS1, NFKB1, NFKBIA, HLA-A, STAT5A, ICAM1, EGR1	3.83	4.14E-03
hsa05134:Legionellosis	IL6, NFKB1, CXCL3, NFKBIA, CXCL1, CXCL2, TNF, IL1B, CXCL8	9.07	6.18E-03
hsa05161:Hepatitis B	EGR3, IL6, PTK2B, NFATC1, TMED7-TICAM2, EGR2, FOS, NFKB1, NFKBIA, STAT5A, JUN, TNF, CXCL8	4.88	1.42E-02
hsa05321:Inflammatory bowel disease (IBD)	IL6, NOD2, NFATC1, IL23A, NFKB1, IL1A, JUN, TNF, IL1B	7.65	2.26E-02
hsa04620:Toll-like receptor signaling pathway	IL6, CCL5, TMED7-TICAM2, FOS, NFKB1, NFKBIA, JUN, TNF, MAP3K8, IL1B, CXCL8	5.65	2.53E-02
hsa05168:Herpes simplex infection	IL6, CCL5, CCL2, PER1, JUN, TNF, SOCS3, HLA-C, FOS, NFKBIA, NFKB1, HLA-A, TRAF1, IL1B	4.16	3.15E-02

hsa05144:Malaria	IL6, CCL2, SELE, CSF3, ICAM1, TNF, IL1B, CXCL8	8.88	3.16E-02

Table S4. List of enriched GO terms, KEGG pathways and their relative genes identified using all the significantly up-regulated genes in A549 cells infected with *A. baumannii* ATCC 19606 and treated with polymyxin B.

GO term/KEGG pathway	Gene symbol	Fold Enrichment	FDR
GO biological process			
GO:0006954~inflammatory response	NFKBIZ, IRAK2, TNFAIP3, REL, CXCL2, CXCR4, BDKRB1, CCL20, CCL24, SERPINA3, PTX3, CXCL3, CXCL1, IL1B, OLR1, IL6, CCL5, TMED7-TICAM2, CCL2, SELE, NFKBID, IL1A, TNF, FOS, ELF3, CSF1, BDKRB2, ZC3H12A, PTGS2, CXCL8	5.52	2.72E-10
GO:0045944~positive regulation of transcription from RNA polymerase II promoter	NOD2, EGR2, VEGFA, NR4A1, CSF3, REL, JUN, RLF, KLF6, UHRF1, NR4A3, KDM6B, INHBA, IRF1, NFKBIA, BHLHE23, POU5F1, ZNF292, IL1B, IL6, FOSL1, ATF3, NFATC1, PER1, MAFF, HMGA2, IL1A, KDM3A, CSRNP1, TNF, FOSB, TFAM, FGF2, FOS, ETS1, ELF3, GATA6, NR4A2, HIF3A, ARID3A, ZC3H12A, EGR1, DDIT3	3.07	1.99E-07
GO:0001666~response to hypoxia	ITGA2, CCL2, VEGFA, KDM3A, CYP1A1, PLAU, CXCR4, EGLN3, DDIT4, CYGB, ADM, ETS1, ANGPTL4, NR4A2, KCNA5, IL1B, EGR1	6.15	3.03E-05
GO:0071222~cellular response to lipopolysaccharide	ZFP36, IL6, CSF2, CCL2, TMED7-TICAM2, TNFAIP3, CSF3, TNF, PLAU, CCL20, NR1D1, ICAM1, ZC3H12A, CXCL8	7.71	5.49E-05
GO:0071456~cellular response to hypoxia	VEGFA, GATA6, NDRG1, STC2, TRPC6, STC1, ICAM1, BDKRB2, BBC3, BNIP3L, PLAU, PTGS2	7.91	5.67E-04
GO:0006366~transcription from RNA polymerase II promoter	NCOR1, EGR2, REL, JUN, CCRN4L, IRF1, HIVEP2, POU5F1, ZNF292, ATF3, FOSL1, NFATC1, MAFF, HMGA2, NFIL3, CSRNP1, TFAM, FOSB, ETS1, FOS, ELF3, GATA6, HIF3A, ARID3A, EGR1	3.41	5.71E-04
GO:0045766~positive regulation of angiogenesis	CCL24, CCL5, ADM, FGF2, VEGFA, ETS1, GATA6, ANGPTL4, IL1A, ZC3H12A, IL1B, CXCL8	7.30	1.29E-03
GO:0006935~chemotaxis	CCL20, CCL24, FOSL1, CCL5, FGF2, CCL2, CKLF, CXCL1, CXCL2, PLAU, CXCL8, CXCR4	6.88	2.31E-03
GO:0030593~neutrophil chemotaxis	CCL20, CCL24, CCL5, CCL2, CXCL3, CKLF, EDN2, IL1B, CXCL8	9.62	6.80E-03
GO:0045893~positive regulation of transcription, DNA-templated	IL6, SERTAD2, NFATC1, EGR2, HMGA2, KDM3A, JUN, RLF, TNF, KLF6, KDM7A, NR1D1, TFAM, INHBA, FGF2, IRF1, ETS1, FOS, ELF3, GATA6, IL1B, DDIT3, EGR1	3.14	7.32E-03
GO:0000122~negative regulation of transcription from RNA polymerase II promoter	NCOR1, KLF10, VEGFA, BHLHE40, REL, ZBTB1, UHRF1, NR4A3, ZNF157, CREBRF, PRDM1, POU5F1, IL1B, ZFP36, ATF3, BHLHE41, PER1, ZNF205, HMGA2, NFIL3, TNF, NR1D1, FOSB, GATA6, NR4A2, EFNA1, DDIT3, EGR1	2.70	8.63E-03
GO:0008285~negative regulation of cell proliferation	KLF10, IL6, FOSL1, NEURL1, PPP1R15A, IL1A, JUN, SPRY1, INHBA, FGF2, ADM,	3.45	1.00E-02

	IRF1, ETS1, NDRG1, CXCL1, BDKRB2, PTGS2, IL1B, CXCL8, IGFBP3		
GO:0006915~apoptotic process	TRAF7, NR4A1, TNFAIP3, SIAH2, PPP1R15A, IL1A, GADD45B, PIM1, MAP3K8, CSRNP1, IER3, BIRC3, CXCR4, EGLN3, C8orf4, IRF1, NFKBIA, HIF3A, TRAF1, BBC3, ZC3H12A, IL1B, IGFBP3, DDIT3	2.98	1.02E-02
GO:0070098~chemokine-mediated signaling pathway	CCL20, CCL24, CCL5, CCL2, CXCL3, CXCL1, CXCL2, CXCL8, CXCR4	8.94	1.19E-02
GO:0042542~response to hydrogen peroxide	OLR1, NR4A3, FOSL1, PPP1R15B, KCNA5, JUN, DUSP1, DDIT3	10.08	2.25E-02
GO:0036499~PERK-mediated unfolded protein response	ATF3, CCL2, IGFBP1, CXCL8, DDIT3	29.39	3.00E-02
GO:0007623~circadian rhythm	EGR3, NR1D1, NCOR1, KLF10, PER1, NFIL3, KLF9, JUN, CCRN4L	7.84	3.18E-02
GO molecular function			
GO:0003700~transcription factor activity, sequence-specific DNA binding	KLF10, EGR2, BHLHE40, REL, JUN, CCRN4L, KLF6, UHRF1, ZNF157, IRF1, ZNF90, CREBRF, HIVEP2, PRDM1, POU5F1, EGR4, EGR3, FOSL1, ATF3, NFATC1, MAFF, ZNF205, NFIL3, KDM3A, CSRNP1, NR1D1, FOSB, TFAM, ETS1, FOS, ELF3, GATA6, KLF9, STAT5A, DDIT3, EGR1	2.64	3.17E-04
GO:0008009~chemokine activity	CCL20, CCL24, CCL5, CCL2, CXCL3, CKLF, CXCL1, CXCL2, CXCL8	12.97	5.32E-04
GO:0001077~transcriptional activator activity, RNA polymerase II core promoter proximal region sequence-specific binding	FOSL1, EGR2, NR4A1, HMGA2, JUN, NR4A3, TFAM, FOSB, IRF1, FOS, ELF3, NR4A2, ZNF292, DDIT3, EGR1	4.55	8.12E-03
GO:0005125~cytokine activity	IL6, CSF2, VEGFA, IL1A, CSF3, TNF, LTB, INHBA, FGF2, GDF15, CKLF, CSF1, IL1B	5.22	1.07E-02
GO:0043565~sequence-specific DNA binding	FOSL1, ATF3, SPNS2, MAFF, NR4A1, ZNF205, NFIL3, JUN, CSRNP1, FOSB, NR4A3, KDM6B, IRF1, ETS1, FOS, ELF3, CREBRF, NR4A2, HIVEP2, POU5F1, DDIT3, EGR1	2.99	2.12E-02
GO:0000982~transcription factor activity, RNA polymerase II core promoter proximal region sequence-specific binding	ATF3, FOSL1, FOSB, ETS1, FOS, JUN	17.65	2.56E-02
GO cellular component			
GO:0005615~extracellular space	VEGFA, ICAM4, CSF3, STC1, CXCL2, PLAU, CCL20, CCL24, PTX3, SERPINA3, GDF15, CXCL3, CXCL1, IGFBP1, TWSG1, ANXA2, IGFL1, IL1B, IGFBP3, IL6, CSF2, CCL5, CCL2, SELE, HILPDA, IL1A, TNF, LTB, ADM, FGF2, CKLF, ANGPTL4, STC2, EDN2, KRT81, ICAM1, CSF1, CXCL8	2.11	1.94E-02
KEGG pathway			
hsa04668:TNF signaling pathway	IL6, NOD2, CSF2, CCL5, CCL2, SELE, TNFAIP3, JUN, CXCL2, TNF, MAP3K8, BIRC3, CCL20, FOS, NFKBIA, CXCL3, CXCL1, ICAM1, TRAF1, CSF1, IL1B, PTGS2	12.26	2.38E-14

hsa05323:Rheumatoid arthritis	IL6, CSF2, CCL5, CCL2, VEGFA, IL1A, JUN, TNF, CCL20, LTB, FOS, ICAM1, CSF1, IL1B, CXCL8	10.07	1.78E-07
hsa04064:NF-kappa B signaling pathway	LTB, TMED7-TICAM2, NFKBIA, TNFAIP3, ICAM1, TRAF1, TNF, PLAU, IL1B, PTGS2, CXCL8, BIRC3	8.15	2.18E-04
hsa04621:NOD-like receptor signaling pathway	IL6, NOD2, CCL5, CCL2, NFKBIA, TNFAIP3, TNF, IL1B, CXCL8, BIRC3	10.74	3.17E-04
hsa05166:HTLV-I infection	ZFP36, IL6, ATF3, CSF2, FOSL1, NFATC1, EGR2, JUN, TNF, HLA-C, FOS, ETS1, NFKBIA, HLA-A, STAT5A, ICAM1, SLC2A1, EGR1	4.15	1.28E-03
hsa05132:Salmonella infection	IL6, CSF2, FOS, CXCL3, CXCL1, IL1A, JUN, CXCL2, IL1B, CXCL8	7.12	1.11E-02
hsa05144:Malaria	IL6, CCL2, SELE, CSF3, ICAM1, TNF, IL1B, CXCL8	9.64	1.85E-02
hsa05134:Legionellosis	IL6, CXCL3, NFKBIA, CXCL1, CXCL2, TNF, IL1B, CXCL8	8.75	3.55E-02
hsa05133:Pertussis	IL6, TMED7-TICAM2, IRF1, FOS, IL1A, JUN, TNF, IL1B, CXCL8	7.09	4.02E-02

Table A2.5. List of enriched GO terms, KEGG pathways and their relative genes identified using the 10% of genes with the highest fold increase in A549 infected with *A. baumannii* ATCC 19606 and treated with polymyxin B.

GO term/KEGG pathway	Gene symbol	Fold Enrichment	FDR
GO biological process			
GO:0006954~inflammatory response	IL6, NFKBIZ, NFKBID, TNFAIP3, IL1A, CXCL2, TNF, CCL20, PTX3, CXCL3, CXCL1, IL1B, CXCL8	21.92	9.66E-11
GO:0006955~immune response	IL6, CCL20, CSF2, LTB, CXCL3, CXCL1, IL1A, CXCL2, TNF, IL1B, CXCL8	16.91	2.92E-07
GO:0071222~cellular response to lipopolysaccharide	IL6, CCL20, CSF2, TNFAIP3, ICAM1, TNF, CXCL8	35.31	3.97E-05
GO:0090023~positive regulation of neutrophil chemotaxis	CXCL3, CXCL1, CXCL2, IL1B, CXCL8	134.51	5.76E-05
GO:0045429~positive regulation of nitric oxide biosynthetic process	IL6, PTX3, ICAM1, TNF, IL1B	71.74	7.91E-04
GO:0070098~chemokine-mediated signaling pathway	CCL20, CXCL3, CXCL1, CXCL2, CXCL8	45.47	5.02E-03
GO:2001240~negative regulation of extrinsic apoptotic signaling pathway in absence of ligand	CSF2, IL1A, TNF, IL1B	69.8	3.17E-02
GO molecular function			
GO:0008009~chemokine activity	CCL20, CXCL3, CXCL1, CXCL2, CXCL8	61.65	1.07E-03
GO:0005125~cytokine activity	IL6, CSF2, LTB, IL1A, TNF, IL1B	20.6	7.91E-03
GO cellular component			
GO:0005615~extracellular space	IL6, CSF2, IL1A, ICAM4, CXCL2, TNF, CCL20, PTX3, LTB, ANGPTL4, CXCL3, STC2, KRT81, CXCL1, ICAM1, IL1B, CXCL8	8.12	4.56E-09
GO:0005576~extracellular region	IL6, CCL20, CSF2, PTX3, CXCL3, ANGPTL4, STC2, CXCL1, IL1A, CXCL2, TNF, IL1B, CXCL8	5.26	9.35E-04
KEGG pathway			
hsa04668:TNF signaling pathway	IL6, CCL20, CSF2, CXCL3, CXCL1, TNFAIP3, ICAM1, CXCL2, TNF, IL1B	32.59	2.85E-09
hsa05323:Rheumatoid arthritis	IL6, CCL20, CSF2, LTB, IL1A, ICAM1, TNF, IL1B, CXCL8	35.34	3.54E-08
hsa05132:Salmonella infection	IL6, CSF2, CXCL3, CXCL1, IL1A, CXCL2, IL1B, CXCL8	33.3	1.31E-06
hsa05134:Legionellosis	IL6, CXCL3, CXCL1, CXCL2, TNF, IL1B, CXCL8	44.79	4.51E-06
hsa04060:Cytokine-cytokine receptor interaction	IL6, CCL20, CSF2, LTB, IL1A, TNF, IL1B, CXCL8	12.02	1.55E-03
hsa04064:NF-kappa B signaling pathway	LTB, TNFAIP3, ICAM1, TNF, IL1B, CXCL8	23.83	2.99E-03
hsa05144:Malaria	IL6, ICAM1, TNF, IL1B, CXCL8	35.26	8.40E-03

hsa04621:NOD-like receptor signaling pathway	IL6, TNFAIP3, TNF, IL1B, CXCL8	31.41	1.34E-02
hsa05133:Pertussis	IL6, IL1A, TNF, IL1B, CXCL8	23.03	4.60E-02

Table A2.6. Differentially expressed genes (log_2 fold-change ≥ |1|, FDR ≤ 0.05) in *A. baumannii* ATCC 19606 following exposure to A549 cells. Statistical significance was calculated using F-statistic with Benjamini Hochberg adjustment to control the FDR.

Locus tag	Gene ID (BioCyc)	Product	Log_2 fold-change	FDR
PRK_01706	HMPREF0010_01868	conserved hypothetical protein	3.75	1.74E-04
PRK_01334	HMPREF0010_03356	conserved hypothetical protein	3.22	2.07E-05
PRK_01211	HMPREF0010_03423	fumarylacetoacetase	2.97	2.25E-03
PRK_03749	HMPREF0010_00185	predicted protein	2.78	8.79E-06
PRK_00971	HMPREF0010_02531	bacterioferritin	2.76	6.07E-04
PRK_00726	HMPREF0010_02733	conserved hypothetical protein	2.72	1.68E-05
PRK_01210	HMPREF0010_03424	GABA permease	2.67	3.55E-03
PRK_02073	HMPREF0010_01303	bacterioferritin	2.66	3.93E-04
PRK_01215	HMPREF0010_03419	4-hydroxyphenylpyruvate dioxygenase	2.65	6.03E-03
PRK_01046	HMPREF0010_02462	predicted protein	2.55	2.25E-03
PRK_00399	HMPREF0010_02172	thiosulfate-binding protein	2.32	7.03E-03
PRK_02998	HMPREF0010_00935	taurine ABC transporter, periplasmic binding protein	2.26	1.73E-02
PRK_01212	HMPREF0010_03422	maleylacetoacetate isomerase	2.14	2.62E-02
PRK_00211	HMPREF0010_02352	outer membrane protein W	2.12	2.25E-03
PRK_01213	HMPREF0010_03421	lactoylglutathione lyase	2.07	3.78E-02
PRK_00923	HMPREF0010_02579	tolA	1.89	1.32E-04
PRK_02627	HMPREF0010_03158	OmpA/MotB	1.77	5.49E-04
PRK_01526	HMPREF0010_04161	tRNA-Ala	1.61	3.76E-02
PRK_01635	HMPREF0010_01936	selenocysteine synthase	1.60	9.79E-04
PRK_00699	HMPREF0010_02760	peroxiredoxin	1.59	3.78E-04
PRK_01311	HMPREF0010_03655	conserved hypothetical protein	1.57	4.09E-02
PRK_00928	HMPREF0010_02574	ornithine-oxo-acid transaminase	1.57	5.17E-04
PRK_01317	HMPREF0010_04161	tRNA-Ala	1.57	5.01E-03
PRK_01272	HMPREF0010_03695	RND type efflux pump	1.57	1.26E-03
PRK_03282	HMPREF0010_00651	aspartate ammonia-lyase	1.56	2.07E-03
PRK_00926	HMPREF0010_02576	succinylglutamic semialdehyde dehydrogenase	1.55	4.34E-03
PRK_01986	HMPREF0010_04106	tRNA-Pro	1.53	2.99E-03
PRK_02237	HMPREF0010_01467	conserved hypothetical protein	1.52	1.95E-02

PRK_01626	HMPREF0010_01945	predicted protein	1.51	9.16E-03
PRK_01119	HMPREF0010_03516	porin	1.49	1.67E-03
PRK_00925	HMPREF0010_02577	succinylarginine dihydrolase	1.49	4.68E-03
PRK_03671	HMPREF0010_00263	small-conductance mechanosensitive channel	1.49	1.18E-03
PRK_01938	HMPREF0010_01636	organic hydroperoxide resistance protein	1.47	6.67E-04
PRK_00929	HMPREF0010_02573	glutamate dehydrogenase/leucine dehydrogenase	1.45	2.27E-02
PRK_01296	HMPREF0010_03671	integral membrane protein TerC family protein	1.43	2.26E-04
PRK_00976	HMPREF0010_02526	beta-propeller domain-containing protein	1.43	1.74E-04
PRK_01432	HMPREF0010_03257	3-deoxy-manno-octulosonate cytidylyltransferase	1.41	1.60E-02
PRK_00934	HMPREF0010_02568	secreted protein	1.40	2.61E-03
PRK_00927	HMPREF0010_02575	arginine N-succinyltransferase	1.40	6.63E-03
PRK_01520	HMPREF0010_03171	TonB-dependent copper receptor	1.39	5.26E-03
PRK_00663	HMPREF0010_02797	TPR repeat-containing SEL1 subfamily protein	1.37	2.35E-04
PRK_03558	HMPREF0010_00375	universal stress protein UspA	1.36	1.54E-03
PRK_03021	HMPREF0010_00913	glutaminase-asparaginase(L-asparagine/L-glutamineamidohydrolase)	1.35	1.98E-02
PRK_02041	HMPREF0010_01271	conserved hypothetical protein	1.35	1.20E-02
PRK_01016	HMPREF0010_02491	conserved hypothetical protein	1.33	1.01E-02
PRK_03831	HMPREF0010_00104	sodium/glutamate symporter	1.30	6.19E-04
PRK_00930	HMPREF0010_02572	GABA permease	1.30	1.01E-02
PRK_00817	HMPREF0010_02681	soluble lytic murein transglycosylase	1.28	1.27E-03
PRK_02330	HMPREF0010_01565	heat shock protein	1.26	1.26E-03
PRK_02019	HMPREF0010_01249	phosphate regulon transcriptional regulatory protein PhoB	1.24	6.19E-04
PRK_00565	HMPREF0010_02888	outer membrane lipoprotein carrier protein LolA	1.23	5.17E-04
PRK_00392	HMPREF0010_02178	periplasmic serine peptidase DegS	1.23	2.59E-03

PRK_00393	HMPREF0010_02178	periplasmic serine peptidase DegS	1.22	4.37E-04
PRK_00977	HMPREF0010_02525	LemA family protein	1.21	1.58E-03
PRK_01312	HMPREF0010_03654	predicted protein	1.21	1.24E-02
PRK_00949	HMPREF0010_02553	hemerythrin HHE cation binding domain-containing protein	1.20	1.37E-02
PRK_01509	HMPREF0010_03182	conserved hypothetical protein	1.20	2.25E-03
PRK_00366	HMPREF0010_02203	conserved hypothetical protein	1.19	4.37E-04
PRK_00720	HMPREF0010_02739	conserved hypothetical protein	1.14	8.48E-03
PRK_02104	HMPREF0010_04110	tRNA-Gln	1.14	1.71E-02
PRK_00924	HMPREF0010_02578	succinylglutamate desuccinylase	1.13	1.98E-02
PRK_02942	HMPREF0010_00993	conserved hypothetical protein	1.13	3.83E-02
PRK_00300	HMPREF0010_02269	conserved hypothetical protein	1.13	4.30E-03
PRK_03600	HMPREF0010_00333	UDP-glucose 4-epimerase	1.13	4.59E-03
PRK_01776	HMPREF0010_01798	conserved hypothetical protein	1.13	1.83E-02
PRK_00941	HMPREF0010_02561	conserved hypothetical protein	1.13	3.03E-02
PRK_03047	HMPREF0010_00887	glutamate/aspartate transporter	1.10	3.30E-02
PRK_02428	HMPREF0010_02957	periplasmic or secreted lipoprotein	1.09	3.98E-03
PRK_02637	HMPREF0010_03761	alkyl hydroperoxide reductase, F subunit	1.09	6.26E-03
PRK_01288	HMPREF0010_03679	protein-disulfide isomerase	1.09	2.61E-03
PRK_00835	HMPREF0010_04144	tRNA-Arg	1.08	1.58E-02
PRK_03537	HMPREF0010_00396	conserved hypothetical protein	1.06	1.93E-02
PRK_03601	HMPREF0010_00332	sulfurtransferase tusD (tRNA 2-thiouridine synthesizing protein D)	1.06	6.40E-03
PRK_00844	HMPREF0010_02658	conserved hypothetical protein	1.04	8.48E-03
PRK_01521	HMPREF0010_03170	conserved hypothetical protein	1.03	6.26E-03
PRK_00071	HMPREF0010_00069	conserved hypothetical protein	1.01	2.32E-02
PRK_01930	HMPREF0010_01644	tRNA(Ile)-lysidine synthetase	-1.01	3.68E-03
PRK_01762	HMPREF0010_01812	conserved hypothetical protein	-1.01	3.14E-03
PRK_03714	HMPREF0010_00218	transcriptional regulator	-1.02	1.61E-02

PRK_01104	HMPREF0010_03531	conserved hypothetical protein	-1.07	2.25E-03
PRK_03224	HMPREF0010_00710	conserved hypothetical protein	-1.08	1.24E-02
PRK_03210	HMPREF0010_00725	PepSY-associated TM helix family protein	-1.10	1.90E-03
PRK_03263	HMPREF0010_00670	Zn-dependent hydrolase	-1.11	2.25E-03
PRK_01820	HMPREF0010_01754	iron-uptake factor	-1.11	4.23E-03
PRK_00725	HMPREF0010_02734	psiE	-1.16	2.61E-03
PRK_03665	HMPREF0010_00269	TonB-dependent siderophore receptor	-1.18	6.59E-03
PRK_00323	HMPREF0010_02246	linoleoyl-CoA desaturase(Delta(6)-desaturase)	-1.18	3.42E-02
PRK_03225	HMPREF0010_00709	FhuE receptor	-1.20	2.25E-03
PRK_02350	HMPREF0010_01586	conserved hypothetical protein	-1.21	6.81E-04
PRK_03201	HMPREF0010_00734	siderophore synthetase component	-1.23	2.22E-02
PRK_01126	HMPREF0010_03509	outer membrane receptor protein	-1.23	8.71E-04
PRK_01749	HMPREF0010_01825	transcriptional regulator	-1.24	2.96E-04
PRK_02284	HMPREF0010_01517	conserved hypothetical protein	-1.33	2.12E-03
PRK_00324	HMPREF0010_02245	flavodoxin reductase family protein 1	-1.39	1.69E-02
PRK_03208	HMPREF0010_00727	ferric aerobactin receptor	-1.41	1.74E-03
PRK_01788	HMPREF0010_01786	tonB	-1.45	9.79E-04
PRK_00970	HMPREF0010_02532	bacterioferritin-associated ferredoxin	-1.45	2.62E-02
PRK_00275	HMPREF0010_02294	non-ribosomal peptide synthetase	-1.52	1.83E-02
PRK_03315	HMPREF0010_00619	isochorismate hydrolase	-1.57	4.55E-03
PRK_03212	HMPREF0010_00723	siderophore biosynthesis protein	-1.70	1.70E-03
PRK_00096	HMPREF0010_00044	predicted protein	-1.82	6.07E-04
PRK_00265	HMPREF0010_02304	2,3-dihydroxybenzoate-AMP ligase	-1.86	1.24E-02
PRK_00097	HMPREF0010_00043	two-component system sensor protein	-1.89	3.09E-05
PRK_01750	HMPREF0010_01824	conserved hypothetical protein	-1.96	1.32E-04
PRK_00277	HMPREF0010_02292	sensory box protein	-1.96	1.32E-04
PRK_03314	HMPREF0010_00620	2-hydroxycyclohexanecarboxyl-CoA dehydrogenase	-2.13	2.60E-04
PRK_00087	HMPREF0010_00053	alpha/beta superfamily hydrolase	-2.21	1.53E-04
PRK_00257	HMPREF0010_02312	isochorismate synthetase	-2.23	1.01E-03

PRK_01342	HMPREF0010_03348	flavodoxin reductase family protein 1	-2.34	4.55E-03
PRK_00276	HMPREF0010_02293	vulnibactin utilization protein viuB	-2.46	2.69E-04

Table A2.7. Differentially expressed genes (log_2 fold-change ≥ |1|, FDR ≤ 0.05) in *A. baumannii* ATCC 19606 following exposure to polymyxin B. Statistical significance was calculated using F-statistic with Benjamini Hochberg adjustment to control the FDR.

Locus tag	Gene ID (BioCyc)	Product	Log_2 fold-change	FDR
PRK_01334	HMPREF0010_03356	conserved hypothetical protein	9.69	1.01E-10
PRK_00726	HMPREF0010_02733	conserved hypothetical protein	9.21	2.88E-11
PRK_03749	HMPREF0010_00185	predicted protein	8.25	2.88E-11
PRK_00720	HMPREF0010_02739	conserved hypothetical protein	7.06	6.57E-10
PRK_01626	HMPREF0010_01945	predicted protein	7.04	3.73E-09
PRK_01046	HMPREF0010_02462	predicted protein	6.46	8.16E-08
PRK_01312	HMPREF0010_03654	predicted protein	6.22	3.89E-09
PRK_00923	HMPREF0010_02579	tolA	6.00	1.93E-09
PRK_03748	HMPREF0010_00186	predicted protein	5.73	3.73E-09
PRK_00565	HMPREF0010_02888	outer membrane lipoprotein carrier protein LolA	5.62	2.99E-10
PRK_01335	HMPREF0010_03355	conserved hypothetical protein	5.24	2.07E-09
PRK_01861	HMPREF0010_01713	macrolide transporter	5.10	3.89E-09
PRK_01862	HMPREF0010_01712	membrane-fusion protein	5.04	1.93E-09
PRK_00071	HMPREF0010_00069	conserved hypothetical protein	4.98	1.04E-08
PRK_01860	HMPREF0010_01714	RND efflux transporter	4.78	3.93E-08
PRK_01509	HMPREF0010_03182	conserved hypothetical protein	4.66	3.29E-09
PRK_00320	HMPREF0010_02249	periplasmic/secreted protein	4.32	4.03E-09
PRK_00934	HMPREF0010_02568	secreted protein	4.31	1.46E-08
PRK_00211	HMPREF0010_02352	outer membrane protein W	4.24	4.72E-07
PRK_00399	HMPREF0010_02172	thiosulfate-binding protein	3.99	9.71E-06
PRK_01311	HMPREF0010_03655	conserved hypothetical protein	3.96	1.13E-05
PRK_02998	HMPREF0010_00935	taurine ABC transporter, periplasmic binding protein	3.87	3.98E-05
PRK_03755	HMPREF0010_00179	biofilm synthesis protein	3.71	1.06E-10
PRK_00895	HMPREF0010_02608	conserved hypothetical protein	3.71	2.22E-08
PRK_00823	HMPREF0010_02675	conserved hypothetical protein	3.56	5.64E-08
PRK_00896	HMPREF0010_02607	toluene tolerance protein Ttg2D	3.50	4.11E-08

PRK_00699	HMPREF0010_02760	peroxiredoxin	3.38	1.04E-08
PRK_02106	HMPREF0010_01333	outer membrane lipoprotein LolB	3.31	2.08E-08
PRK_03685	HMPREF0010_00247	conserved hypothetical protein	3.24	7.15E-07
PRK_00300	HMPREF0010_02269	conserved hypothetical protein	3.14	1.52E-07
PRK_02152	HMPREF0010_01378	type VI secretion system OmpA/MotB	3.04	3.93E-06
PRK_01776	HMPREF0010_01798	conserved hypothetical protein	3.02	7.32E-07
PRK_01333	HMPREF0010_03357	amino-acid N-acetyltransferase	2.97	4.12E-07
PRK_00491	HMPREF0010_02077	transglycosylase SLT domain-containing protein	2.96	2.93E-06
PRK_01542	HMPREF0010_02025	rossmann fold nucleotide-binding protein	2.94	3.30E-07
PRK_03750	HMPREF0010_00184	luciferase family monooxygenase	2.89	1.46E-07
PRK_00497	HMPREF0010_02071	multidrug resistance protein mexB	2.87	1.22E-07
PRK_02330	HMPREF0010_01565	heat shock protein	2.81	2.88E-06
PRK_02279	HMPREF0010_01511	conserved hypothetical protein	2.74	7.99E-06
PRK_01119	HMPREF0010_03516	porin	2.73	2.51E-05
PRK_00663	HMPREF0010_02797	TPR repeat-containing SEL1 subfamily protein	2.56	5.03E-08
PRK_00897	HMPREF0010_02606	toluene tolerance efflux transporter	2.50	1.50E-06
PRK_03753	HMPREF0010_00181	glycosyltransferase	2.46	2.94E-06
PRK_00719	HMPREF0010_02740	two component signal transduction system	2.44	7.50E-07
PRK_00446	HMPREF0010_02124	kinase sensor component lipoprotein releasing system, transmembrane protein LolE	2.39	1.50E-06
PRK_02614	HMPREF0010_03145	peptidase family M48 family protein	2.34	9.92E-06
PRK_03671	HMPREF0010_00263	small-conductance mechanosensitive channel	2.29	2.54E-06
PRK_00445	HMPREF0010_02125	lipoprotein releasing system, ATP-binding protein	2.28	3.93E-06
PRK_00919	HMPREF0010_02583	conserved hypothetical protein	2.23	1.07E-06
PRK_01632	HMPREF0010_01939	conserved hypothetical protein	2.20	1.83E-07
PRK_00817	HMPREF0010_02681	soluble lytic murein transglycosylase	2.16	1.37E-05
PRK_02041	HMPREF0010_01271	conserved hypothetical protein	2.16	2.51E-05

PRK_01339	HMPREF0010_03351	thiol-disulfide isomerase and thioredoxin	2.15	2.21E-07
PRK_01073	HMPREF0010_02437	2-hydroxymuconic semialdehyde dehydrogenase	2.10	2.54E-06
PRK_01020	HMPREF0010_02487	sulfate transporter	2.09	7.01E-04
PRK_03324	HMPREF0010_00610	conserved hypothetical protein	2.08	6.49E-06
PRK_00321	HMPREF0010_02248	transcriptional regulator lysR family	2.05	1.02E-06
PRK_01394	HMPREF0010_03295	nicotinate-nucleotide diphosphorylase (carboxylating)	2.03	3.87E-06
PRK_03600	HMPREF0010_00333	UDP-glucose 4-epimerase	1.99	2.43E-05
PRK_00718	HMPREF0010_02741	phosphate regulon transcriptional regulatory protein PhoB	1.96	1.84E-05
PRK_02942	HMPREF0010_00993	conserved hypothetical protein	1.95	1.06E-04
PRK_00949	HMPREF0010_02553	hemerythrin HHE cation binding domain-containing protein	1.94	2.66E-05
PRK_03601	HMPREF0010_00332	sulfurtransferase tusD (tRNA 2-thiouridine synthesizing protein D)	1.94	7.99E-06
PRK_02052	HMPREF0010_01282	conserved hypothetical protein	1.91	2.42E-04
PRK_00409	HMPREF0010_02162	conserved hypothetical protein	1.90	2.12E-05
PRK_00645	HMPREF0010_02815	entericidin EcnA/B family protein	1.87	7.04E-04
PRK_00944	HMPREF0010_02558	conserved hypothetical protein	1.84	2.87E-06
PRK_02312	HMPREF0010_01545	lipoprotein	1.84	2.60E-05
PRK_00059	HMPREF0010_00081	peptidase family M48 family protein	1.83	1.50E-06
PRK_01174	HMPREF0010_03461	MATE efflux family protein	1.78	6.61E-06
PRK_02237	HMPREF0010_01467	conserved hypothetical protein	1.72	1.52E-03
PRK_01830	HMPREF0010_01744	phosphogluconate dehydratase	1.72	1.05E-04
PRK_02759	HMPREF0010_01177	peptidase M15 family protein	1.71	1.13E-05
PRK_01229	HMPREF0010_03406	predicted protein	1.70	7.98E-04
PRK_01531	HMPREF0010_02036	transglycosylase SLT domain-containing protein	1.67	6.21E-04
PRK_00938	HMPREF0010_02564	copper/zinc superoxide dismutase	1.66	8.61E-06
PRK_02627	HMPREF0010_03158	OmpA/MotB	1.63	5.84E-05
PRK_00622	HMPREF0010_02833	conserved hypothetical protein	1.63	4.72E-06

PRK_02943	HMPREF0010_00992	catalase HPII(Hydroxyperoxidase II)	1.62	9.65E-05
PRK_00920	HMPREF0010_02582	conserved hypothetical protein	1.58	1.84E-05
PRK_01432	HMPREF0010_03257	3-deoxy-manno-octulosonate cytidylyltransferase	1.54	8.91E-04
PRK_00217	HMPREF0010_02347	acyl-coenzyme A dehydrogenase(ACDH)	1.53	7.20E-05
PRK_00681	HMPREF0010_02779	3-demethylubiquinone-9 3-methyltransferase	1.53	2.69E-05
PRK_02428	HMPREF0010_02957	periplasmic or secreted lipoprotein	1.50	8.95E-05
PRK_01987	HMPREF0010_01217	adenosine deaminase	1.47	6.29E-04
PRK_01698	HMPREF0010_01876	conserved hypothetical protein	1.46	1.84E-04
PRK_00075	HMPREF0010_00065	alternative sigma factor RpoH	1.45	9.50E-05
PRK_01248	HMPREF0010_03386	membrane-fusion protein	1.45	1.35E-04
PRK_03114	HMPREF0010_00820	transcriptional regulator	1.45	1.27E-04
PRK_00941	HMPREF0010_02561	conserved hypothetical protein	1.45	6.33E-04
PRK_01785	HMPREF0010_01789	2-hydroxymuconic semialdehyde dehydrogenase	1.44	6.53E-03
PRK_01203	HMPREF0010_03432	histidine ammonia-lyase	1.43	1.01E-03
PRK_00571	HMPREF0010_02882	multidrug efflux protein AdeI	1.43	1.90E-03
PRK_00697	HMPREF0010_02762	conserved hypothetical protein	1.42	2.69E-05
PRK_00730	HMPREF0010_02729	conserved hypothetical protein	1.42	4.74E-05
PRK_01878	HMPREF0010_01696	aconitate hydratase 1	1.42	5.45E-04
PRK_00564	HMPREF0010_02889	conserved hypothetical protein	1.41	2.14E-06
PRK_03558	HMPREF0010_00375	universal stress protein UspA	1.40	4.47E-05
PRK_00369	HMPREF0010_02200	outer membrane lipoprotein blc	1.39	5.08E-04
PRK_00572	HMPREF0010_02881	multidrug efflux protein AdeJ	1.35	6.08E-03
PRK_00570	HMPREF0010_02883	membrane-associated phospholipid phosphatase	1.35	4.47E-04
PRK_01934	HMPREF0010_01640	conserved hypothetical protein	1.33	2.84E-06
PRK_01202	HMPREF0010_03433	GABA permease	1.31	9.43E-04
PRK_00251	HMPREF0010_02318	beta-lactamase	1.30	4.45E-05
PRK_01288	HMPREF0010_03679	protein-disulfide isomerase	1.30	2.66E-05
PRK_03449	HMPREF0010_00484	catechol 1,2-dioxygenase	1.29	2.58E-04

PRK_00405	HMPREF0010_02166	carbapenem-associated resistance protein	1.29	1.84E-03
PRK_00432	HMPREF0010_02138	RNA polymerase sigma-70 factor	1.28	3.12E-04
PRK_00392	HMPREF0010_02178	periplasmic serine peptidase DegS	1.26	1.72E-04
PRK_03447	HMPREF0010_00486	muconate cycloisomerase I	1.26	7.10E-04
PRK_00424	HMPREF0010_02146	TolQ	1.26	1.88E-04
PRK_00816	HMPREF0010_02682	ribonuclease I	1.25	3.04E-04
PRK_01201	HMPREF0010_03434	imidazolonepropionase	1.24	2.25E-03
PRK_01247	HMPREF0010_03387	acr family drug resistance transporter	1.23	7.04E-04
PRK_02927	HMPREF0010_01007	cyclohexanecarboxyl-CoA dehydrogenase	1.23	4.79E-02
PRK_01924	HMPREF0010_01650	integration host factor, alpha subunit	1.22	8.53E-04
PRK_00643	HMPREF0010_02817	conserved hypothetical protein	1.22	3.26E-04
PRK_00297	HMPREF0010_02272	peptidase M23B	1.22	2.51E-05
PRK_00433	HMPREF0010_02137	conserved hypothetical protein	1.22	3.43E-04
PRK_03771	HMPREF0010_00163	conserved hypothetical protein	1.21	1.12E-04
PRK_03001	HMPREF0010_00932	alpha-ketoglutarate-dependent taurine dioxygenase	1.21	4.74E-03
PRK_02075	HMPREF0010_01305	alpha,alpha-trehalose-phosphate synthase (UDP-forming)	1.21	9.67E-04
PRK_00478	HMPREF0010_02090	ydeP	1.21	1.33E-04
PRK_02304	HMPREF0010_01537	sulfate adenylyltransferase subunit 2	1.20	1.94E-04
PRK_01194	HMPREF0010_03441	phosphatidylglycerophosphatase A	1.17	2.85E-04
PRK_01289	HMPREF0010_03678	transglycosylase-associated protein	1.17	1.31E-03
PRK_02019	HMPREF0010_01249	phosphate regulon transcriptional regulatory protein PhoB	1.17	6.59E-04
PRK_01831	HMPREF0010_01743	KHG/KDPG aldolase	1.16	7.97E-05
PRK_03320	HMPREF0010_00614	coenzyme PQQ biosynthesis protein C	1.16	5.38E-04
PRK_01146	HMPREF0010_03489	conserved hypothetical protein	1.16	2.86E-03
PRK_00393	HMPREF0010_02178	periplasmic serine peptidase DegS	1.15	6.38E-05
PRK_00573	HMPREF0010_02880	outer membrane protein oprM	1.14	3.29E-03
PRK_02615	HMPREF0010_03146	tas	1.14	2.38E-04

PRK_00428	HMPREF0010_02142	peptidoglycan-associated lipoprotein	1.13	9.90E-05
PRK_03631	HMPREF0010_00302	glutamate synthase	1.13	7.97E-05
PRK_01797	HMPREF0010_01777	prolipoprotein diacylglyceryl transferase	1.10	9.24E-06
PRK_01690	HMPREF0010_01884	conserved hypothetical protein	1.10	2.16E-04
PRK_03438	HMPREF0010_00495	non-heme chloroperoxidase	1.09	3.72E-04
PRK_02177	HMPREF0010_01403	multidrug resistance transmembrane protein	1.09	3.27E-04
PRK_00425	HMPREF0010_02145	TolR	1.09	1.94E-04
PRK_02176	HMPREF0010_01402	multidrug resistance secretion protein	1.09	2.56E-04
PRK_03712	HMPREF0010_00220	conserved hypothetical protein	1.08	2.65E-04
PRK_01204	HMPREF0010_03431	urocanate hydratase	1.08	1.82E-02
PRK_00427	HMPREF0010_02143	tol-Pal system beta propeller repeat protein TolB	1.08	1.94E-04
PRK_00933	HMPREF0010_02569	conserved hypothetical protein	1.07	5.38E-04
PRK_00824	HMPREF0010_02674	xanthine phosphoribosyltransferase	1.07	4.74E-04
PRK_01839	HMPREF0010_01735	tail-specific protease	1.06	1.14E-04
PRK_03790	HMPREF0010_00144	glucose/sorbosone dehydrogenase	1.04	5.99E-04
PRK_03537	HMPREF0010_00396	conserved hypothetical protein	1.04	2.81E-03
PRK_00814	HMPREF0010_02684	conserved hypothetical protein	1.04	7.72E-03
PRK_00976	HMPREF0010_02526	beta-propeller domain-containing protein	1.03	7.74E-05
PRK_00426	HMPREF0010_02144	TolA	1.03	3.83E-04
PRK_01303	HMPREF0010_03663	lytic murein transglycosylase B	1.02	1.10E-04
PRK_03532	HMPREF0010_00401	cyd operon protein YbgE family protein	1.01	2.78E-02
PRK_02241	HMPREF0010_01471	peptidyl-prolyl cis-trans isomerase	1.01	2.98E-04
PRK_03770	HMPREF0010_00164	transglutaminase	1.01	7.46E-05
PRK_03506	HMPREF0010_00427	FAD/FMN-binding/pyridine nucleotide-disulphide oxidoreductase	1.01	1.25E-03
PRK_00087	HMPREF0010_00053	alpha/beta superfamily hydrolase	-1.01	1.91E-03
PRK_03224	HMPREF0010_00710	conserved hypothetical protein	-1.01	1.44E-03
PRK_03731	HMPREF0010_00201	K+-transporting ATPase, B subunit	-1.01	1.09E-02

PRK_02867	HMPREF0010_01068	conserved hypothetical protein	-1.02	6.00E-04
PRK_01431	HMPREF0010_03259	conserved hypothetical protein	-1.03	4.45E-05
PRK_02145	HMPREF0010_01371	threonine efflux system	-1.03	3.55E-04
PRK_02197	HMPREF0010_01420	conserved hypothetical protein	-1.03	6.70E-04
PRK_00939	HMPREF0010_02563	inner membrane protein yccS	-1.03	2.10E-04
PRK_00289	HMPREF0010_02280	conserved hypothetical protein	-1.03	1.36E-04
PRK_02199	HMPREF0010_01428	conserved hypothetical protein	-1.03	1.62E-03
PRK_01750	HMPREF0010_01824	conserved hypothetical protein	-1.06	3.96E-04
PRK_02439	HMPREF0010_02968	outer membrane receptor protein	-1.06	5.25E-04
PRK_02181	HMPREF0010_01406	predicted protein	-1.06	2.00E-04
PRK_02409	HMPREF0010_02938	outer membrane receptor protein	-1.06	3.04E-04
PRK_02573	HMPREF0010_03102	conserved hypothetical protein	-1.06	1.36E-03
PRK_01828	HMPREF0010_01746	phosphate acetyltransferase	-1.06	5.92E-03
PRK_00277	HMPREF0010_02292	sensory box protein	-1.07	2.85E-04
PRK_00281	HMPREF0010_02287	conserved hypothetical protein	-1.07	1.51E-02
PRK_00177	HMPREF0010_02387	predicted protein	-1.07	4.23E-02
PRK_02200	HMPREF0010_01429	CRISPR-associated protein	-1.07	2.44E-03
PRK_00156	HMPREF0010_02409	P-loop protein	-1.07	4.00E-02
PRK_01152	HMPREF0010_03483	ribosomal subunit interface protein	-1.08	4.04E-02
PRK_01258	HMPREF0010_03376	predicted protein	-1.08	5.08E-04
PRK_03212	HMPREF0010_00723	siderophore biosynthesis protein	-1.08	1.93E-02
PRK_03208	HMPREF0010_00727	ferric aerobactin receptor	-1.09	5.43E-03
PRK_00906	HMPREF0010_02596	conserved hypothetical protein	-1.09	3.76E-04
PRK_02483	HMPREF0010_03012	curli production assembly/transport component CsgG	-1.09	1.64E-02
PRK_03665	HMPREF0010_00269	TonB-dependent siderophore receptor	-1.10	1.90E-03
PRK_01452	HMPREF0010_03237	pyrimidine utilization protein C	-1.10	1.82E-02
PRK_00174	HMPREF0010_02390	conserved hypothetical protein	-1.10	3.18E-02
PRK_02788	HMPREF0010_01144	predicted protein	-1.11	3.09E-03
PRK_02751	HMPREF0010_02430	conserved hypothetical protein	-1.12	2.10E-05

PRK_02448	HMPREF0010_02975	predicted protein	-1.13	8.39E-03
PRK_00499	HMPREF0010_03763	Zn-dependent hydrolase	-1.13	1.99E-04
PRK_00989	HMPREF0010_02514	type IV pilus assembly protein PilM	-1.14	2.51E-05
PRK_02300	HMPREF0010_01533	conserved hypothetical protein	-1.14	3.19E-04
PRK_00916	HMPREF0010_02586	conserved hypothetical protein	-1.14	1.14E-04
PRK_01762	HMPREF0010_01812	conserved hypothetical protein	-1.15	5.38E-05
PRK_02479	HMPREF0010_03007	transmembrane protein	-1.15	2.69E-05
PRK_00608	HMPREF0010_02847	outer membrane transporter	-1.17	5.65E-03
PRK_00734	HMPREF0010_02725	conserved hypothetical protein	-1.20	8.53E-05
PRK_00744	HMPREF0010_02715	signal transduction histidine kinase	-1.20	4.77E-06
PRK_02371	HMPREF0010_01607	conserved hypothetical protein	-1.21	2.85E-04
PRK_01384	HMPREF0010_03304	predicted protein	-1.25	3.43E-04
PRK_00621	HMPREF0010_02834	intracellular protease 1(Intracellular protease I)	-1.26	1.86E-03
PRK_00130	HMPREF0010_00009	predicted protein	-1.27	1.92E-05
PRK_03660	HMPREF0010_00274	universal stress protein family	-1.27	5.31E-03
PRK_02594	HMPREF0010_03123	conserved hypothetical protein	-1.30	2.58E-04
PRK_00028	HMPREF0010_00108	transcriptional regulator	-1.31	1.99E-03
PRK_00749	HMPREF0010_02711	LemA family protein	-1.34	4.83E-05
PRK_00134	HMPREF0010_00006	phage anti-repressor protein AntB	-1.34	1.60E-02
PRK_02284	HMPREF0010_01517	conserved hypothetical protein	-1.34	1.40E-03
PRK_01821	HMPREF0010_01753	outer membrane receptor for monomeric catechols	-1.36	9.82E-04
PRK_03314	HMPREF0010_00620	2-hydroxycyclohexanecarboxyl-CoA dehydrogenase	-1.36	6.65E-04
PRK_00253	HMPREF0010_02316	outermembrane protein	-1.36	1.77E-03
PRK_03315	HMPREF0010_00619	isochorismate hydrolase	-1.37	2.95E-03
PRK_02685	HMPREF0010_03604	conserved hypothetical protein	-1.39	1.27E-04
PRK_01082	HMPREF0010_02428	conserved hypothetical protein	-1.39	2.85E-04
PRK_03732	HMPREF0010_00200	K+-transporting ATPase, A subunit	-1.39	7.22E-04
PRK_00261	HMPREF0010_02308	ABC transporter	-1.45	1.03E-02
PRK_00264	HMPREF0010_02305	isochorismate hydrolase	-1.45	9.38E-03
PRK_00259	HMPREF0010_02310	acinetobactin biosynthesis protein	-1.47	9.27E-03

PRK_03715	HMPREF0010_00217	conserved hypothetical protein	-1.48	2.39E-03
PRK_02311	HMPREF0010_01544	isocitrate lyase	-1.48	1.31E-03
PRK_01142	HMPREF0010_03493	TonB-dependent receptor protein	-1.49	2.44E-02
PRK_02749	HMPREF0010_02428	conserved hypothetical protein	-1.49	2.54E-04
PRK_01019	HMPREF0010_02488	2-hydroxycyclohexanecarboxyl-CoA dehydrogenase	-1.52	3.59E-03
PRK_00270	HMPREF0010_02299	phosphonate ABC transporter, ATP-binding protein	-1.61	7.13E-03
PRK_02399	HMPREF0010_02928	conserved hypothetical protein	-1.63	4.02E-04
PRK_00269	HMPREF0010_02300	BauB	-1.64	1.40E-02
PRK_00263	HMPREF0010_02306	acinetobactin biosynthesis protein	-1.67	3.32E-03
PRK_00275	HMPREF0010_02294	non-ribosomal peptide synthetase	-1.73	3.04E-03
PRK_00132	HMPREF0010_00007	predicted protein	-1.82	1.19E-04
PRK_00845	Unannotated	Unannotated	-1.84	9.82E-04
PRK_03354	HMPREF0010_00580	type III restriction enzyme	-1.84	2.75E-06
PRK_00524	HMPREF0010_02049	conserved hypothetical protein	-1.86	2.18E-05
PRK_00274	HMPREF0010_02295	non-ribosomal peptide synthetase	-2.04	4.31E-03
PRK_00257	HMPREF0010_02312	isochorismate synthetase	-2.20	4.63E-04

Table A2.8. Differentially expressed genes (log2 fold-change ≥ |1|, FDR ≤ 0.05) in *A. baumannii* ATCC 19606 (co-cultured with A549 cells) following exposure to polymyxin B. Statistical significance was calculated using F statistic with Benjamini Hochberg adjustment to control the FDR.

Locus tag	Gene ID (BioCyc)	Product	Log_2 fold-change	FDR
PRK_01334	HMPREF0010_03356	conserved hypothetical protein	6.88	1.66E-09
PRK_00726	HMPREF0010_02733	conserved hypothetical protein	6.17	1.02E-09
PRK_03749	HMPREF0010_00185	predicted protein	5.79	1.02E-09
PRK_00720	HMPREF0010_02739	conserved hypothetical protein	5.76	3.99E-09
PRK_01312	HMPREF0010_03654	predicted protein	5.72	1.20E-08
PRK_01626	HMPREF0010_01945	predicted protein	5.13	7.79E-08
PRK_01862	HMPREF0010_01712	membrane-fusion protein	5.10	1.66E-09
PRK_01335	HMPREF0010_03355	conserved hypothetical protein	5.06	3.40E-09
PRK_03748	HMPREF0010_00186	predicted protein	4.96	1.50E-08
PRK_00565	HMPREF0010_02888	outer membrane lipoprotein carrier protein LolA	4.86	1.04E-09
PRK_01861	HMPREF0010_01713	macrolide transporter	4.82	8.78E-09
PRK_00071	HMPREF0010_00069	conserved hypothetical protein	4.63	3.06E-08
PRK_01860	HMPREF0010_01714	RND efflux transporter	4.46	9.70E-08
PRK_00923	HMPREF0010_02579	tolA	4.16	2.70E-08
PRK_03755	HMPREF0010_00179	biofilm synthesis protein	3.97	1.02E-09
PRK_01046	HMPREF0010_02462	predicted protein	3.94	1.45E-05
PRK_00934	HMPREF0010_02568	secreted protein	3.78	9.70E-08
PRK_02106	HMPREF0010_01333	outer membrane lipoprotein LolB	3.77	1.24E-08
PRK_00896	HMPREF0010_02607	toluene tolerance protein Ttg2D	3.74	8.78E-09
PRK_01509	HMPREF0010_03182	conserved hypothetical protein	3.72	2.86E-08
PRK_00320	HMPREF0010_02249	periplasmic/secreted protein	3.67	2.70E-08
PRK_00895	HMPREF0010_02608	conserved hypothetical protein	3.63	1.50E-08
PRK_01542	HMPREF0010_02025	rossmann fold nucleotide-binding protein	3.63	6.65E-08
PRK_00823	HMPREF0010_02675	conserved hypothetical protein	3.54	3.58E-08
PRK_03685	HMPREF0010_00247	conserved hypothetical protein	3.50	2.24E-06
PRK_02279	HMPREF0010_01511	conserved hypothetical protein	3.24	1.40E-05

PRK_00211	HMPREF0010_02352	outer membrane protein W	3.19	2.04E-05
PRK_01333	HMPREF0010_03357	amino-acid N-acetyltransferase	3.14	5.95E-07
PRK_00491	HMPREF0010_02077	transglycosylase SLT domain-containing protein	3.01	2.38E-06
PRK_01311	HMPREF0010_03655	conserved hypothetical protein	3.01	4.04E-04
PRK_00497	HMPREF0010_02071	multidrug resistance protein mexB	2.97	1.33E-07
PRK_01020	HMPREF0010_02487	sulfate transporter	2.92	3.37E-04
PRK_00719	HMPREF0010_02740	two component signal transduction system	2.87	1.77E-07
PRK_03750	HMPREF0010_00184	kinase sensor component luciferase family monooxygenase	2.67	2.38E-06
PRK_01339	HMPREF0010_03351	thiol-disulfide isomerase and thioredoxin	2.63	1.60E-08
PRK_03324	HMPREF0010_00610	conserved hypothetical protein	2.60	2.41E-06
PRK_01776	HMPREF0010_01798	conserved hypothetical protein	2.55	2.25E-05
PRK_00663	HMPREF0010_02797	TPR repeat-containing SEL1 subfamily protein	2.46	1.30E-07
PRK_02152	HMPREF0010_01378	type VI secretion system OmpA/MotB	2.44	1.05E-05
PRK_00446	HMPREF0010_02124	lipoprotein releasing system, transmembrane protein LolE	2.35	1.03E-06
PRK_00300	HMPREF0010_02269	conserved hypothetical protein	2.33	5.05E-06
PRK_00897	HMPREF0010_02606	toluene tolerance efflux transporter	2.33	1.66E-06
PRK_01632	HMPREF0010_01939	conserved hypothetical protein	2.30	1.47E-06
PRK_00445	HMPREF0010_02125	lipoprotein releasing system, ATP-binding protein	2.24	1.02E-05
PRK_00699	HMPREF0010_02760	peroxiredoxin	2.21	4.09E-06
PRK_00919	HMPREF0010_02583	conserved hypothetical protein	2.20	9.38E-06
PRK_02052	HMPREF0010_01282	conserved hypothetical protein	2.18	8.31E-05
PRK_03753	HMPREF0010_00181	glycosyltransferase	2.17	9.72E-05
PRK_01073	HMPREF0010_02437	2-hydroxymuconic semialdehyde dehydrogenase	2.15	2.38E-06
PRK_03239	HMPREF0010_00694	predicted protein	2.09	4.17E-05
PRK_02759	HMPREF0010_01177	peptidase M15 family protein	2.02	3.00E-06
PRK_01119	HMPREF0010_03516	porin	2.02	1.22E-04

PRK_02312	HMPREF0010_01545	lipoprotein	1.96	2.44E-05
PRK_00718	HMPREF0010_02741	phosphate regulon transcriptional regulatory protein PhoB	1.95	4.48E-05
PRK_02614	HMPREF0010_03145	peptidase family M48 family protein	1.95	2.71E-05
PRK_02330	HMPREF0010_01565	heat shock protein	1.94	2.79E-05
PRK_00920	HMPREF0010_02582	conserved hypothetical protein	1.89	3.32E-05
PRK_00681	HMPREF0010_02779	3-demethylubiquinone-9 3-methyltransferase	1.88	4.41E-05
PRK_00321	HMPREF0010_02248	transcriptional regulator lysR family	1.87	2.57E-05
PRK_00217	HMPREF0010_02347	acyl-coenzyme A dehydrogenase(ACDH)	1.87	4.92E-05
PRK_00409	HMPREF0010_02162	conserved hypothetical protein	1.86	1.33E-05
PRK_01104	HMPREF0010_03531	conserved hypothetical protein	1.85	8.32E-06
PRK_00059	HMPREF0010_00081	peptidase family M48 family protein	1.84	3.57E-06
PRK_01723	HMPREF0010_01851	ribosomal protein L31	1.80	5.67E-05
PRK_02041	HMPREF0010_01271	conserved hypothetical protein	1.77	1.11E-03
PRK_02943	HMPREF0010_00992	catalase HPII(Hydroxyperoxidase II)	1.72	4.78E-04
PRK_03712	HMPREF0010_00220	conserved hypothetical protein	1.71	6.01E-05
PRK_01987	HMPREF0010_01217	adenosine deaminase	1.68	1.40E-03
PRK_01174	HMPREF0010_03461	MATE efflux family protein	1.65	9.83E-05
PRK_03600	HMPREF0010_00333	UDP-glucose 4-epimerase	1.63	1.34E-04
PRK_03601	HMPREF0010_00332	sulfurtransferase tusD (tRNA 2-thiouridine synthesizing protein D)	1.59	1.55E-04
PRK_00944	HMPREF0010_02558	conserved hypothetical protein	1.59	7.13E-05
PRK_03447	HMPREF0010_00486	muconate cycloisomerase I	1.58	1.51E-03
PRK_00075	HMPREF0010_00065	alternative sigma factor RpoH	1.57	1.85E-05
PRK_02671	HMPREF0010_03618	pyrroline-5-carboxylate reductase	1.57	3.08E-03
PRK_01247	HMPREF0010_03387	acr family drug resistance transporter	1.56	1.09E-03
PRK_03775	HMPREF0010_00159	conserved hypothetical protein	1.55	3.52E-03
PRK_00645	HMPREF0010_02815	entericidin EcnA/B family protein	1.55	3.44E-03
PRK_00938	HMPREF0010_02564	copper/zinc superoxide dismutase	1.55	3.98E-05

PRK_00399	HMPREF0010_02172	thiosulfate-binding protein	1.54	2.48E-02
PRK_00817	HMPREF0010_02681	soluble lytic murein transglycosylase	1.54	1.37E-04
PRK_00814	HMPREF0010_02684	conserved hypothetical protein	1.53	3.54E-03
PRK_03771	HMPREF0010_00163	conserved hypothetical protein	1.53	2.13E-04
PRK_01227	HMPREF0010_03408	glucose/sorbosone dehydrogenase	1.50	7.88E-04
PRK_01830	HMPREF0010_01744	phosphogluconate dehydratase	1.49	1.23E-03
PRK_00576	HMPREF0010_02877	conserved hypothetical protein	1.49	4.06E-03
PRK_00949	HMPREF0010_02553	hemerythrin HHE cation binding domain-containing protein	1.48	2.03E-03
PRK_03770	HMPREF0010_00164	transglutaminase	1.47	3.54E-05
PRK_02075	HMPREF0010_01305	alpha,alpha-trehalose-phosphate synthase (UDP-forming)	1.47	2.32E-03
PRK_01289	HMPREF0010_03678	transglycosylase-associated protein	1.47	1.51E-03
PRK_01394	HMPREF0010_03295	nicotinate-nucleotide diphosphorylase (carboxylating)	1.46	4.72E-04
PRK_01248	HMPREF0010_03386	membrane-fusion protein	1.44	1.41E-03
PRK_00824	HMPREF0010_02674	xanthine phosphoribosyltransferase	1.44	6.18E-05
PRK_03598	HMPREF0010_00335	conserved hypothetical protein	1.44	4.43E-03
PRK_00643	HMPREF0010_02817	conserved hypothetical protein	1.43	1.09E-03
PRK_00622	HMPREF0010_02833	conserved hypothetical protein	1.42	2.05E-04
PRK_01303	HMPREF0010_03663	lytic murein transglycosylase B	1.41	6.30E-06
PRK_00432	HMPREF0010_02138	RNA polymerase sigma-70 factor	1.41	6.99E-05
PRK_03438	HMPREF0010_00495	non-heme chloroperoxidase	1.40	4.26E-04
PRK_00313	HMPREF0010_02256	sphX	1.39	9.34E-03
PRK_03671	HMPREF0010_00263	small-conductance mechanosensitive channel	1.37	7.88E-04
PRK_01878	HMPREF0010_01696	aconitate hydratase 1	1.37	1.04E-03
PRK_00428	HMPREF0010_02142	peptidoglycan-associated lipoprotein	1.37	1.84E-05
PRK_03506	HMPREF0010_00427	FAD/FMN-binding/pyridine nucleotide-disulphide oxidoreductase	1.36	1.51E-03

PRK_02942	HMPREF0010_00993	conserved hypothetical protein	1.36	7.88E-03
PRK_00424	HMPREF0010_02146	TolQ	1.35	5.06E-04
PRK_00725	HMPREF0010_02734	psiE	1.35	4.72E-04
PRK_02004	HMPREF0010_01234	methionine synthase II	1.34	6.07E-03
PRK_03537	HMPREF0010_00396	conserved hypothetical protein	1.33	2.63E-03
PRK_00627	HMPREF0010_02828	phospholipase	1.33	2.65E-03
PRK_00348	HMPREF0010_02221	NADH dehydrogenase	1.32	2.24E-04
PRK_00369	HMPREF0010_02200	outer membrane lipoprotein blc	1.32	3.36E-03
PRK_00405	HMPREF0010_02166	carbapenem-associated resistance protein	1.31	9.20E-04
PRK_00572	HMPREF0010_02881	multidrug efflux protein AdeJ	1.31	4.06E-03
PRK_01883	HMPREF0010_01691	conserved hypothetical protein	1.31	3.74E-03
PRK_03449	HMPREF0010_00484	catechol 1,2-dioxygenase	1.30	2.12E-03
PRK_03142	HMPREF0010_00792	6-pyruvoyl-tetrahydropterin synthase	1.29	4.72E-04
PRK_00571	HMPREF0010_02882	multidrug efflux protein AdeI	1.26	2.67E-03
PRK_03785	HMPREF0010_00149	conserved hypothetical protein	1.22	1.92E-02
PRK_00478	HMPREF0010_02090	ydeP	1.21	1.17E-03
PRK_01934	HMPREF0010_01640	conserved hypothetical protein	1.21	3.85E-05
PRK_00907	HMPREF0010_02595	acyl-CoA dehydrogenase domain-containing protein	1.20	1.44E-02
PRK_01194	HMPREF0010_03441	phosphatidylglycerophosphatase A	1.20	1.37E-03
PRK_01171	HMPREF0010_03464	conserved hypothetical protein	1.20	1.13E-02
PRK_00433	HMPREF0010_02137	conserved hypothetical protein	1.19	9.20E-04
PRK_02042	HMPREF0010_01272	TM helix repeat-containing protein	1.18	4.06E-03
PRK_01724	HMPREF0010_01850	protein kinase	1.18	1.10E-04
PRK_03001	HMPREF0010_00932	alpha-ketoglutarate-dependent taurine dioxygenase	1.18	3.00E-02
PRK_02428	HMPREF0010_02957	periplasmic or secreted lipoprotein	1.16	1.17E-03
PRK_03790	HMPREF0010_00144	glucose/sorbosone dehydrogenase	1.16	2.69E-03
PRK_01531	HMPREF0010_02036	transglycosylase SLT domain-containing protein	1.16	3.02E-02
PRK_02331	HMPREF0010_01566	extradiol ring-cleavage dioxygenase	1.16	2.65E-03
PRK_00849	HMPREF0010_02654	flavin-containing monooxygenase FMO	1.15	1.35E-03

PRK_02241	HMPREF0010_01471	peptidyl-prolyl cis-trans isomerase	1.15	1.35E-03
PRK_00634	HMPREF0010_02820	sulfite reductase	1.14	4.66E-05
PRK_02969	HMPREF0010_00965	glutathione S-transferase domain-containing protein	1.14	5.80E-04
PRK_01146	HMPREF0010_03489	conserved hypothetical protein	1.14	4.85E-03
PRK_02003	HMPREF0010_01233	conserved hypothetical protein	1.12	4.06E-02
PRK_02155	HMPREF0010_01381	phosphomannomutase/phosphoglucomutase(PMM/PGM)	1.12	6.21E-04
PRK_03199	HMPREF0010_00736	conserved hypothetical protein	1.11	5.90E-03
PRK_00933	HMPREF0010_02569	conserved hypothetical protein	1.11	3.54E-03
PRK_00730	HMPREF0010_02729	conserved hypothetical protein	1.11	2.69E-03
PRK_01698	HMPREF0010_01876	conserved hypothetical protein	1.11	8.95E-03
PRK_01839	HMPREF0010_01735	tail-specific protease	1.09	3.85E-05
PRK_01699	HMPREF0010_01875	chaperone DnaJ	1.08	2.12E-04
PRK_02053	HMPREF0010_01283	ATPase	1.08	2.05E-04
PRK_02615	HMPREF0010_03146	tas	1.08	1.57E-03
PRK_01785	HMPREF0010_01789	2-hydroxymuconic semialdehyde dehydrogenase	1.07	2.18E-02
PRK_01690	HMPREF0010_01884	conserved hypothetical protein	1.07	6.69E-04
PRK_00434	HMPREF0010_02136	conserved hypothetical protein	1.06	1.15E-02
PRK_00249	HMPREF0010_02320	xanthine dehydrogenase, small subunit	1.05	6.91E-03
PRK_01924	HMPREF0010_01650	integration host factor, alpha subunit	1.04	3.54E-03
PRK_00697	HMPREF0010_02762	conserved hypothetical protein	1.04	5.28E-04
PRK_02005	HMPREF0010_01235	pyrimidine utilization flavin reductase F	1.04	4.01E-03
PRK_03511	HMPREF0010_00422	ATP-dependent Clp protease ATP-binding subunit ClpA	1.03	9.71E-04
PRK_00570	HMPREF0010_02883	membrane-associated phospholipid phosphatase	1.03	3.30E-03
PRK_02235	HMPREF0010_01465	MarC family integral membrane protein	1.03	2.69E-02
PRK_00573	HMPREF0010_02880	outer membrane protein oprM	1.02	3.84E-03
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PRK_03558	HMPREF0010_00375	universal stress protein UspA	1.02	3.61E-03
PRK_01229	HMPREF0010_03406	predicted protein	1.02	3.06E-02
PRK_01067	HMPREF0010_02443	glutathione-dependent formaldehyde-activating enzyme family protein	1.01	1.17E-03
PRK_03705	HMPREF0010_00227	tRNA delta(2)-isopentenylpyrophosphate transferase	1.01	1.09E-04
PRK_03114	HMPREF0010_00820	transcriptional regulator	1.01	1.08E-02
PRK_03737	HMPREF0010_00196	molybdopterin synthase sulfurylase MoeB	1.01	4.45E-03
PRK_01288	HMPREF0010_03679	protein-disulfide isomerase	1.01	1.97E-03
PRK_01832	HMPREF0010_01742	high-affinity gluconate transporter	1.00	1.27E-03
PRK_00253	HMPREF0010_02316	outermembrane protein	-1.00	1.10E-02
PRK_00734	HMPREF0010_02725	conserved hypothetical protein	-1.03	1.73E-03
PRK_03021	HMPREF0010_00913	glutaminase-asparaginase(L-asparagine/L-glutamineamidohydrolase)	-1.04	3.92E-02
PRK_02594	HMPREF0010_03123	conserved hypothetical protein	-1.05	3.62E-03
PRK_00263	HMPREF0010_02306	acinetobactin biosynthesis protein	-1.06	3.79E-02
PRK_00651	HMPREF0010_02809	type IV pilus response regulator protein PilH	-1.06	1.86E-04
PRK_03314	HMPREF0010_00620	2-hydroxycyclohexanecarboxyl-CoA dehydrogenase	-1.07	6.61E-03
PRK_00227	HMPREF0010_02337	NIF3 family protein	-1.07	1.75E-03
PRK_01157	HMPREF0010_03478	nucleoside-binding outer membrane protein	-1.10	3.23E-03
PRK_03208	HMPREF0010_00727	ferric aerobactin receptor	-1.10	3.26E-03
PRK_02751	HMPREF0010_02430	conserved hypothetical protein	-1.11	1.14E-04
PRK_03732	HMPREF0010_00200	K+-transporting ATPase, A subunit	-1.11	7.41E-03
PRK_00289	HMPREF0010_02280	conserved hypothetical protein	-1.11	7.98E-04
PRK_00749	HMPREF0010_02711	LemA family protein	-1.11	1.98E-03
PRK_02485	HMPREF0010_03014	lipoprotein	-1.11	4.31E-02
PRK_03224	HMPREF0010_00710	conserved hypothetical protein	-1.13	4.35E-03
PRK_02685	HMPREF0010_03604	conserved hypothetical protein	-1.16	3.36E-03
PRK_00524	HMPREF0010_02049	conserved hypothetical protein	-1.17	2.27E-03

PRK_00608	HMPREF0010_02847	outer membrane transporter	-1.18	3.54E-03
PRK_02439	HMPREF0010_02968	outer membrane receptor protein	-1.19	3.32E-04
PRK_03524	HMPREF0010_00409	ferrichrome-iron receptor protein	-1.22	9.83E-05
PRK_03481	HMPREF0010_00452	conserved hypothetical protein	-1.23	3.86E-04
PRK_00275	HMPREF0010_02294	non-ribosomal peptide synthetase	-1.23	2.48E-02
PRK_03315	HMPREF0010_00619	isochorismate hydrolase	-1.23	8.32E-03
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PRK_00727	HMPREF0010_02732	conserved hypothetical protein	-1.33	1.17E-04
PRK_03665	HMPREF0010_00269	TonB-dependent siderophore receptor	-1.34	1.45E-03
PRK_00264	HMPREF0010_02305	isochorismate hydrolase	-1.34	1.43E-02
PRK_00989	HMPREF0010_02514	type IV pilus assembly protein PilM	-1.36	5.81E-05
PRK_00259	HMPREF0010_02310	acinetobactin biosynthesis protein	-1.38	3.77E-02
PRK_00261	HMPREF0010_02308	ABC transporter	-1.38	1.81E-02
PRK_02399	HMPREF0010_02928	conserved hypothetical protein	-1.46	3.58E-03
PRK_00087	HMPREF0010_00053	alpha/beta superfamily hydrolase	-1.46	6.56E-04
PRK_03715	HMPREF0010_00217	conserved hypothetical protein	-1.47	2.32E-03
PRK_02284	HMPREF0010_01517	conserved hypothetical protein	-1.64	1.49E-04
PRK_02483	HMPREF0010_03012	curli production assembly/transport component CsgG	-1.72	3.55E-03
PRK_00260	HMPREF0010_02309	ABC transporter, CydDC cysteine exporter (CydDC-E) family	-1.75	1.09E-02
PRK_01925	HMPREF0010_01649	predicted protein	-1.85	1.21E-03
PRK_03337	HMPREF0010_00597	fimbrial subunit	-1.85	7.98E-04
PRK_03336	HMPREF0010_00598	pili assembly chaperone	-1.91	2.07E-03
PRK_00132	HMPREF0010_00007	predicted protein	-1.92	1.14E-04
PRK_03354	HMPREF0010_00580	type III restriction enzyme	-1.93	1.32E-05
PRK_01821	HMPREF0010_01753	outer membrane receptor for monomeric catechols	-2.10	2.25E-05
PRK_00268	HMPREF0010_02301	ferric anguibactin receptor	-2.25	1.48E-02

PRK_01142	HMPREF0010_03493	TonB-dependent receptor protein	-2.29	1.60E-03
PRK_00274	HMPREF0010_02295	non-ribosomal peptide synthetase	-2.34	1.77E-03
PRK_00270	HMPREF0010_02299	phosphonate ABC transporter, ATP-binding protein	-2.55	1.34E-03
PRK_00269	HMPREF0010_02300	BauB	-2.63	1.55E-03

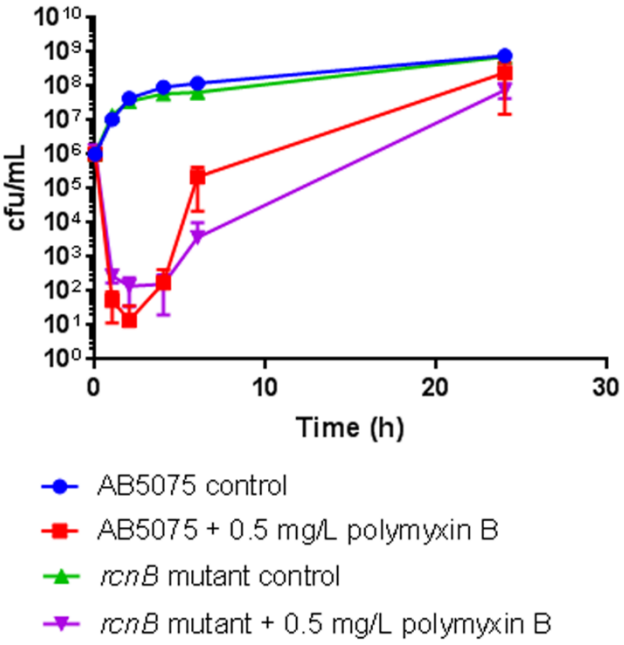

Figure A2.1 Time-kill assay with 0.5 mg/L polymyxin B against *A. baumannii* AB5075 wildtype and its transposon-inserted *rcnB* mutant. Error bars are means ± SDs from three biological replicates.

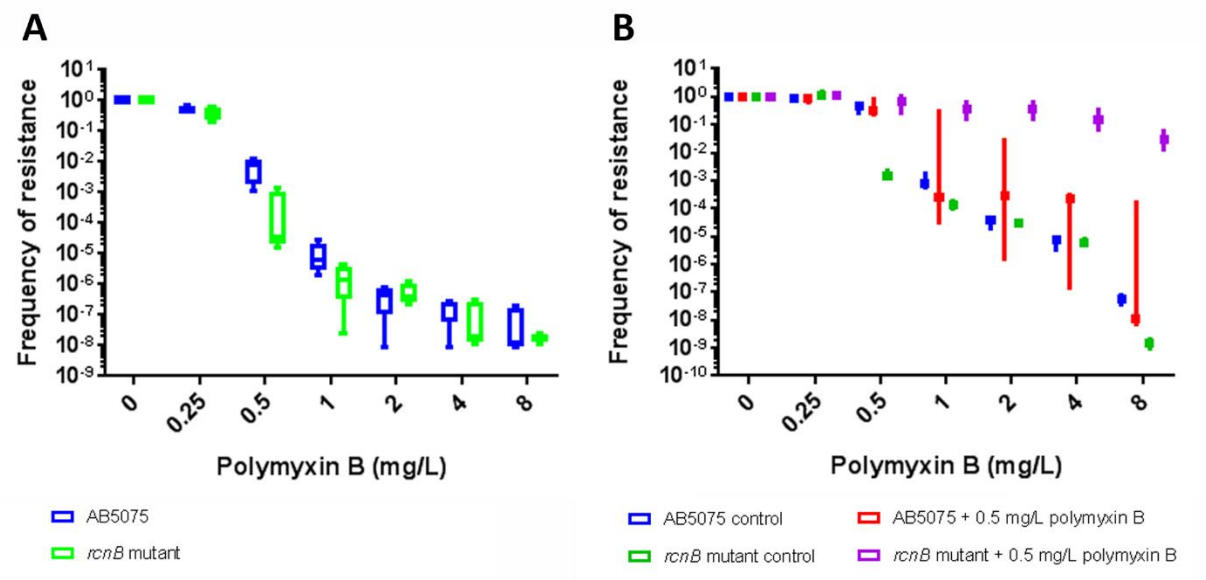

Figure A2.2 PAPs of *A. baumannii* AB5075 and its transposon-inserted *rcnB* mutant with different concentration for polymyxin B (mg/L) at (A, n = 4) baseline and (B, n = 3) 24 h following the time-kill assay. Box and whiskers indicate upper and lower quartiles and the range of data.

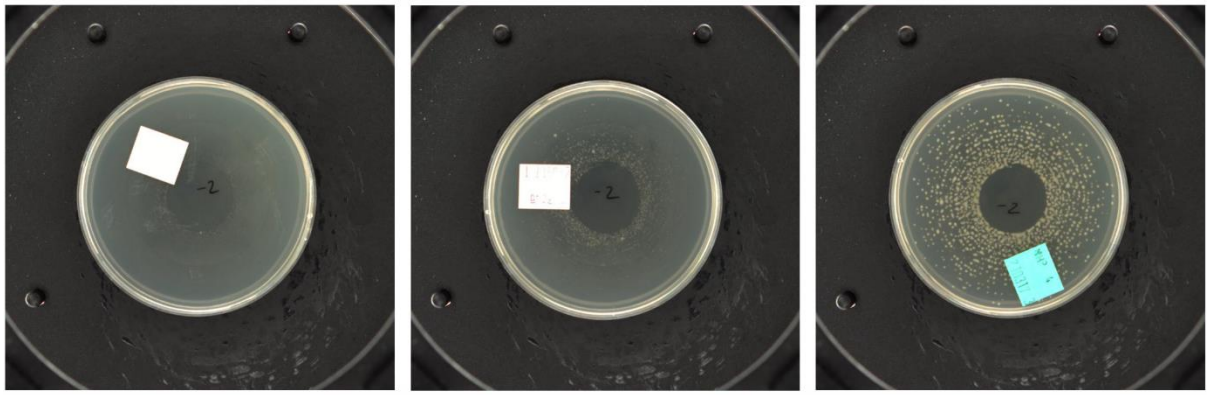

Figure A2.3 Morphology of *A. baumannii* mutant (rcnB) on Mueller-Hinton agar containing different concentration of polymyxin B at 24 h following polymyxin B treatment.

APPENDIX 3: PUBLICATIONS IN SUPPORT OF THIS THESIS

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Pharmacokinetics/pharmacodynamics of colistin and polymyxin B: are we there yet?

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ABSTRACT

The polymyxin antibiotics [colistin and polymyxin B (PMB)] are increasingly used as a last-line option for the treatment of infections caused by extensively drug-resistant Gram-negative bacteria. Despite having similar structures and antibacterial activity in vitro, the two clinically available polymyxins have very different pharmacological properties, as colistin (polymyxin E) is intravenously administered to patients in the form of an inactive prodrug colistin methanesulphonate (sodium). This review will discuss recent progress in the pharmacokinetics/pharmacodynamics and toxicity of colistin and PMB, the factors that affect their pharmacological profiles, and the challenges for the effective use of both polymyxins. Strategies are proposed for optimising their clinical utility based upon the recent pharmacological studies in vitro, in animals and patients. In the 'bad bugs, no drugs' era, polymyxins are a critically important component of the antibiotic armamentarium against difficult-to-treat Gram-negative 'superbugs'. Rational approaches to the use of polymyxins must be pursued to increase their effectiveness and to minimise resistance and toxicity.

1. Introduction

Colistin (polymyxin E) and polymyxin B (PMB) are lipopeptide antibiotics with activity against many Gram-negative bacteria [1,2]. The polymyxins were approved for clinical use in the late 1950s but fell out of favour during the mid-1970s owing to concerns over their potential to cause nephrotoxicity and neurotoxicity [3]. Over the last two decades, clinical interest in polymyxins has increased due to the emergence of extensively drug-resistant Gram-negative bacteria coupled with the dry antibiotic development pipeline [1]. Colistin and PMB are currently considered a last-line defence against the problematic Gram-negative 'superbugs', notably carbapenem-resistant Enterobacteriaceae, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, which are classified under 'Urgent' or 'Serious' threat level by the US Centers for Disease Control and Prevention (CDC) [4]. It is used against these pathogens that will be the focus of this mini-review.

Colistin and PMB possess very similar chemical structures, differing only by one amino acid at position 6 in the peptide ring, with a D-leucine and D-phenylalanine, respectively [5]. Not surprisingly, they have very similar antimicrobial spectra and resistance mechanisms [6]. A major difference between the polymyxins is the form in which they are administered parenterally. Colistin is administered in the form of an inactive prodrug, colistin methanesulphonate (CMS) (a polyanion at physiological pH), while PMB (a polycation at physiological pH) is administered directly as its active form [1]. The different chemical forms administered have significant impacts on their pharmacokinetics and toxicity [7]. For optimal use of CMS/colistin and PMB, it is important to understand their pharmacological differences. In this mini-review, we will discuss the latest progress in the pharmacokinetics/pharmacodynamics and toxicity of colistin and PMB as well as the challenges for optimal use of both polymyxins.

2. Different labelling of polymyxin products

Undoubtedly, a major contributing factor to the confusion surrounding the effective use of CMS is differences in the dosing terminology [2]. In many parts of the world, such as Europe and India, International Units (IU) are used, whereas in North and South

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America, Southeast Asia and Oceania colistin base activity (CBA) is used [1,2]. One million IU (MIU) of CMS is equal to ca. 80 mg of CMS or 34 mg of CBA; a more detailed discussion on differences in labelling and dosage recommendations can be found in our previous reviews [1,2]. Understanding the labelling differences is critical for the optimal use of CMS in patients. For PMB, which is available in North and South America, Southeast Asia and Japan, all products are labelled using IU (1 mg = 10 000 IU).

3. Minimum inhibitory concentrations (MICs) and mode of action

As CMS is an inactive prodrug of colistin, colistin sulphate should be used in MIC measurements for colistin [1]. To date, SENTRY Antimicrobial Surveillance Program (2006–2009) is the largest surveillance programme examining the MICs of the polymyxins. The compiled data from this programme showed that PMB and colistin have similar in vitro activities (MIC_{90}, ≤0.5–1 mg/L) against *P. aeruginosa*, *A. baumannii* and *Klebsiella pneumoniae*, with very low resistance rates globally (<0.1–1.5%) [8]. However, questions have been raised regarding the susceptibility testing methods used for polymyxins, including their potential adsorption to plastic devices used in the MIC measurement and poor diffusion of polymyxins in agar [9]. In this regard, polysorbate 80 (P-80) was initially proposed to improve the broth microdilution MIC results for colistin and PMB as it can prevent the binding of polymyxins to plastic panels. However, its use was contraindicated by the Clinical and Laboratory Standards Institute (CLSI) owing to potential synergism between P-80 and the polymyxins [9,10]. In the most recent CLSI protocol, P-80 is not recommended in the measurement of colistin and PMB MICs. Presently, broth microdilution is regarded as the best method for polymyxin susceptibility testing. Susceptibility breakpoints for colistin and PMB set by the CLSI for *P. aeruginosa*, *Acinetobacter* spp. and other non-Enterobacteriaceae are identical, where an MIC of ≤2 mg/L is regarded as susceptible [11]. The susceptibility breakpoints of colistin by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) are ≤4 mg/L for *Pseudomonas* spp. and ≤2 mg/L for *Acinetobacter* spp. and Enterobacteriaceae [12]. However, as will be discussed in Section 5 below on pharmacodynamics, data from recent pharmacokinetic/pharmacodynamic (PK/PD) studies suggest the breakpoints for the above Gram-negative pathogens could be even lower. Consequently, a joint CLSI and EUCAST Working Group is currently re-evaluating the existing breakpoints [1,9].

The precise mechanism of action of the polymyxins is currently unclear. However, it is believed that activity is related, in part, to disruption of the bacterial outer and inner membranes via a 'self-promoted uptake' mechanism [13]. The initial step involves binding of the positively charged polymyxins to negatively charged lipopolysaccharide (LPS) on the outer membrane of Gram-negative bacteria both via electrostatic and hydrophobic interactions (Fig. 1) [5]. Bacteria can become resistant to polymyxins by modifications of the negatively charged phosphate groups of lipid A [14] or by loss of LPS [15]. For more details, we direct the reader to the review in this Theme Issue on the mechanism of polymyxin resistance.

4. Pharmacokinetics of polymyxins

4.1. Colistin methanesulphonate/colistin

The positively charged colistin exhibits a markedly different PK profile to that of the sulfomethylated derivative [1]. CMS is eliminated predominantly by the kidneys, whereas colistin is mainly cleared by a route other than renal excretion [2]. Following parenteral administration of CMS, colistin is generally formed slowly, with the plasma concentration increasing slowly. Plachouras et al. [16] showed that it can take >36 h to reach a colistin steady-state plasma concentration of 2 mg/L with intravenous (i.v.) administration of 3 MIU CMS every 8 h (q8h) in patients with good renal function. This finding highlights that the low initial exposure to formed colistin is a significant PK/PD challenge for optimising CMS use in patients. This dilemma can be partially counteracted with the use of a loading dose. In studies that evaluated CMS loading doses of 6 MIU and 9 MIU, the average colistin plasma concentrations reached 1.34 mg/L and 2.65 mg/L, respectively, at 8 h after the loading dose, with the likelihood of earlier eradication of the infecting bacteria [17,18]. In critically ill patients, kidney function and renal replacement therapy (RRT) have a dramatic impact on the pharmacokinetics of CMS and formed colistin [19,20]. One of the largest population PK studies reported thus far in critically ill patients involved 105 patients with varying degrees of renal function [creatinine clearance (CL_{Cr}) of 3–169 mL/min/1.73 m^2], including 12 patients on intermittent

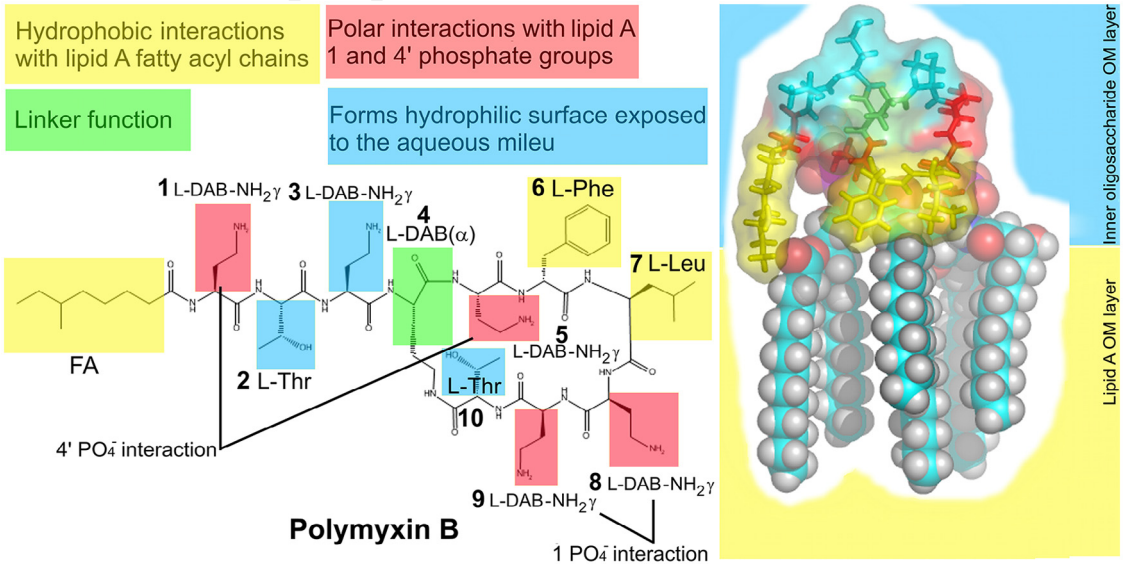

Fig. 1. Schematic diagram showing key contacts involved in the complex formation between polymyxin B and the lipid A component of lipopolysaccharide. FA, *N*-terminal fatty acyl chain; OM, outer membrane. Figure reproduced from Velkov et al. [5] with permission. Published 2010 by the American Chemical Society.

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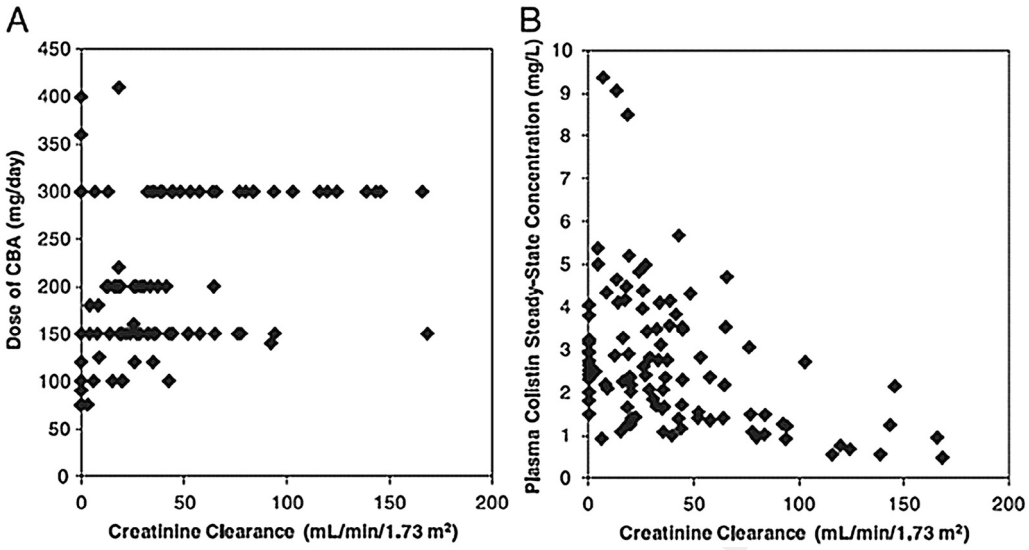

Fig. 2. Relationship of physician-selected daily dose of colistin base activity (CBA) (A) and the resultant average steady-state plasma colistin concentration (B) versus creatinine clearance (CL_{Cr}) in 105 critically ill patients. CL_{Cr} was calculated using the Jelliffe equation [21]. Figure reproduced from Garonzik et al. [19] with permission. Published 2011 by the American Society for Microbiology.

haemodialysis and 4 on continuous RRT (CRRT) [19]. Even though there was only a ca. 5.5-fold range in the daily doses (2.5–13.7 MIU), substantial interpatient variation (0.48–9.38 mg/L, ca. 19.5-fold) in the average steady-state plasma colistin concentration ($C_{ss,avg}$) was observed in the 105 patients. Significant interpatient variation was observed even among patients with similar CL_{Cr} and those receiving the same daily dose of CMS (Fig. 2). In patients on RRT, both CMS and formed colistin were cleared [19,20]. Clearly, given that the plasma concentration of formed colistin is highly influenced by renal function, it is essential that the dosage regimen of CMS is adjusted in patients with varying renal function to ensure that appropriate colistin exposure is obtained. In patients with a CL_{Cr} of >80 mL/min, only 65–75% of patients receiving the approved updated dose recommended by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) achieved a $C_{ss,avg}$ of formed colistin ≥1 mg/L [22]. As the MIC_{90} for colistin is ≤0.5–1 mg/L against *P. aeruginosa*, *A. baumannii* and *K. pneumoniae* [8], it would be clinically useful to administer the maximal CMS dose in patients with CL_{Cr} > 80 mL/min, ideally in combination with another antibiotic that may provide synergistic bacterial killing [19,23]. As colistin is ca. 50% unbound in human plasma [23] (and unpublished data), a colistin $C_{ss,avg}$ of ca. 2 mg/L is necessary for effective treatment of bacteria with an MIC of 1 mg/L. For patients on RRT, in order to achieve a colistin $C_{ss,avg}$ of 2 mg/L, the current recommendation suggests a CMS loading dose of 9 MIU followed at 24 h by 1 MIU every 12 h (q12h) for patients on intermittent haemodialysis, and 4.3 MIU q8h or 6.3 MIU q12h for patients on CRRT [19]. Furthermore, haemodialysis patients should aim to have their dialysis performed towards the end of the CMS dosing interval to avoid excessive removal of CMS from the body. After dialysis, a CMS dose of 1.7 MIU is required to replenish the removed CMS.

Currently, little is known about the pharmacokinetics of CMS and formed colistin in extravascular sites. In critically ill patients with and without central nervous system (CNS) infection, the distribution of colistin into the cerebral spinal fluid (CSF) appears to be very low following i.v. CMS administration. In a study by Ziaka et al. [24], the CSF concentrations of formed colistin (at 1, 4 and 8 h) following i.v. administration of 3 MIU CMS q8h were only ca. 7% of the total serum colistin concentrations in patients without CNS infection and ca. 11% in patients with external ventricular drain-associated ventriculitis (EVDV). When a combination of i.v. (3 MIU CMS q8h) and intraventricular (0.125 MIU CMS once daily) CMS was administered to patients with EVDV, concentrations of formed colistin in the CSF were ca. 1.45, 0.84 and 0.62 mg/L, respectively, at 1, 4 and 8 h and were >40% of the total colistin serum concentration at each time point [24]. It is evident that the combination of i.v. and intraventricular CMS may be useful for the treatment of CNS infection caused by Gram-negative bacteria; however, further clinical studies are required.

A recent study in cystic fibrosis (CF) patients showed that the concentration of formed colistin in sputum following i.v. administration of CMS is minimal. When six patients with CF were administered an i.v. CMS dose of 5 MIU at 3 days post-nebulisation of 4 MIU of CMS, the formed colistin concentrations in the sputum over 12 h were similar to their carryover concentrations in the pre-dose sputum (0.12–0.72 mg/L) [25]. Higher concentrations (>10-fold) of formed colistin in the sputum were achieved via inhalation (4 MIU/day of CMS). After a single inhalation dose, an average maximum colistin concentration of ca. 6.0 mg/L was achieved in the sputum at ca. 3 h for 2 MIU of CMS and ca. 12.8 mg/L at ca. 4.6 h for 4 MIU of CMS [25]. However, plasma concentrations of CMS and formed colistin were very low following inhalation. Following a single nebulisation dose of CMS at 2 MIU or 4 MIU, the maximum plasma CMS concentrations were 0.22 ± 0.055 mg/L at ca. 1.3 h and 0.33 ± 0.092 mg/L at ca. 1.9 h, respectively, with <3% of the nebulised CMS dose recovered in the urine by 24 h. In a study comparing the intrapulmonary and systemic pharmacokinetics of formed colistin in critically ill patients following administration of 2 MIU of CMS via inhalation, the steady-state colistin concentrations in the epithelial lining fluid were much higher than the steady-state plasma colistin concentrations (9.53–1137 mg/L vs. 0.15–0.73 mg/L) [26]. These findings highlight the potential to administer CMS by inhalation for the treatment of Gram-negative bacterial pneumonia, maximising the exposure of formed colistin in the lungs while minimising plasma concentrations and associated systemic toxicity. Clearly, further PK/PD studies are warranted for optimising the use of inhaled CMS.

4.2. Polymyxin B

Compared with CMS, only a very small number of studies have examined the pharmacokinetics of PMB following i.v. administration. One study involving eight critically ill patients showed that

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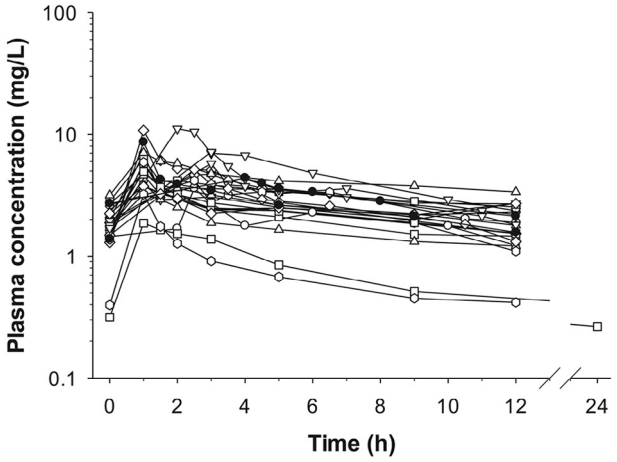

Fig. 3. Plasma concentration–time profiles of polymyxin B in 24 critically ill patients. Concentrations from patients undergoing continuous venovenous haemodialysis are shown by filled symbols. Figure reproduced from Sandri et al. [29] with permission. Published 2013 by Oxford University Press.

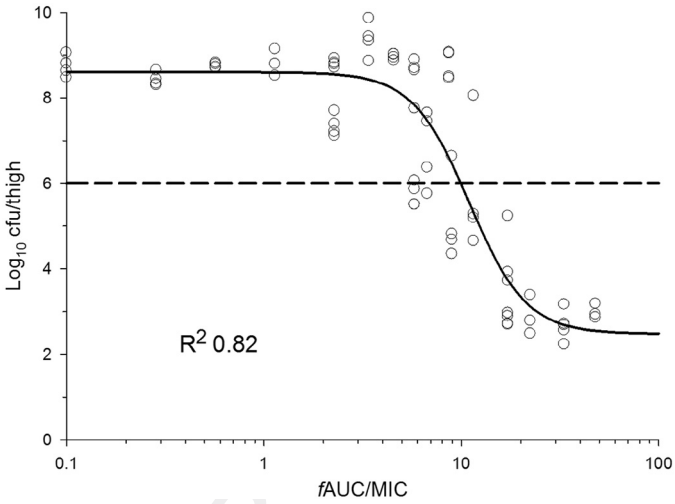

Fig. 4. Relationship between bacterial load in the thighs of neutropenic mice at 24 h and the ratio of the area under the unbound (free) concentration–time curve to the MIC (*f*AUC/MIC) of colistin for *Pseudomonas aeruginosa* ATCC 27853. Figure adapted from Cheah et al. [23] with permission. Published 2015 by Oxford University Press.

PMB is mainly eliminated by non-renal pathway(s), with <1% recovered in the unchanged form in urine [27], which is very similar to colistin in rats [28]. The largest population PK study to date involved 24 critically ill patients with a wide range of kidney function (CL_{Cr} of 10–143 mL/min), including two patients on CRRT [29]. With i.v. doses ranging from 0.45 mg/kg/day to 3.38 mg/kg/day (i.e. ca. 7.5-fold), the PMB $C_{ss,avg}$ ranged from 0.68 mg/L to 4.88 mg/L (ca. 7.2-fold) (Fig. 3) and the median urinary recovery (4.04%) was very low. The PMB clearance scaled by total body weight from this study showed minimal interpatient variability in the PMB $C_{ss,avg}$ (range, 0.02–0.06 L/h/kg; ca. 3-fold), a finding in marked contrast to the influence of renal function on the $C_{ss,avg}$ of plasma colistin following administration of CMS as discussed above. Thus, renal function does not markedly affect PMB plasma concentrations and should not be used for dose adjustment. In the two patients on CRRT, 12.2% and 5.62% of the dose was recovered as unchanged PMB in the dialysates during the 12-h dosing interval [29]. Similar to colistin, PMB is cleared during dialysis; however, dosage adjustments are currently not recommended for patients on CRRT owing to limited clinical data. A National Institutes of Health (NIH)-funded clinical study is investigating the PK, PD and toxicodynamic (TD) relationships of i.v. PMB in critically ill patients, which aims to develop scientifically-based dosing recommendations for this important polymyxin antibiotic (NCT02682355, http://www.clinicaltrials.gov). In addition, little is known about the distribution of PMB into extravascular sites following i.v. administration, and studies in this area will be essential to determine the usefulness of i.v. PMB for the treatment of infections such as pneumonia and meningitis.

In summary, the pharmacokinetics of CMS/colistin is influenced by renal function, with dosage regimens requiring adjustment in different types of patients. However, such an adjustment is not required for PMB, which is mainly cleared by non-renal pathway(s). As it is difficult to achieve a $C_{ss,avg}$ of even 1 mg/L in patients with good renal function following i.v. administration of CMS [19], PMB may be a better option for treatment of bloodstream infections, with less interpatient variability and higher $C_{ss,avg}$ [7,29]. Since CMS is mainly eliminated by the kidneys with high levels of colistin produced in the urinary tract, it may be a better option than PMB for the treatment of urinary tract infections. Inhaled CMS has been successfully employed for the treatment of lung infections caused by *P. aeruginosa* in patients with CF over the last three decades [30]. Given that inhaled PMB has been associated with a greater incidence of local airway irritation compared with CMS [31], CMS may be a better choice for inhalation. Nevertheless, prospective randomised controlled clinical studies are warranted to compare the efficacy of both polymyxins for the treatment of different types of infections.

5. Pharmacodynamics of polymyxins

Most studies examining the pharmacodynamics of the polymyxins have been conducted using colistin [23,32–34]. In in vitro studies, colistin shows rapid concentration-dependent killing against *A. baumannii*, *K. pneumoniae* and *P. aeruginosa*, with a minimal post-antibiotic effect at clinically achievable concentrations [32–34]. However, despite rapid initial killing, re-growth often occurs quickly (as early as within 2 h of the initial exposure). PMB displays very similar pharmacodynamics to that of colistin, with similar rapid killing against *A. baumannii*, *K. pneumoniae* and *P. aeruginosa* in vitro, followed by rapid re-growth [35–37]. In polymyxin-heteroresistant strains, amplification of polymyxin-resistant subpopulations has been shown to play an important role in the rapid emergence of resistance [38–40]. An inoculum effect has been reported both with colistin and PMB in vitro [37,40].

Using *P. aeruginosa* and *A. baumannii* in neutropenic mouse thigh and lung infection models, the PK/PD index that best describes the antimicrobial activity of colistin is the ratio of the area under the unbound (free) concentration–time curve to the MIC (*f*AUC/MIC) (Fig. 4) [23]; for *P. aeruginosa*, this has also been demonstrated in vitro [34]. Owing to the potential binding of polymyxins to the plasticware or ultrafiltration membranes, our group identified that ultrafiltration can be problematic [28], and ultracentrifugation and rapid equilibrium dialysis methods are superior for measuring plasma binding of polymyxins [23]. Our recent PK/PD study using ultracentrifugation and rapid equilibrium dialysis methods in neutropenic mice showed that the unbound fraction of colistin of 0.084 is ca. 6-fold lower than in humans (ca. 0.5) [23] (and unpublished data). For three strains of *P. aeruginosa* [ATCC 27853, PAO1 and a multidrug-resistant (MDR) clinical isolate] and three strains of *A. baumannii* (ATCC 19606 and two MDR clinical isolates), an *f*AUC/MIC value of 7.4–13.7 and 7.4–17.6, respectively, was required for a 2 $\log_{10}$ reduction in bacterial load in the thigh of neutropenic mice. In the neutropenic mouse lung infection model, subcutaneous colistin was substantially less effective at killing *P. aeruginosa* and *A. baumannii*

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compared with in the thigh infection model [23]. With the highest tolerable dose (40 mg/kg administered 6- or 8-hourly with cumulative daily doses of 120–160 mg/kg), 2 $\log_{10}$ killing in the lungs was not achievable for all six of the tested strains. The lower antibacterial activity in the lungs relative to the thigh is most likely due to limited drug exposure in the lungs following parenteral administration. Currently available data from animal and clinical studies suggest that colistin (and CMS) may have limited efficacy against respiratory tract infections [23,25].

Limited studies to date have examined the PK/PD index driving the activity of PMB. Given the similarity in the structure, it is very likely that *f*AUC/MIC is the most predictive PK/PD parameter for parenteral PMB [37]. In patients with good renal function, however, administration of PMB is very likely to generate higher *f*AUC/MIC values than CMS because: (i) CMS distribution is influenced by kidney function while PMB is not; and (ii) CMS conversion to colistin in vivo is slow and incomplete. To optimise the clinical use of PMB, more PD studies are needed.

6. Toxicodynamics of polymyxins

In the early years of their use, polymyxin-associated neurotoxicity occurred in patients with an incidence as high as 27% following parenteral administration [3,41]. However, recent retrospective clinical studies have not shown neurotoxicity to be a major concern [42,43]. Nephrotoxicity is by far the most common and dose-limiting side effect associated with parenteral polymyxins, with incidence rates in patients as high as 60% [44,45]. However, the rate of nephrotoxicity in patients receiving i.v. polymyxins is somewhat variable and depends on the definition of nephrotoxicity employed [e.g. RIFLE (risk, injury, failure, loss, and end-stage kidney disease) and AKIN (Acute Kidney Injury Network) scoring systems] [46].

Nephrotoxicity has been observed both with colistin and PMB following parenteral administration [46–49]. Recent TD analyses of colistin showed that patients with colistin $C_{ss,avg} > 2.5$ mg/L and patients with $CL_{Cr} > 80$ mL/min are more likely to develop nephrotoxicity [47,48]. The minimum colistin plasma concentration was also identified as an independent risk factor for nephrotoxicity, which occurred in the majority of patients when the minimum colistin plasma concentration was ≥2.2 mg/L (odds ratio = 4.6 on Day 7) [47]. For PMB, a daily dose of ≥150 mg (hazard ratio = 1.92) has been identified as the risk factor of nephrotoxicity [49]. A retrospective study showed the earliest onset of nephrotoxicity reported for i.v. CMS or PMB occurred 2 days after initiation of therapy, with the majority of cases occurring after 15 days of therapy [46]. Fortunately, polymyxin-associated nephrotoxicity was, however, reversible in most patients [47,50].

With regard to the mechanism of polymyxin-induced nephrotoxicity, cell culture and animal studies have demonstrated that colistin and PMB accumulate in renal tubular cells possibly through active uptake mechanisms mediated by megalin and PEPT2 transporters [51,52]. The resultant extremely high intracellular concentration of polymyxins in renal tubular cells causes dramatic changes in the morphology of mitochondria, loss of cytoplasmic membrane potential, apoptosis and cell cycle arrest [53,54]. The precise mechanisms of the uptake of polymyxins by renal tubular cells and subsequent cell death remain unanswered. However, elucidating these mechanisms is crucial for optimising their use in patients, development of novel approaches to attenuate polymyxin-induced nephrotoxicity, and the discovery of safer new-generation polymyxins.

7. Conclusions

Significant progress in understanding the pharmacology of polymyxins has been made over the past 15 years, although many gaps still remain. Scientifically-based dosing recommendations have now been developed for i.v. administration of CMS in critically ill patients and more recent studies are generating valuable insights for PMB. It is evident now that only the dose of CMS/colistin, not PMB, should be adjusted according to the patient's renal function. As CRRT can efficiently eliminate both colistin and PMB, further clinical PK/PD/TD studies are warranted in order to optimise their use in this type of patient. Other high-priority research areas include evaluation of the efficacy of i.v. CMS/colistin and PMB for the treatment of respiratory tract infections and clinical PK/PD/TD studies of intrathecal and intraventricular administration of both polymyxins for the treatment of meningitis. While we await the development of novel antibiotics for the treatment of infections caused by Gram-negative 'superbugs', every effort must be made to optimise the clinical use of the polymyxins to maximise their efficacy while minimising the emergence of resistance and toxicity.

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Agents of Last Resort
Polymyxin Resistance

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KEYWORDS

• Colistin • Polymyxin *B* • Polymyxins • Resistance • Gram-negative

KEY POINTS

- Polymyxin resistance is a major public health threat, as the polymyxins represent "last-line" therapeutics for Gram-negative pathogens resistant to essentially all other antibiotics.
- Improved understanding of mechanisms of, and risk factors for, polymyxin resistance, as well as infection prevention and stewardship strategies, together with optimization of dosing of polymyxins including in combination regimens, can help to limit the emergence and dissemination of polymyxin resistance.

INTRODUCTION

The polymyxins, colistin (also known as polymyxin E) and polymyxin B, have a unique and interesting history. Originally introduced in the 1950s for the treatment of infections due to Gram-negative organisms, the polymyxins fell out of favor by the mid-1970s because of high rates of nephrotoxicity (approaching 50%) and neurotoxicity and the advent of less toxic alternatives, notably the antipseudomonal aminoglycosides. By the mid-1990s the polymyxins were reintroduced into clinical practice, not because of an enhanced safety profile, but rather due to the development of extensively drug-resistant (XDR) Gram-negative bacilli resistant to all other treatment options.[1,2] The polymyxins now serve a critical role in the antimicrobial armamentarium, as they are one of few, and sometimes the only, antimicrobial agent retaining activity against carbapenem-resistant *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and Enterobacteriaceae (CRE), organisms that frequently cause life-threatening infections in the most vulnerable of patient populations. These pathogens have been recognized by the Centers for Disease Control and Prevention as serious or

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urgent threats to human health and mortality rates in invasive infections due to these pathogens can exceed 50%.[2,3] The relatively dry antimicrobial pipeline for the treatment of infections caused by these organisms magnifies the importance of the polymyxins. Given the critical role of the polymyxins in the care of hospitalized patients, an understanding of both the epidemiology of polymyxin resistance as well as strategies to prevent resistance are paramount. Therefore, this article introduces similarities and differences between the two clinically available polymyxins, discusses the mechanism of action and resistance to these agents, describes the clinical epidemiology of polymyxin-resistant organisms, and finally suggests strategies to minimize the development and spread of polymyxin resistance.

Colistin (also known as polymyxin E) and polymyxin B are nearly structurally identical, differing by only one amino acid at position 6 (Fig. 1). They are considered to be very similar microbiologically and cross-resistance exists. Both polymyxins are products of fermentation and therefore are multicomponent mixtures. Colistin and polymyxin B have two major components (colistin A and B; polymyxin B1 and B2) that slightly differ at the site of the *N*-terminal fatty acyl tail.[4] The polymyxins are

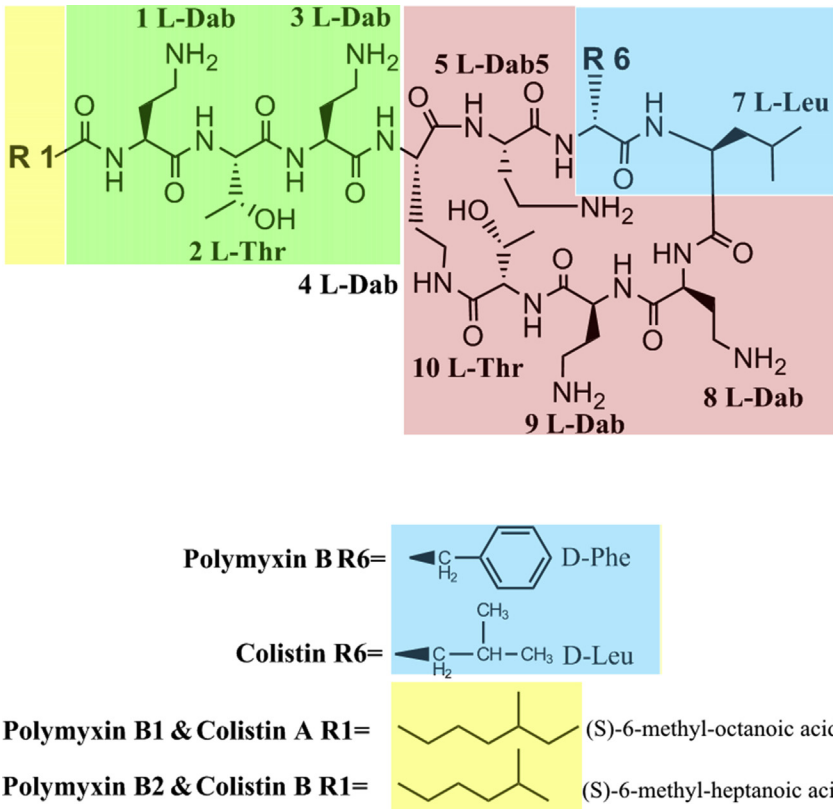

Fig. 1. Chemical structures of polymyxin B and colistin. The functional segments of polymyxins are colored as follows: yellow, fatty acyl chain; green, linear tripeptide segment; red, the polar residues of the heptapeptide; blue, the hydrophobic motif within the heptapeptide ring. (*Reprinted* with permission from Velkov T, Thompson PE, Nation RL, et al. Structure–activity relationships of polymyxin antibiotics. J Med Chem 2010;53(5):1898. Copyright © 2010 American Chemical Society.)

amphipathic molecules, consisting of both hydrophilic and hydrophobic regions (see Fig. 1) and these properties are essential to their antimicrobial activity (described later in this article). Although polymyxin B is administered directly as its sulfate salt, colistin is administered in the form of its inactive prodrug colistimethate sodium (CMS, also known as colistin methanesulfonate).[5] CMS is synthesized by sulfomethylation of active colistin, and although CMS is considered to exist in its fully pentamethanesulfonated form, recent analyses have shown that the material reconstituted for use in patients likely exists as a combination of up to 32 fully or partially methanesulfonated derivatives.[6] As is described in detail later in this article, the administration of colistin as an inactive prodrug has a significant impact on the pharmacokinetics of colistin in patients and is an important differentiator between the two polymyxins. Both polymyxins are associated with nephrotoxicity rates in the 30% to 50% range,[1] and all strategies for optimal use need to be taken in the context of the dose, and subsequent concentration-dependent toxicity that may be seen.

MECHANISM OF ACTION

The precise mechanism of antibacterial activity of polymyxins is not completely understood; however, the general current view is that polymyxins kill bacteria by disrupting the bacterial outer and inner membranes through the "self-promoted uptake" pathway.[7] The initial binding target of polymyxins is the lipopolysaccharides (LPS) in the outer membrane of Gram-negative bacteria, with both electrostatic and hydrophobic interactions being important.[4] Electrostatic interaction via the positively charged diaminobutyric acid (Dab) residues of the polymyxin (see Fig. 1) and the negatively charged phosphate groups on the lipid A moiety of LPS leads to displacement of divalent cations (Mg^{2+} and Ca^{2+}) that bridge the lipid A phosphoesters, thereby destabilizing the outer membrane.[8] This event allows the polymyxin to insert its hydrophobic regions (fatty acyl tail and amino acids at positions 6 and 7) into the bacterial outer membrane to interact with the fatty acyl chains of lipid A; this hydrophobic interaction causes further outer membrane disruption that promotes the uptake of the polymyxin.[7,9] It has been proposed that after transiting the outer membrane, polymyxins mediate the fusion of the inner leaflet of the outer membrane with the outer leaflet of the cytoplasmic membrane, which induces phospholipid exchange and causes osmotic imbalance that leads to cell death.[10] The amphipathic property of polymyxins (ie, presence of both cationic and hydrophobic regions) is necessary for the killing of Gram-negative bacteria. Polymyxin B nonapeptide (ie, polymyxin B lacking the fatty acyl tail and the Dab residue at position 1) and colistimethate (in which the Dab residues are masked by negatively charged methanesulfonate moieties) do not possess antibacterial activity.[5,11] In addition to their membrane-disrupting effect in Gram-negative bacteria, binding of polymyxins to lipid A also neutralizes the toxicity of endotoxins.[12,13]

A secondary antibacterial mechanism of polymyxins is thought to be via inhibition of the nicotinamide adenine dinucleotide oxidase enzyme family. This inhibitory activity has been observed in *Escherichia coli*, *Klebsiella pneumoniae*, *A baumannii*,[14] and *Mycobacterium smegmatis.*[15]

MECHANISMS OF RESISTANCE

As reviewed previously, the interaction of polymyxins with LPS is essential for their antimicrobial activity. This explains why polymyxin B and colistin are not active against Gram-positive bacteria. In Gram-negative bacteria, which are intrinsically resistant to polymyxins, this interaction is diminished due to LPS that has lower binding affinity for polymyxins. In these LPS molecules, lipid A usually contains modified phosphate groups,

thereby decreasing their overall net negative charge.[16–18] Likewise, in bacteria that are susceptible to polymyxins, resistance is usually acquired through LPS modifications.[19]

Arguably, the modification of LPS that most commonly leads to polymyxin resistance in *P aeruginosa* involves the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) to the phosphate groups in lipid A.[19] This modification is usually controlled by the *arn* (*pmr*) operon, which is regulated by the PmrA/PmrB and PhoP/PhoQ 2-component systems (TCSs).[20] These systems can also be activated by changes in the environment (eg, high Fe^{3+} concentration, low Mg^{2+} or Ca^{2+} concentrations, and low pH) and the lipid A modification can lead to decreased bridging of adjacent lipid A molecules via divalent cations.[21–23] PmrB and PhoQ are cytoplasmic membrane-bound sensor kinases that phosphorylate their respective regulator proteins PmrA and PhoP on activation. Once phosphorylated, PmrA and PhoP promote the upregulation of the *arn* operon leading to the addition of L-Ara4N to the phosphate groups of lipid A.[24] Resistance to polymyxins can develop when mutations occur in the PmrA/PmrB and PhoP/PhoQ systems.[25] Addition of phosphoethanolamine (PEtN) to lipid A, which also decreases the negative charge has also been identified in the modification of LPS of polymyxin-resistant *P aeruginosa*. This modification is controlled by the ColR/ColS TCS, which is upregulated in the presence of excess extracellular Zn^{2+}.[26]

In *A baumannii*, where L-Ara4N biosynthesis and attachment genes are generally lacking, polymyxin resistance is often achieved from the modification of LPS by the addition of PEtN to lipid A.[27] This modification can be caused by mutations in *pmrA* and/or *pmrB* that induce the autoregulation of the promoter region of the *pmrCAB* operon.[25] Recent findings from polymyxin-resistant *A baumannii* clinical isolates indicate that the modification of LPS with galactosamine (GalN) also contributes to polymyxin resistance, although the precise regulatory pathway is not yet understood.[28] Apart from LPS modifications, *A baumannii* also possesses a unique polymyxin resistance mechanism that involves the complete loss of LPS.[29] This phenotype can be caused by mutations in lipid A biosynthesis genes. In these polymyxin-resistant *A baumannii* isolates, genes responsible for transport of phospholipids/lipoproteins and production of poly-β-1,6-*N*-acetylglucosamine are upregulated to compensate for the missing LPS in the outer leaflet of the outer membrane.[30]

In *K pneumoniae*, resistance to polymyxins may involve several different strategies. One of these involves the modification of lipid A by the addition of either L-Ara4N or PEtN.[25] These modifications are caused by mutations in *pmrA*, *pmrB*, or *phoQ* genes that upregulate the PhoP/PhoQ and PmrA/PmrB systems.[31–33] It has also been reported that the upregulation of the PhoP/PhoQ and PmrA/PmrB systems can be caused by deletion in the *mgrB* locus.[34] Another polymyxin resistance mechanism in *K pneumoniae* is overproduction of surface capsular polysaccharides (CPS). It is believed that the CPS may act as a barrier to limit the interaction of polymyxins with lipid A,[35] by "trapping" polymyxins.[36] It is also reported that the AcrAB-TolC efflux pump may play a role in polymyxin resistance in *K pneumoniae*.[37]

Phenotypically, resistance to polymyxins also can be developed from polymyxin-heteroresistant bacteria. The minimum inhibitory concentrations (MICs) of polymyxins in these bacteria are $\leq$2 mg/L; however, there is a subpopulation of bacterial cells that can survive in the presence of more than 2 mg/L polymyxins. This leads to the amplification of the resistant subpopulation in the presence of polymyxin alone and the eventual development of polymyxin resistance.[38] Recent studies indicate that polymyxin heteroresistance in *P aeruginosa* is infrequent[39]; however, it is very common in both multidrug-resistant *K pneumoniae*[40] and *A baumannii*.[38,41]

Laboratory studies have indicated that resistance to polymyxins may compromise the resistance to other classes of antibiotics.[42,43] In a study with *A baumannii* that

compared the antibiograms of multi-drug resistant (MDR) colistin-susceptible clinical isolates with those of the respective laboratory-generated colistin-resistant paired strains,[42] the polymyxin-resistant strains were more susceptible to other antibiotics compared with their parent polymyxin-susceptible strains. These findings suggested that polymyxin combinations may be useful to prevent polymyxin resistance in MDR bacteria. However, the clinical relevance of this finding remains to be determined, as in clinical practice most polymyxin-resistant isolates are usually resistant to a broad range of other antibiotics.

Resistance to polymyxins may also come at a fitness cost. *A baumannii* isolates with polymyxin resistance usually grow at a much slower rate and are less capable of causing infection.[44,45] Studies that compared the fitness cost of lipid A modification and LPS loss in *A baumannii* isolates showed that reduction in biological fitness associated with LPS loss was greater than with PEtN addition.[44,46] Impaired virulence in *A baumannii* is also linked to reduced expression of metabolic proteins and of the OmpA porin.[47] Significant biological fitness cost due to polymyxin resistance has yet to be observed in *P aeruginosa* and *K pneumoniae*.

CLINICAL EPIDEMIOLOGY OF POLYMYXIN-RESISTANT GRAM-NEGATIVE BACILLI

As previously discussed, the primary clinical role for the polymyxins is for the treatment of infections due to carbapenem-resistant *A baumannii, P aeruginosa*, or CRE (most notably carbapenem-resistant *K pneumoniae*), as no other reliable treatment options are available. Fortunately, colistin has excellent in vitro activity in this setting, and most isolates are susceptible at the susceptibility breakpoint of 2 mg/L or lower concentration. However, there are regional variations in susceptibility rates and clinicians should be aware of local susceptibility data. Although it is not the focus of this article, it is important for the reader to be aware of a few important points. First, not all published analyses have used the same susceptibility breakpoint for colistin to define resistance. Second, the current susceptibility breakpoints might not be ideal from a pharmacokinetic/pharmacodynamic standpoint. Third, there are unique complexities that exist with regards to the determination of the colistin MIC via conventional methods.[48] Because of these issues, Clinical and Laboratory Standards Institute (CLSI) and The European Committee on Antimicrobial Susceptibility Testing (EUCAST) have formed a joint Working Group to examine MIC testing methods and breakpoints for the polymyxins, and the work of that group is being informed by data from recent preclinical and clinical pharmacokinetic/pharmacodynamic studies.[49] For the purposes of this section, a susceptibility breakpoint of 2 mg/L is used. Polymyxin B susceptibility is not routinely performed and colistin is used as a categorical surrogate for susceptibility.

Published data regarding rates of colistin-resistant *P aeruginosa* are scarce; however, most published rates are between 0% and 4%.[50,51] Nonetheless, this finding is not universal and there are notable variations regionally. A recent analysis from India assessing *P aeruginosa* isolates found that 8 (8%) of 95 were resistant to colistin.[52] Furthermore, in 2002 Schulin[53] published susceptibility data to colistin from 385 *P aeruginosa* isolates in patients with cystic fibrosis from Germany and found colistin resistance (MIC >2 mg/L) in 35 (15%) of 229 nonmucoid strains and 5 (3%) of 156 mucoid strains, for an overall resistance rate of 10.4%.

Despite the widespread nature of carbapenem resistance in A baumannii and the increasingly common use of polymyxins as one of the only therapeutic options, widespread polymyxin resistance in this organism has not been reported. Data from the Sentry Antimicrobial Surveillance database, which include isolates from the United

States, Europe, Latin America, and the Asia-Pacific region, have shown resistance between 0.9% and 3.3% from 2001 to 2011.[54–56] Although some individual reports have shown higher numbers, rarely do rates exceed 5%,[57] and when they do, there are notable limitations. Many studies reporting high rates of colistin resistance include isolates that are carbapenem susceptible and/or are not all-inclusive studies of every *Acinetobacter* isolate in the institution. For example, a frequently cited report that showed colistin resistance to be 16.7% is limited because it included only 18 isolates, 3 of which were colistin resistant.[58] Additionally, the vast majority (17/18) were actually susceptible to carbapenems. Similarly, although Ko and colleagues[59] reported an extremely high rate of colistin resistance of 31% in 214 *A baumannii* isolates in Korea, of the 83 polymyxin-resistant strains, only 5 were resistant to imipenem. Although Arroyo and colleagues[60] reported a rate of colistin resistance of 19.1% in Spain (21/115 isolates), it is unclear how these isolates were selected and whether or not they represented all isolates in their institution. Similarly, in another report published by the same group in Spain that described a 41% rate of colistin resistance, the analysis did not consist of all *Acinetobacter* isolates from their institution and was specifically chosen to assess the in vitro activities of various other antimicrobials against both multidrug and pan-drug–resistant isolates.[61] Although these studies might overstate the incidence of colistin resistance in carbapenem-resistant *A baumannii*, they clearly demonstrate that colistin-resistant *A baumannii* exists in various geographic locales and some reports have shown the incidence to be increasing, albeit still at low overall numbers.[62] The most alarming epidemiologic trend with regard to carbapenem-resistant Gram-negative bacilli has been the rise and worldwide spread of CRE, primarily, but not exclusively, driven by the *K pneumoniae* carbapenemase (KPC) enzyme.[63] Although KPC is most commonly produced in *K pneumoniae*, it can be produced by other Enterobacteriaceae as well as nonfermenting organisms. Rates of KPC production among clinical isolates of *K pneumoniae* vary worldwide, but staggering numbers have been reported in some regions. For example, in Italy, surveillance data pertaining to *K pneumoniae* bloodstream isolates demonstrated a rise in carbapenem resistance from 1% to 2% in 2006 to 2009, to 30% in 2011.[63] Furthermore, a recent publication from Italy showed a continual climb in rates of carbapenem resistance in *K pneumoniae* bloodstream infections from a rate of 3% in 2009 to 42% in 2011 and to 66% in 2013.[64] Similar rates have been reported in neighboring Greece.[63]

Unfortunately, but perhaps unsurprisingly, immediately following the rise of KPCs worldwide, case reports and series describing clusters and outbreaks of colistin-resistant KPC-producers began to appear in the literature.[65] Additionally, rates of colistin resistance in *Klebsiella* spp from surveillance studies have varied greatly and interpretation of these studies is complicated because many of them do not focus solely on KPC-producing isolates.[65] However, the rates of colistin resistance in *K pneumoniae*, unlike what has been described with other carbapenem-resistant organisms, appear to be increasing at a much higher rate. Surveillance data examining rates of colistin resistance among carbapenem-resistant as well as carbapenem-susceptible *Klebsiella* isolates generally place the rate at $\leq$7%.[65] However, data from Greece from the mid to late 2000s place the rate at 10.5% to 20.0%.[66,67] Additionally, 2 reports, 1 from Austria, and 1 from the Netherlands, showed rates of approximately 50% of colistin resistance in extended-spectrum β-lactamase (ESBL)-producing *Klebsiella*, although these studies were done in the setting of oral colistin administration for selective gut decontamination.[68,69] Most concerning, however, have been reports of extremely high rates of colistin resistance from regions in which KPC-producers have become endemic. Rates of colistin resistance in carbapenemase-producing *Klebsiella* have ranged from 14% to 25% in

Greece.[70–74] In Italy, reported rates have been even higher. Multiple publications have reported colistin resistance exceeding 30% in carbapenem-resistant *K pneumoniae.*[65] One recent study of *Klebsiella* resistance in bloodstream infections in an Italian hospital reported 66% of strains to be carbapenem resistant, and 57% to 65% of those carbapenem-resistant *K pneumoniae* strains were also resistant to colistin.[64] To put these resistant rates in clinical perspective, if a patient was to develop a *Klebsiella* spp bloodstream infection, there would be approximately a 43% chance that it would be both colistin-resistant and carbapenem-resistant. Similarly, data examining 191 carbapenemase-producing Enterobacteriaceae in 21 hospitals in Italy (187 *K pneumoniae*, 4 *E coli*) from November 2013 to April 2014 reported 76 (43%) to also be colistin-resistant.[75]

Although most available data assessing rates of colistin resistance in Gram-negative bacilli represent nonclinical surveillance data, there are a few reports assessing risk factors for isolation of colistin-resistant Gram-negative bacilli. Although polymyxin exposure is frequently identified as a risk factor, this finding is not universal. Qureshi and colleagues[76] described the characteristics of 20 patients with colistin-resistant *A baumannii* isolated from their institution over a 7-year period. Nineteen (95%) of 20 patients had prior genetically related colistin-susceptible isolates and significant prior intravenous and inhaled colistin exposure was present in all but 1 of the 20 patients. Similarly, Papadimitriou-Olivgeris and colleagues[74] described their experience in 254 patients who were not colonized with colistin-resistant KPC-producing isolates on admission to the intensive care unit (ICU). Of the patients, 62 (24.4%) became colonized with colistin-resistant KPC-producing (CRKPC) organisms while in the ICU, with the primary risk factor for isolation being colistin exposure (odds ratio 13.5, 95% confidence interval 6.1–30.2). Other risk factors for isolation of colistin-resistant KPC producers were corticosteroid use and number of CRKPC-positive patients treated in nearby beds per day, suggesting the importance of horizontal transmission as well. Interestingly, Meletis and colleagues[77] evaluated colistin use over time and its association with colistin-resistant Gram-negative bacilli. Colistin use increased significantly over the period of the study from 7 defined daily doses (DDD) per 1000 patient days in 2007 to 27 DDD per 1000 patient days in 2013 and a likewise significant increase in colistin-resistant KPC was seen from 0% in 2007 to 2010, to 16% in 2010 to 2013. This increase was most notable among ICU isolates, where CRKPC was reported in 20 (22%) of 92 isolates. What is most interesting is that although there was a dramatic increase in colistin-resistant KPC over the study period, there was no parallel increase in colistin resistance in carbapenem-resistant *A baumannii* or *P aeruginosa*. Rates of colistin-resistant carbapenem-resistant *A baumannii* were 0% over the entire study period, and rates of colistin resistance in *P aeruginosa* actually decreased from 5% in 2007 to 2010, to 2% in 2010 to 2013. This finding is consistent with the overall data presented in this section that colistin resistance in KPC producers seems to be developing at an alarming rate, whereas colistin resistance rates in the nonfermenters remain relatively low and stable.

These findings are interesting in light of a recent publication by Giani and colleagues[64] in which the investigators described their experience with an outbreak of 93 bloodstream infections with colistin-resistant KPC over a 4-year period, in an area in Italy where KPC is endemic (the investigators report that two-thirds of all *Klebsiella* were carbapenem resistant, and carbapenem resistance was largely mediated by KPC). Data on previous colistin exposure were available for 38 patients, 35 (92%) of whom did not receive colistin before isolation of their colistin-resistant pathogen. Of the 59 patients in whom genotyping was performed, the mgrB gene deletion was present in 50 (85%) of 59 isolates; and in a subset of 19 subjects for whom colistin data were available,

18 (95%) had not had prior colistin exposure. Although the outbreak was initially tied to increased colistin utilization at the institution, the continued spread in the absence of colistin exposure suggested clonal expansion of a single strain (ie, patient-to-patient spread) and also suggested that this particular mechanism may not have been associated with decreased strain fitness of survival. This finding is in line with another report that associates mgrB inactivation with a lack of fitness cost in *A baumannii*.[46] Taken together, these results suggest that colistin-resistant carbapenem-resistant *K pneumoniae* could become more widespread.

In summary, although rates of colistin resistance among carbapenem-resistant *A baumannii*, *P aeruginosa*, and *K pneumoniae* remain relatively low, there are trends emerging that increased polymyxin exposure in institutions for the treatment of these pathogens is leading to the predictable emergence of resistance. Additionally, particularly in *K pneumoniae*, there is mounting evidence that a stable form of resistance is emerging that might be seen in the absence of polymyxin exposure with clonal expansion throughout a given unit or hospital. These findings, when taken together, stress the critical need for optimal strategies for the use of polymyxins, as well as infection control and antimicrobial stewardship programs to preserve these critical, last-line agents. Therefore, the rest of this article focuses on such strategies.

STRATEGIES TO MINIMIZE POLYMYXIN RESISTANCE

As discussed previously, there are two polymyxins currently being used in the clinic: colistin and polymyxin B.[78] Colistin is more widely used and is administered parenterally in the form of an inactive prodrug, the sodium salt of colistin methanesulfonate (CMS, also known as colistimethate).[78] A parenteral formulation of polymyxin B (as its sulfate salt) is available in a number of countries, including the United States, but is not available in Europe, Australia, and several other countries.[9,79] Polymyxin B is administered directly in its active antibacterial form, whereas CMS requires conversion in vivo to generate the active entity, colistin. This difference in the form administered to patients has a major effect on the clinical pharmacologic profile of the 2 polymyxins, an understanding of which is critical to their optimal clinical use.[80]

Because of the lack of new antibiotics and potential for development of resistance with polymyxin monotherapy, it is important that both polymyxins are used optimally to maximize their efficacy and minimize resistance and nephrotoxicity. Unfortunately, as polymyxins were approved for clinical use before the introduction of the contemporary drug development and regulatory approval processes, the prescribing information of both polymyxin products has been limited and not supported by solid pharmacologic data. Fortunately, this situation has been changing over the past decade. Indeed, the polymyxins have been the first of the "old" antibiotics to be subjected to a "redevelopment" process, largely led by academic and clinical researchers. To optimize their dosage regimens, it is essential to understand their pharmacokinetics (PK), pharmacodynamics (PD), and toxicodynamics (TD), and the relationships between exposure and desired/undesired responses (ie, PK/PD and PK/TD).[81–84] There are a number of approaches to minimize resistance development to polymyxins, in particular optimizing their dosage regimens in patients using PK/PD/TD, employment of rational combinations, and limiting clinical use to patients with MDR/XDR Gram-negative infections.

OPTIMIZING DOSING REGIMENS

Currently, there are two different labeling systems in use for parenteral CMS.[78] In Europe, the international unit (IU) is used for CMS, whereas colistin base activity

(CBA) is used in North America, South America, and Southeast Asia. For more information on the conversion between the number of IU and milligrams of CBA, please refer to our reviews and a recent editorial.[78,85,86] One million IU is equivalent to approximately 30 mg of CBA. It is crucial that clinicians are aware of the labeling differences and proper conversions are achieved before implementing at the local level dosage regimens reported in journal articles.[85,86]

Over the past decade, significant preclinical and clinical pharmacologic data have been generated to inform clinicians on optimizing the use of colistin and polymyxin B in patients. The PK/PD index that best predicts the activity of colistin was recently identified as the ratio of the area under the plasma concentration versus time curve across 24 hours to the minimum inhibitory concentration (AUC/MIC). This was first described using an in vitro PK/PD study with colistin against *P aeruginosa*.[82] In vivo studies using murine thigh and lung infection models have confirmed this finding.[81] The data from the recent mouse thigh infection studies, when translated to the clinic after accounting for interspecies differences in plasma protein binding, suggest that the average steady-state plasma colistin concentration ($C_{ss,avg}$) required for good antibacterial effect in a patient corresponds to the MIC of the organism causing the infection.[81] It is important, however, to keep in mind that the risk of nephrotoxicity in patients increases as the plasma colistin concentration increases, especially at concentrations above approximately 2.5 mg/L.[83,84,87] Thus, there is substantial overlap in the plasma concentrations associated with the desired and undesired effects of the drug; it is very clear that colistin is an antibiotic with a very narrow therapeutic window. Because the colistin MIC may not be known at initiation of therapy, a "target" plasma colistin $C_{ss,avg}$ of 2 mg/L would seem appropriate, especially in view of the known link between inadequate initial antibiotic therapy and clinical outcome.[88]

In terms of PK of colistin, as mentioned previously, it is important to note that colistin is used parenterally as the inactive prodrug CMS. Because CMS converts to colistin in vitro and in vivo,[78,89] it was not possible in the past to accurately determine the PK of CMS and formed colistin using microbiological assays. Liquid chromatographic analytical methods made possible the separate measurement of CMS and formed colistin in biological fluids.[90,91] It is evident now that CMS and colistin have very different PK; CMS is eliminated mainly by the kidney, whereas the colistin formed in the body is eliminated via nonrenal pathway(s).[78,89,92] An analysis of patients in the ICU who were given intravenous CMS for treatment of infections caused by Gram-negative bacteria showed that, due to the slow conversion of CMS to colistin, the plasma concentration of formed colistin increased slowly following the first few intravenous doses.[93] In these patients, in whom 3×10^6 IU (ie, ~90 mg CBA) CMS was given every 8 hours, a plasma colistin $C_{ss,avg}$ of 2 mg/L was not reached until after 3 doses or more. In a subsequent study, patients who received a higher first dose (ie, a loading dose) of 6×10^6 IU (~180 mg CBA) achieved the desired bactericidal concentration much faster than those who did not.[94] These findings indicate that a loading dose may contribute to improvement of the clinical outcomes.

The largest study on the population PK of CMS and formed colistin in critically ill patients to date was conducted by Garonzik and colleagues.[95] The study included 105 patients; 89 not receiving renal support and with large variation in creatinine clearance (range 3–169 mL/min/1.73 m^2), 12 on intermittent hemodialysis, and 4 on continuous renal replacement therapy (CRRT). The physician-selected daily intravenous dose among all patients ranged from 75 to 410 mg CBA. The plasma colistin $C_{ss,avg}$ ranged from 0.48 to 9.38 mg/L. The findings in this study highlighted several key points. First, a high daily dose of CMS did not always produce desirable colistin plasma concentrations, because of the influence of renal function on the disposition of CMS and the

fraction of each dose of the prodrug available for conversion to colistin in the body. The plasma concentration of formed colistin was generally lower in patients with good renal function, as CMS was more rapidly cleared by the kidney and only a small fraction of the dose was retained in the body and available for conversion to colistin. In patients with good renal function, it is important to consider active combination therapy with colistin (discussed later in this article), especially if the MIC of the infecting pathogen is near the current susceptibility breakpoint. Second, there was a large degree of interpatient variability in the apparent clearance of formed colistin and, consequently, the plasma colistin $C_{ss,avg}$ achieved from the same daily dose. This large variability was observed even among patients with similar renal function, possibly related to brand-to-brand and batch-to-batch variability across CMS parenteral products in the rate and extent of conversion of CMS to colistin.[6] Third, the findings supported previous PK data from patients on CRRT and intermittent hemodialysis, which indicated that renal replacement therapy has significant impact on the plasma concentration of formed colistin.[96,97] As a result, patients receiving CRRT require daily doses of CMS similar to those used in patients with normal kidney function; and patients on intermittent hemodialysis should be dosed on nondialysis days, as for an anuric patient, but receive a supplemental dose at the end of each dialysis session.[95] In summary, the most important outcome of the largest population PK study was the development of dosing algorithms to calculate the loading and daily maintenance doses of CMS to be administered to patients with various degrees of renal function and in those who require either intermittent or continuous renal supportive therapy.[95]

As noted previously, a major difference between polymyxin B and colistin is that the former is administered as its active form. To date, there is less information known about the clinical pharmacology of polymyxin B. In a study that involved 8 critically ill patients, polymyxin B was infused over 60 minutes with doses ranging from 0.5 to 1.5 mg/kg every 12 to 48 hours. The plasma polymyxin B concentrations were analyzed from blood samples of all patients and urine samples of 4 patients. In this study, the peak plasma concentrations of polymyxin B at the end of the infusion ranged from 2.38 to 13.9 mg/L. Only 0.04% to 0.86% of the dose was recovered as unchanged form in urine.[98] This study showed that, like colistin in rats,[99] polymyxin B is eliminated mainly by nonrenal pathway(s) and the status of the renal function would be expected to have little impact on the total body clearance of polymyxin B. In a more recent study on the population PK of polymyxin B, a total of 24 critically ill patients were included. Two patients received CRRT, whereas the rest exhibited a wide range of creatinine clearance (range 10–143 mL/min). The intravenous doses of polymyxin B administered ranged from 0.45 to 3.38 mg/kg per day. The total body clearance of polymyxin B was very similar among all patients, with the population mean of 0.0276 L/h/kg. Median urinary recovery of polymyxin B was very low at 4.04%. This study confirmed the previous finding from this research group that polymyxin B is largely nonrenally cleared and that the daily dose required to achieve a given average steady-state plasma concentration of polymyxin B is not dependent on renal function. The study also indicated that although a loading dose of polymyxin B is less critical than for CMS, steady-state can be achieved more quickly with the addition of a loading dose.[100]

Due to the different formulations of the parenteral products of colistin and polymyxin B, the 2 products are considered pharmacokinetically as “chalk and cheese” rather than “peas in a pod.”[80] In most clinical applications, polymyxin B would be regarded as having superior clinical pharmacologic properties; for example, it is possible to more quickly and reliably achieve and maintain plasma concentrations that are likely

to be effective across a wide range of renal function. Relatively little pharmacodynamic and clinical work has been published with polymyxin B to date and comparative data between the two agents are lacking.[49] Colistin may be a better option for treatment of urinary tract infection, as CMS is extensively eliminated by the renal pathway and degrades to colistin within the urinary tract. For inhalation, CMS is less irritating than colistin[101] and very likely polymyxin B also, although there appear to be no direct comparisons of CMS and polymyxin B administered by inhalation. It is therefore important to optimize dosage regimens of each drug according to patient characteristics, as these factors will influence their distribution in the body.

Currently, there are limited data available regarding the impact of dosing of polymyxins and the development of polymyxin resistance. An in vitro study that examined the effect of once-daily, twice-daily, and thrice-daily dosing of colistin on the emergence of colistin resistance in *P aeruginosa* suggested the 8-hourly regimen to be the most effective at minimizing emergence of resistance.[102] Similar observations were obtained for another in vitro study that investigated polymyxin B against *P aeruginosa*.[103] These results, however, have not yet been confirmed in clinical studies.

Little information is available regarding the distribution of polymyxins into extravascular sites. In cerebral spinal fluid (CSF), colistin concentrations were found to be relatively low compared with the plasma concentration following intravenous administration of CMS.[104,105] Similar findings were also obtained for colistin concentrations in sputum and bronchoalveolar lavage fluid.[106–108] A combination of intravenous and intraventricular administration of CMS in critically ill patients with central nervous system infection showed an overall higher mean CSF colistin concentration than intravenous or intraventricular administration alone.[105] In cystic fibrosis and mechanically ventilated critically ill patients, inhalational delivery of CMS resulted in significantly higher colistin concentrations in the sputum and epithelial lining fluid, respectively, compared with intravenous administration.[108,109] Based on the current literature, alternative routes of dosing combined with intravenous administration of CMS may be useful for the treatment of extravascular infections.

COMBINATION THERAPY

Based on recent animal PK/PD and clinical PK data, colistin combination therapy is likely to be beneficial in patients infected by a causative pathogen with an MIC greater than 1 mg/L, or in patients with moderate-to-good renal function receiving intravenous CMS.[81,95] Given the high incidence of polymyxin heteroresistance in *K pneumoniae* and *A baumannii,*[40,41] polymyxin combinations may be useful in the prevention of polymyxin resistance development in these pathogens. The presence of a second antibiotic is potentially beneficial, as it may help eliminate the subpopulation that is resistant to the other antibiotic.[110] Additionally, when two antibiotics are used, they may target different cellular pathways that can lead to overall enhanced antimicrobial activity.[110] It has also been proposed that, as polymyxins disrupt the outer membrane of Gram-negative bacteria, they can promote the entry of other antibiotics into the Gram-negative bacterial cells.[111] Unfortunately, many polymyxin combinations used in the clinic have been chosen empirically. Such an approach does not take into consideration the rationalities of antibiotic combinations discussed previously. A more systematic and rational approach to the choice of a secondary antibiotic to use in the combination with polymyxins should include consideration of the following: the effect of the second antibiotic on the polymyxin-resistant subpopulation and vice versa; whether the target for the second antibiotic is intracellular; and the changes in global bacterial response to the combination treatment.

The synergistic activity of antibiotic combination therapy is often assessed in vitro with fractional inhibitory concentration (FIC) index and E-test methods.[112,113] These methods, however, only provide information regarding the activity for a single time point; therefore, they are not very informative and the results can be variable. The more desirable in vitro methods for the assessment of antibiotic combination therapy are static concentration or dynamic (ie, fluctuating concentrations to mimic dosage regimens in patients) killing kinetics assays; these assays are more useful than the FIC and E-test methods, as they examine the antimicrobial activity over time.[112,113]

A number of different antibiotics have been investigated for their combinations with polymyxins; however, the most common combinations are with carbapenems and rifampicin. A systematic review and meta-analysis of polymyxin combinations with carbapenems showed that in in vitro time-kill studies synergism occurred for *P aeruginosa*, *A baumannii*, and *K pneumoniae*; however, it occurred more frequently against *A baumannii* (143 [77%] of 186 isolates) than *P aeruginosa* (68 [50%] of 136 isolates) and *K pneumoniae* (64 [44%] of 146 isolates).[114] For all 3 species, polymyxins combined with doripenem produced the highest synergy. For *A baumannii*, the combination of polymyxins with meropenem was more active than with imipenem, whereas against *P aeruginosa* the converse was the case. In addition to enhanced initial bacterial killing, the combinations also suppressed the development of resistance to polymyxins.[114] For combinations of polymyxins with rifampicin, time-kill studies showed synergy against 14 (100%) of 14 carbapenemase-producing *K pneumoniae* isolates,[115] and 160 (57%) of 280 *A baumannii* isolates.[113] In a mouse pneumonia model with multidrug-resistant *P aeruginosa*, a combination of colistin (intranasal) and rifampicin (oral) provided maximum survival protection compared with either drug alone.[116]

Apart from the enhanced killing, in vitro studies have also demonstrated that the combinations of polymyxins and carbapenems or rifampicin successfully suppressed the emergence of polymyxin resistance. Suppression of polymyxin resistance development was observed for colistin combined with doripenem against *P aeruginosa*,[117] including biofilm-embedded MDR *P aeruginosa*,[118] and *K pneumoniae*.[119] The combination of colistin and rifampicin has been shown to suppress the development of polymyxin resistance in *A baumannii*.[120]

Although the results from preclinical studies are promising, the potential benefit of polymyxin combinations in patients remains unclear. Many reports relating to polymyxin combinations describe observational studies, usually involving small numbers of patients and being nonrandomized. For example, in a clinical study, the benefit of combining colistin with another antibiotic was evaluated in 70 patients with ventilator-associated pneumonia.[121] Of the total number of patients, 17 patients were administered intravenous colistin alone, 20 patients were administered intravenous colistin and sulbactam, and 33 patients were administered intravenous colistin and a carbapenem. The clinical and microbiological responses from the investigation showed no significant difference statistically ($P>.05$), although both responses were higher in the carbapenem combination group.[121] In a recent analysis conducted on all clinical studies that compared colistin monotherapy with colistin-based combination therapy for the treatment of carbapenem-resistant Gram-negative bacteria, the findings indicated that both colistin alone and colistin/carbapenem combination produced similar outcome.[122] The investigators of the analysis, however, indicated that there are potential sources of bias in the original studies, including selection bias (different criteria required for the selection of polymyxin monotherapy or combination therapy), small study size (does not permit adjustment for other risk factors), potentially suboptimal dosing strategies, and the appropriateness of the initial empirical antibiotic treatment.

Regarding the potential benefit of polymyxin and rifampicin combinations in patients, a recent multicenter randomized controlled trial (RCT) was conducted comparing the clinical outcome of colistin and rifampicin with colistin alone in 210 patients with serious infections due to extensively drug-resistant *A baumannii*.[123] In this study, the patients were randomly allocated (1:1) to either CMS alone (2 MIU [~60 mg CBA] every 8 hours intravenously), or CMS (at the same dose specified) plus rifampicin (600 mg intravenously every 12 hours). The primary end point of the study was overall 30-day mortality and the secondary end points were infection-related death, microbiologic eradication, and length of hospitalization. The results showed a significant increase in microbiologic eradication rate for the colistin/rifampicin combination group; however, no difference was observed for infection-related death and length of hospitalization.[123] The multicenter RCT conducted by Durante-Mangoni and colleagues[123] also included polymyxin resistance emergence as one of its secondary outcome measures, but no difference between the combination and monotherapy groups was found. In a single-center RCT, the benefit of using colistin combined with rifampicin over colistin alone was evaluated in 43 patients with ventilator-associated pneumonia.[124] In this study, 22 patients were administered only colistin intravenously (300 mg CBA [~10 MIU] per day), and the other 21 patients were administered colistin intravenously (at the same dose) combined with rifampicin (600 mg/day) nasogastrically. Similar to the multicenter RCT,[123] the time to microbiological clearance was significantly shorter in the combination group. The findings also showed that clinical, laboratory, radiological, and microbiological response rates were better in the combination group; however, they were not statistically significant (P>.05). At present, the available clinical data do not support the combination of colistin and rifampicin because of the lack of improved clinical outcomes with the combination therapy. Some degree of synergy between colistin and fosfomycin against *A baumannii* and *P aeruginosa* isolates, including those resistant to carbapenems, has been observed in in vitro studies, most typically using the checkerboard technique for determination of FIC index.[125–127] However, the role of this combination in the clinic remains unclear. A recent preliminary open-label RCT compared this combination against colistin alone in 94 patients (47 in each group) infected with carbapenem-resistant *A baumannii*; approximately 75% of patients in both groups had ventilator-associated pneumonia.[126,128] There was a significantly more favorable microbiological response for the colistin-fosfomycin group. However, favorable clinical outcomes, mortality at the end of study treatment, and mortality at 28 days were not significantly different, nor was there a significant difference in survival time between the patients who received combination therapy and monotherapy.

As noted previously, multiple factors may have contributed to the lack of significant benefit observed with polymyxin combinations in clinical studies. In addition, as previously discussed, colistin is administered in the clinic as CMS, thus often leads to suboptimal plasma exposure at initiation of therapy and during the treatment course. To rapidly achieve a desirable plasma colistin $C_{ss,avg}$ (ie, 2 mg/L), a loading dose is usually required. Most clinical studies that have compared colistin combination therapy with colistin monotherapy did not include a loading dose and many studies involved administration of daily maintenance doses that were most likely suboptimal. To understand the real benefit of polymyxin combination treatments, future RCTs should include a loading dose and/or use higher daily maintenance doses to achieve optimal plasma polymyxin concentrations throughout the treatment course. Furthermore, several clinical studies were underpowered and/or suffered from the ethical constraints involved in conducting RCTs in critically ill patients. With regard to the latter, in most studies, patients in both the polymyxin monotherapy and combination

groups received multiple other antibiotics in addition to the index second antibiotic under consideration.[123] Because of the last-line status of the polymyxins, in many studies it is likely (but usually not reported) that the time from diagnosis of infection to the initiation of polymyxin in the polymyxin monotherapy and combination groups differed, very likely favoring the monotherapy group. Most studies have had multiple endpoints, but many have neglected to identify the potential benefit of polymyxin combinations to prevent the development of polymyxin resistance. Future studies should also be appropriately designed to evaluate the emergence of polymyxin resistance following combination and monotherapy. Although the clinical benefit of polymyxin combinations remains unproven, it may be beneficial to use polymyxin combination therapy considering the polymyxin-associated nephrotoxicity and PK/PD considerations as reviewed previously. Well-designed and appropriately powered RCTs are required to examine the potential advantage of polymyxin combination therapy versus polymyxin monotherapy. Currently, 2 such RCTs of the colistin and carbapenem combination are being conducted in Europe (NCT01732250) and the United States (NCT01597973). These studies are expected to be completed in approximately September 2016 for Europe and September 2017 for the United States.

PREVENTION OF POLYMYXIN RESISTANCE

Infection Control

Polymyxin MIC testing is typically performed only for XDR pathogens, and thus, most identified polymyxin-resistant pathogens are XDR and, as a result, patients often have already been placed in enhanced infection control precautions. As the polymyxins are a "last-line" therapeutic option, polymyxin-resistant XDR pathogens represent an urgent threat, and an outbreak could lead to temporary closure of a hospital ward or floor. Patients colonized with polymyxin-resistant MDR or XDR pathogens should be managed as an infection-control emergency and serious efforts should be made to prevent hospital spread of these pathogens.

In addition to standard precautions (eg, hand hygiene), enhanced infection control precautions for patients colonized with polymyxin-resistant MDR pathogens often involve contact precautions (ie, use of gowns and gloves and dedicated medical equipment, such as stethoscopes) and placement of a patient in a private room.[127,129] Extrapolating from experience in controlling CRE,[130] cohorting patients colonized with polymyxin-resistant MDR pathogens and when hospital resources permit, cohorting health care workers caring for those patients (so that certain health care workers care for colonized and/or infected patients only) are warranted in outbreaks or in hyperendemic settings. Active surveillance screening (eg, of rectal swabs for CRE), coupled with contact precautions, has been useful in containing MDR Gram-negative pathogens, including CRE,[131] and could be used in a similar way to identify patients asymptomatically colonized with polymyxin-resistant MDR pathogens. Chlorhexidine bathing of patients has also been reported to be effective in reducing risk for spread of MDR pathogens.[131,132] Prevention bundles used to effectively control CRE have included active surveillance, contact precautions, chlorhexidine bathing, and cohorting of patients. A similar bundle of strategies would likely be effective in preventing the spread of polymyxin-resistant Gram-negative pathogens.[131–133]

Antimicrobial Stewardship

Antimicrobial stewardship strategies are an important component of prevention strategies to limit the emergence of antimicrobial resistance among Gram-negative

bacteria.[134] Although avoidance of polymyxin use whenever possible will likely help to prevent the emergence and spread of polymyxin resistance, timely and appropriate use can have a positive impact on clinical outcomes. In some instances, when patients are at increased risk for infection due to XDR-GNB pathogens, empiric polymyxin use is warranted. Certain patient characteristics, such as a prior history of XDR-GNB infection or admission from a long-term acute-care center where XDR-GNB pathogens are common, in addition to assessment of level of acute severity of illness, can help to identify patients who have increased risk for life-threatening XDR-GNB infection, and who might be appropriate candidates for empiric polymyxin therapy. Using formal clinical scores to identify patients at high risk for infection due to an XDR pathogen and who is an appropriate candidate for empiric polymyxin therapy have, unfortunately, not been shown to be accurate or effective.[134] As an alternative to empiric polymyxin therapy, rapid diagnostics can be used to more quickly identify XDR-GNB pathogens and more rapidly implement polymyxin therapy.

Additionally, negative results from rapid diagnostic tests can be used to quickly discontinue polymyxins. If polymyxins are empirically prescribed, then rapid de-escalation should be practiced whenever possible to limit unnecessary polymyxin use. De-escalation is modification of empiric therapy (when appropriate) based on a patient's clinical status and available culture results.[135] Typically, de-escalation occurs at approximately day 3 of antimicrobial therapy. If patients have microbiologic data indicating that an XDR pathogen is not present, then more often than not, polymyxin therapy can be stopped. De-escalation can help to limit unnecessary polymyxin use and prevent emergence of polymyxin resistance. If a full course of polymyxins is needed to treat an infection, then the duration of therapy should be monitored and the shortest effective duration should be prescribed. Careful attention to the "day of polymyxin therapy," and to the patient's clinical response to therapy, can help to minimize the duration of therapy whenever possible, to avoid unnecessarily long polymyxin courses and to prevent the emergence of polymyxin resistance.

Finally, as polymyxins represent a last-line therapeutic option, and resistance to these agents will in many cases leave clinicians with no viable treatment alternatives, the use of polymyxins for selective gut decontamination strategies for ESBL-producing organisms or other Gram-negative pathogens, should be avoided. Multiple analyses looking at ESBL gut decontamination strategies with colistin showed both a failure to eradicate the ESBL-producing pathogens and even more concerning, an astounding rise in the rate of colistin resistance from essentially zero to greater than 50%.[68,69]

SUMMARY

Polymyxin resistance is a major public health threat, as the polymyxins represent "last-line" therapeutics for Gram-negative pathogens resistant to essentially all other antibiotics. Improved understanding of mechanisms of, and risk factors for, polymyxin resistance, as well as infection prevention and stewardship strategies, together with optimization of dosing of polymyxins including in combination regimens, can help to limit the emergence and dissemination of polymyxin resistance.

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ORIGINAL ARTICLE

Anthelmintic closantel enhances bacterial killing of polymyxin B against multidrug-resistant *Acinetobacter baumannii*

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Polymyxins, an old class of antibiotics, are currently used as the last resort for the treatment of multidrug-resistant (MDR) *Acinetobacter baumannii*. However, recent pharmacokinetic and pharmacodynamic data indicate that monotherapy can lead to the development of resistance. Novel approaches are urgently needed to preserve and improve the efficacy of this last-line class of antibiotics. This study examined the antimicrobial activity of novel combination of polymyxin B with anthelmintic closantel against *A. baumannii*. Closantel monotherapy (16 mg l^{-1}) was ineffective against most tested *A. baumannii* isolates. However, closantel at 4–16 mg l^{-1} with a clinically achievable concentration of polymyxin B (2 mg l^{-1}) successfully inhibited the development of polymyxin resistance in polymyxin-susceptible isolates, and provided synergistic killing against polymyxin-resistant isolates (MIC $\geqslant$ 4 mg l^{-1}). Our findings suggest that the combination of polymyxin B with closantel could be potentially useful for the treatment of MDR, including polymyxin-resistant, *A. baumannii* infections. The repositioning of non-antibiotic drugs to treat bacterial infections may significantly expedite discovery of new treatment options for bacterial 'superbugs'.
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INTRODUCTION

The past two decades has seen a substantial increase in Gram-negative 'superbugs' resistant to almost all clinically available antibiotics.[1] This dire situation is exacerbated by a lack of novel antibiotics in the drug discovery pipeline, leaving the world in a vulnerable state against these life-threatening bacteria.[1] 'Old' polymyxin class of antibiotics, polymyxin B and E (the latter also known as colistin), are now used as a last line of defense against Gram-negative 'superbugs'.[2] Of these pathogens, *Acinetobacter baumannii* is one of the most problematic, causing a range of infections in the nosocomial setting and in injured military personnel.[3] Although polymyxins largely remain effective against problematic Gram-negative bacteria such as *A. baumannii*, recent pharmacokinetic and pharmacodynamic data on polymyxins suggest that caution is required with monotherapy due to emergence of resistance.[4,5] Worryingly, there have been increasing reports of infections caused by *A. baumannii* which are resistant to all available antibiotics, including polymyxins.[6,7] The emergence of polymyxin-resistant *A. baumannii* highlights the urgent need to investigate novel approaches for maintaining and improving the clinical efficacy of polymyxins.

The use of synergistic combinations of non-antibiotic drugs with antibiotics is emerging as a potentially valuable and cost-effective approach to improve the clinical efficacy of currently available antibiotics against problematic multidrug-resistant (MDR) bacterial pathogens.[8] The aim of the present study was to investigate bacterial killing and the rapid emergence of polymyxin resistance in *A. baumannii* using clinically relevant concentrations of polymyxin B in combination with the non-antibiotic closantel.

MATERIALS AND METHODS

Bacterial strains and MIC measurements

Eight strains of *A. baumannii* representing a mixture of polymyxin-susceptible (that is, MIC $\leqslant$ 2 mg l^{-1}) and polymyxin-resistant (that is, MIC $\geqslant$ 4 mg l^{-1}) strains, including MDR strains, were employed in this study (Table 1). Of the four polymyxin-susceptible isolates, FADDI-AB009 and 2949 were polymyxin heteroresistant; polymyxin heteroresistance was defined as a polymyxin-susceptible isolate (that is, MIC $\leqslant$ 2 mg l^{-1}) with subpopulations able to grow in the presence of $>$2 mg l^{-1} polymyxin B or colistin.[9] *A. baumannii* ATCC 19606 was purchased from the American Type Culture Collection (Rockville, MD, USA) and the polymyxin-resistant variant FADDI-AB065 was from a previous study;[10] polymyxin resistance of FADDI-AB065 is conferred by complete loss of lipopolysaccharide (LPS) from the outer membrane.[10] FADDI-AB009 was provided by The Alfred Hospital (Melbourne, Australia) and its polymyxin-resistant variant FADDI-AB085 was produced by plating onto Mueller-Hinton agar (Oxoid, Adelaide, Australia) containing 10 mg l^{-1} of colistin sulfate (Sigma-Aldrich, Castle Hill, Australia). In addition,

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Table 1 MICs for polymyxin B and closantel against the *A. baumannii* strains examined in this study

			MICs ($mg\,l^{-1}$)		
Strain	*MDR*[a]	*Polymyxin susceptibility*[b]	*Polymyxin B*	*Closantel*	*Closantel in the presence of 2 $mg\,l^{-1}$ polymyxin B*[c]
ATCC 19606	No	S	0.5	>128	NP[d]
FADDI-AB009[e]	No	S (HR)	0.5	>128	NP
2382	Yes	S	0.5	>128	NP
2949[e]	Yes	S (HR)	1	>128	NP
FADDI-AB065	No	R	128	0.5	0.5
FADDI-AB085	No	R	32	0.5	0.5
2384	Yes	R	8	>128	1
2949A	Yes	R	64	>128	2

[a]Multidrug resistance (MDR) was defined as non-susceptible to $\geqslant 1$ treating agent in $\geqslant 3$ antimicrobial categories.[12]
[b]CLSI breakpoints (S, susceptible; R, resistant): Polymyxin B, S $\leqslant 2\,mg\,l^{-1}$, R $\geqslant 4\,mg\,l^{-1}$; breakpoints are not available for closantel.
[c]Closantel MICs in the presence of $2\,mg\,l^{-1}$ of polymyxin B.
[d]Not performed (NP) for polymyxin-susceptible isolates.
[e]Polymyxin heteroresistant (HR). Heteroresistance to polymyxins was defined as the existence, in an isolate for which the polymyxin B or colistin MIC was $\leqslant 2\,mg\,l^{-1}$, of subpopulations able to grow in the presence of $>2\,mg\,l^{-1}$ polymyxin B or colistin.[9]

two pairs of polymyxin-susceptible and -resistant isolates were obtained from two patients at the University of Pittsburgh Medical Center before (susceptible) and following (resistant) colistin treatment: 2382 vs 2384 and 2949 vs 2949A.[11] Polymyxin resistance in isolates 2384 and 2949A is conferred by the modifications of lipid A.[11] All four isolates from the University of Pittsburgh Medical Center are MDR (defined as non-susceptible to $\geqslant 1$ treating agent in $\geqslant 3$ antimicrobial categories).[12]

MICs to polymyxin B (Sigma-Aldrich, Castle Hill, NSW, Australia; Batch number BCBD1065V) and closantel (Sigma-Aldrich; Batch number SZBC320XV) were determined for all isolates in three replicates on separate days using broth microdilution in cation-adjusted Mueller-Hinton broth (CAMHB; Ca^{2+} at $23.0\,mg\,l^{-1}$ and Mg^{2+} at $12.2\,mg\,l^{-1}$; Oxoid, Hampshire, UK).[13] Stock solutions of polymyxin B and closantel were prepared immediately before each experiment. Polymyxin B was dissolved in Milli-Q water (Millipore, North Ryde, Australia) and sterilized by passage through a 0.20-μm cellulose acetate syringe filter (Millipore, Bedford, MA, USA). Closantel was first dissolved in dimethyl sulfoxide (Sigma-Aldrich) and then in Milli-Q water to make 10% (v/v). The solution was further serially diluted in filter-sterilized Milli-Q water to the desired final concentration; preliminary studies demonstrated the final concentration of dimethyl sulfoxide (2.5%, v/v) to which the bacteria were exposed had no effect on their growth. All assays were performed in 96-well microtiter plates (Techno Plas, St Marys, SA, Australia) in CAMHB with a bacterial inoculum of $\sim 5\times10^5$ c.f.u. ml^{-1}. Plates were incubated at 37 °C for 20 h. MICs were determined as the lowest concentrations that inhibited the visible growth of the bacteria. For polymyxin-resistant isolates, MICs of closantel in the presence of $2\,mg\,l^{-1}$ of polymyxin B were also determined (that is, polymyxin B at the specified concentrations was added to each well of the 96-well plate).

Baseline polymyxin population analysis profiles

The possible existence of polymyxin-resistant subpopulations at baseline ($t=0$ h) was determined with population analysis profiles (PAPs) as described previously.[14] In brief, bacterial cell suspensions (50 μl) of $\sim 10^8$ c.f.u. ml^{-1} were appropriately diluted with 0.9% saline and plated onto Mueller-Hinton agar plates (Media Preparation Unit, University of Melbourne, Parkville, Australia) containing polymyxin B (0, 0.5, 1, 2, 4 and $8\,mg\,l^{-1}$) using an automatic spiral plater (WASP, Don Whitley Scientific, West Yorkshire, UK). Colonies were counted after 24-h incubation at 37 °C using a ProtoCOL colony counter (Synbiosis, Cambridge, UK).

Static time-kill studies

Time-kill studies with polymyxin B and closantel alone, and in combination, were conducted. For monotherapy, polymyxin B was used at $2\,mg\,l^{-1}$ and closantel at $16\,mg\,l^{-1}$. Three polymyxin B/closantel combinations were investigated using polymyxin B at $2\,mg\,l^{-1}$ combined with closantel at 2, 4 or $16\,mg\,l^{-1}$ (dimethyl sulfoxide at 2.5% (v/v) was used for all treatments). Before each experiment, isolates were subcultured onto nutrient agar plates (Media Preparation Unit) and incubated overnight at 35 °C. One colony was then selected and grown overnight in 20 ml CAMHB at 37 °C; from this colony an early log-phase culture was obtained. Each drug was added alone or in combination to 20 ml of a log-phase broth culture of $\sim 5\times10^5$ c.f.u. ml^{-1} to yield the desired concentrations. Each 20-ml culture was placed in a sterile 50-ml polypropylene tube (Greiner Bio-One, Frickenhausen, Germany) containing 20 ml of CAMHB and incubated in a shaking water bath at 37 °C (shaking speed, 150 r.p.m. min^{-1}). Serial samples (0.5 ml) were removed aseptically at 0, 0.5, 1, 2, 4, 6 and 24 h for viable-cell counting; the samples were appropriately diluted in 0.9% saline and 50 μl of the resultant bacterial cell suspension was spirally plated onto nutrient agar. In order to examine the rapid emergence of polymyxin-resistant subpopulations, samples at 24 h were additionally plated onto Mueller-Hinton agar containing polymyxin B at 4 $mg\,l^{-1}$. Enumeration was performed after 24 h of incubation as described above. Microbiological responses of combination therapy relative to monotherapy were examined descriptively and via the log change method, that is, comparing the change in Log_{10} c.f.u. ml^{-1} from 0 h (c.f.u.$_0$) to time t (4 and 24 h; c.f.u.$_t$) as shown: log change = Log_{10}(c.f.u.$_t$) − Log_{10}(c.f.u.$_0$). Synergy was defined as $\geqslant 2$ Log_{10} c.f.u. ml^{-1} killing for the combination relative to the most active corresponding monotherapy at a specified time.[15]

Quantification of antibacterial activity

The antibacterial activity of polymyxin B and closantel, both individually and in combination, was quantified using a recently reported empirical modeling approach[16] which characterizes the rate of bacterial killing in addition to the suppression of bacterial regrowth. An empirical model (Equation 1) was fitted to the time-kill experimental data and estimates were obtained for the parameters A, B, C, K_d and K_r that describe the magnitude of bacterial killing, magnitude of bacterial regrowth, time delay of bacterial regrowth and the rates of bacterial killing and regrowth, respectively.

$$\mathrm{Log}_{10}\left(\frac{\text{c.f.u.}}{\text{ml}}\right) = A\cdot e^{-K_d\cdot t} + \frac{B}{1+e^{-K_r\cdot(t-C)}} \quad (1)$$

Estimation was performed by non-linear regression using the solver add-in in Microsoft Excel and the parameter estimates were subsequently used to calculate a model-derived time to 2 Log_{10} killing (T2LK; Equation 2) and time to 3 Log_{10} regrowth (T3LR; Equation 3). The T2LK was used as a measure of bacterial killing, whereas the T3LR was used as a measure of the suppression of bacterial regrowth. T3LR was constrained to <24 h to account for the duration

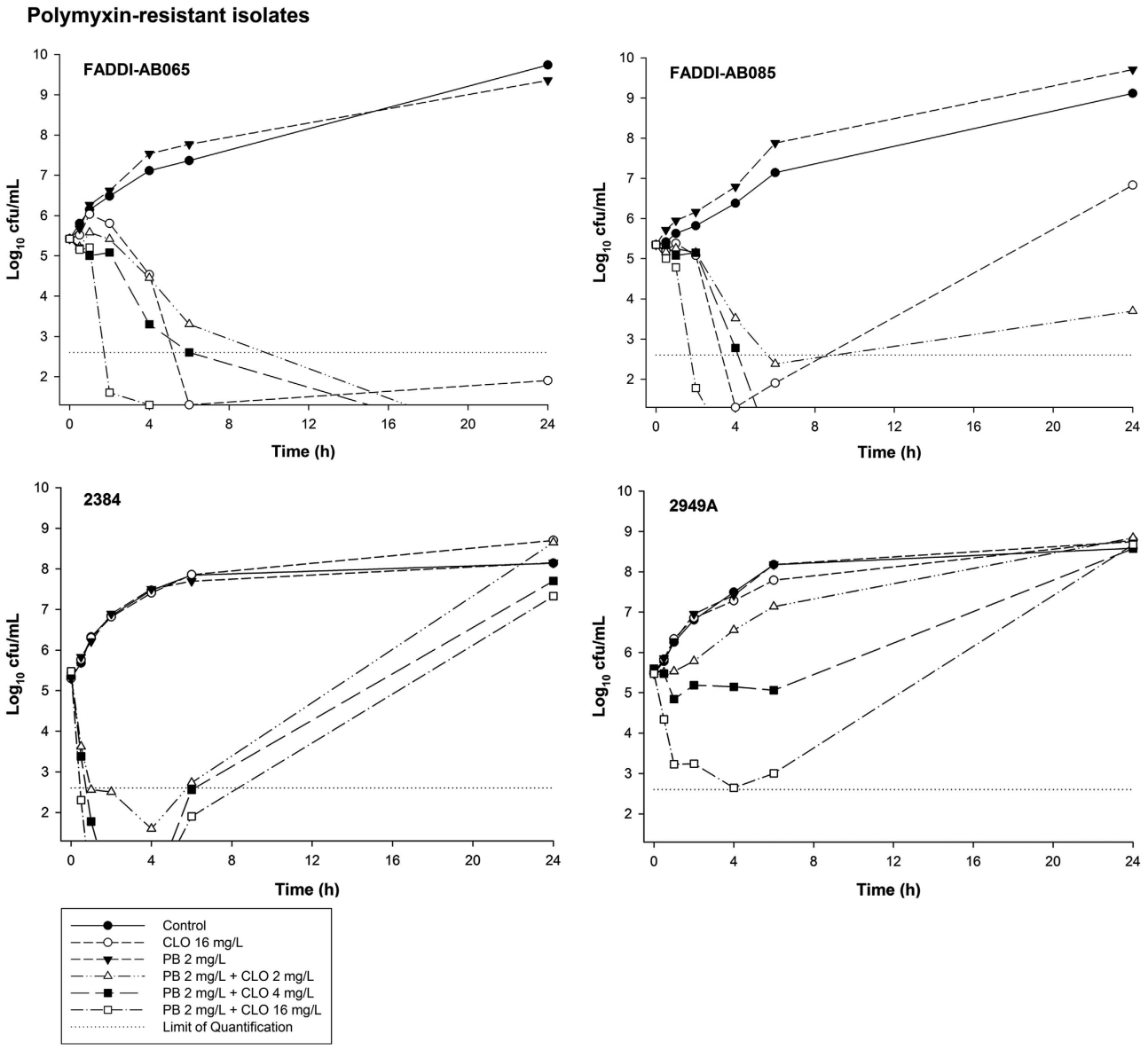

Figure 1 Time-kill curves for polymyxin B (PB) and closantel (CLO) monotherapy and combination therapy against polymyxin-resistant *A. baumannii* isolates FADDI-AB065, FADDI-AB085, 2384 and 2949A. The *y* axis starts from the limit of detection and the limit of quantification is indicated by the horizontal dotted line.

of the time-kill study.

$$t_{x-\mathrm{Log}_{10}\mathrm{kill}} = -\frac{1}{K_\mathrm{d}} \cdot \ln\left(1 - \frac{x_{\mathrm{Log}_{10}\mathrm{killing}}}{A}\right) \quad (2)$$

$$t_{x-\mathrm{Log}_{10}\mathrm{regrowth}} = C + \frac{1}{K_\mathrm{r}} \cdot \ln\left(\frac{x_{\mathrm{Log}_{10}\mathrm{regrowth}}}{B - x_{\mathrm{Log}_{10}\mathrm{regrowth}}}\right) \quad (3)$$

RESULTS

MICs and PAPs

MICs of each drug alone plus MICs to closantel in the presence of polymyxin B (2 mg l^{-1}), as well as results for baseline PAPs, are shown in Table 1. Closantel alone was inactive (MIC > 128 mg l^{-1}) against the majority of isolates. However, an MIC of closantel of 0.5 mg l^{-1} was achieved against two polymyxin-resistant strains (FADDI-AB065 and FADDI-AB085); for these two strains, closantel MICs were unaffected by the addition of polymyxin B (2 mg l^{-1}). The addition of polymyxin B substantially reduced closantel MICs in the two remaining polymyxin-resistant isolates (2384 and 2949A; Table 1). The varying susceptibility to polymyxin B of subpopulations within the polymyxin-susceptible isolates before polymyxin B treatment was evident in the PAPs. Two isolates (2949 and FADDI-AB009) considered susceptible based upon polymyxin B MIC results were heteroresistant, containing subpopulations able to grow in the presence of > 2 mg l^{-1} polymyxin B (Table 1). For the polymyxin-resistant isolates, virtually the entire bacterial population was highly resistant to polymyxin B and grew in the presence of 8 mg l^{-1} polymyxin B.

Table 2 Proportion of polymyxin-resistant subpopulations of examined isolates before and after 24-h treatment with polymyxin B (PB) alone, closantel (CLO) alone, and polymyxin B plus closantel

	Proportion of polymyxin B-resistant subpopulations able to grow on agar supplemented with 4 mg l^{-1} polymyxin B						
		After 24 h treatment					
A. baumannii *strains*	*Baseline*	*Control*	*PB 2.0 mg l^{-1}*	*CLO 16 mg l^{-1}*	*PB 2.0 mg l^{-1} + CLO 2.0 mg l^{-1}*	*PB 2.0 mg l^{-1} + CLO 4.0 mg l^{-1}*	*PB 2.0 mg l^{-1} + CLO 16 mg l^{-1}*
Polymyxin susceptible							
ATCC 19606	ND[a]	3.33×10^{-8}	ND	ND	ND	NG[b]	NG
FADDI-AB009[c]	5.00×10^{-7}	5.00×10^{-6}	NG	1.00×10^{-6}	NG	NG	NG
2382	ND	ND	NG	ND	NG	NG	NG
2949[c]	3.33×10^{-5}	1.67×10^{-5}	9.17×10^{-1}	4.17×10^{-6}	5.91×10^{-3}	NG	NG
Polymyxin resistant							
FADDI-AB065	8.96×10^{-1}	7.46×10^{-1}	1.86	1.00	NG	NG	NG
FADDI-AB085	1.52	1.29	2.14	1.12×10^{-2}	1.77	NG	NG
2384	4.75×10^{-1}	2.90×10^{-1}	1.97×10^{-1}	5.95×10^{-1}	4.82×10^{-1}	4.89×10^{-1}	2.55×10^{-2}
2949A	1.01	1.74	1.62	1.38	1.13	1.42	1.31

[a]No polymyxin-resistant subpopulations detected (ND).
[b]No growth detected after 24 h (NG).
[c]Polymyxin B heteroresistant isolates.

Time-kill studies and rapid emergence of polymyxin resistance

Time-kill profiles for polymyxin B and closantel monotherapy and combination therapy against polymyxin-resistant isolates are shown in Figure 1. Against the closantel-susceptible isolates FADDI-AB065 and FADDI-AB085, polymyxin B monotherapy (2 mg l^{-1}) resulted in no bacterial killing, whereas closantel monotherapy (16 mg l^{-1}) resulted in rapid killing between 2 and 4 h (T2LK: 178 and 113 min for FADDI-AB065 and FADDI-AB085, respectively). Minimal regrowth was observed at 24 h for FADDI-AB065 (<2 Log$_{10}$ c.f.u. ml^{-1}), although substantial regrowth occurred for FADDI-AB085 (>6 Log$_{10}$ c.f.u. ml^{-1}, T3LR = 19.3 h; Figure 1). Despite subsequent regrowth at 24 h, the polymyxin-resistant subpopulations of FADDI-AB085 treated with closantel monotherapy (16 mg l^{-1}) were ~1:100 compared with control, treatment with polymyxin B monotherapy (2 mg l^{-1}) and treatment with polymyxin B/closantel 2 mg l^{-1} combination (Table 2). Against the remaining polymyxin-resistant isolates 2384 and 2949A, no bacterial killing was observed with either polymyxin B or closantel monotherapy, with growth mirroring that of the controls over 24 h (Figure 1). Combination therapy of polymyxin B and closantel was highly effective against isolates FADDI-AB065 and FADDI-AB085. For FADDI-AB065, all combinations of polymyxin B and closantel resulted in complete inhibition, with no viable colonies detected at 24 h. For FADDI-AB085, complete inhibition was achieved with combinations of polymyxin B and closantel at concentration 4 and 16 mg l^{-1}. Against the isolates 2384 and 2949A, even though regrowth was at or close to control values by 24 h with all polymyxin B/closantel combinations, rapid and extensive bacterial killing was observed soon after the commencement of the combination therapy. Against isolate 2949A, polymyxin B plus closantel at 16 mg l^{-1} was synergistic at 4 h (T2LK: 80.7 min), with an additional ~4.5 Log$_{10}$ kill compared with polymyxin B monotherapy observed with the highest closantel concentration (Figure 1). For isolates 2384, rapid and extensive bacterial killing was observed with all polymyxin B/closantel combinations with a minimum of ~5 Log$_{10}$ greater killing compared with monotherapy at 4 h (T2LK: 46.7, 20.1 and 11.7 min for polymyxin B 2 mg l^{-1} plus closantel 2, 4 and 16 mg l^{-1}, respectively; Figure 1). Within 2 h of initiation of therapy, no viable bacteria were detected with the polymyxin B/closantel (4 and 16 mg l^{-1}) combinations; the killing at 4 h in these cases was ~7.5 Log$_{10}$ more than with equivalent monotherapy.

Time-kill profiles for polymyxin B and closantel monotherapy and combination therapy against polymyxin-susceptible isolates are shown in Figure 2. The proportions of polymyxin-resistant isolates before and after 24 h of treatment with each regimen are shown in Table 2. Against all polymyxin-susceptible isolates, polymyxin B monotherapy (2 mg l^{-1}) resulted in rapid bacterial killing to below the limit of detection within 0.5–1 h, with no viable colonies detected up to 6 h. For FADDI-AB009 and 2382, no regrowth was observed at 24 h. However, regrowth occurred at 24 h with the remaining two isolates (Figure 2). For heteroresistant isolate 2949, the proportion of polymyxin-resistant subpopulations markedly increased at 24 h following polymyxin B monotherapy, with virtually the entire population able to grow on Mueller-Hinton agar containing 4 mg l^{-1} polymyxin B (Table 2); the substantial bacterial killing observed at this time with all other susceptible isolates precludes meaningful comparison of polymyxin-susceptible and -resistant subpopulations. For isolates ATCC 19606 and 2949 (the isolates where regrowth at 24 h was observed), the addition of closantel at 4 and 16 mg l^{-1} to polymyxin B was synergistic at 24 h, preventing regrowth despite closantel having no discernible antibacterial activity as monotherapy against any polymyxin-susceptible isolate (that is, growth with closantel monotherapy was essentially indistinguishable from that of the control). Regrowth similar to that which occurred with polymyxin B monotherapy was observed with the polymyxin B/closantel 2 mg l^{-1} combination against isolates ATCC 19606 and 2949. However, with this combination the rapid emergence of polymyxin-resistant subpopulations was ~100 times lower than polymyxin B monotherapy for isolate 2949 (Table 2). Antimicrobial activity for the combination of polymyxin B and closantel against polymyxin-susceptible isolates, quantified by the model-derived T2LK, did not differ significantly compared with polymyxin B alone (mean ± s.d.: 11.5 ± 2.60 vs 10.5 ± 0.73 min, $P = 0.47$). Notably, against isolate 2949, the bacterial regrowth was markedly suppressed following combination therapy

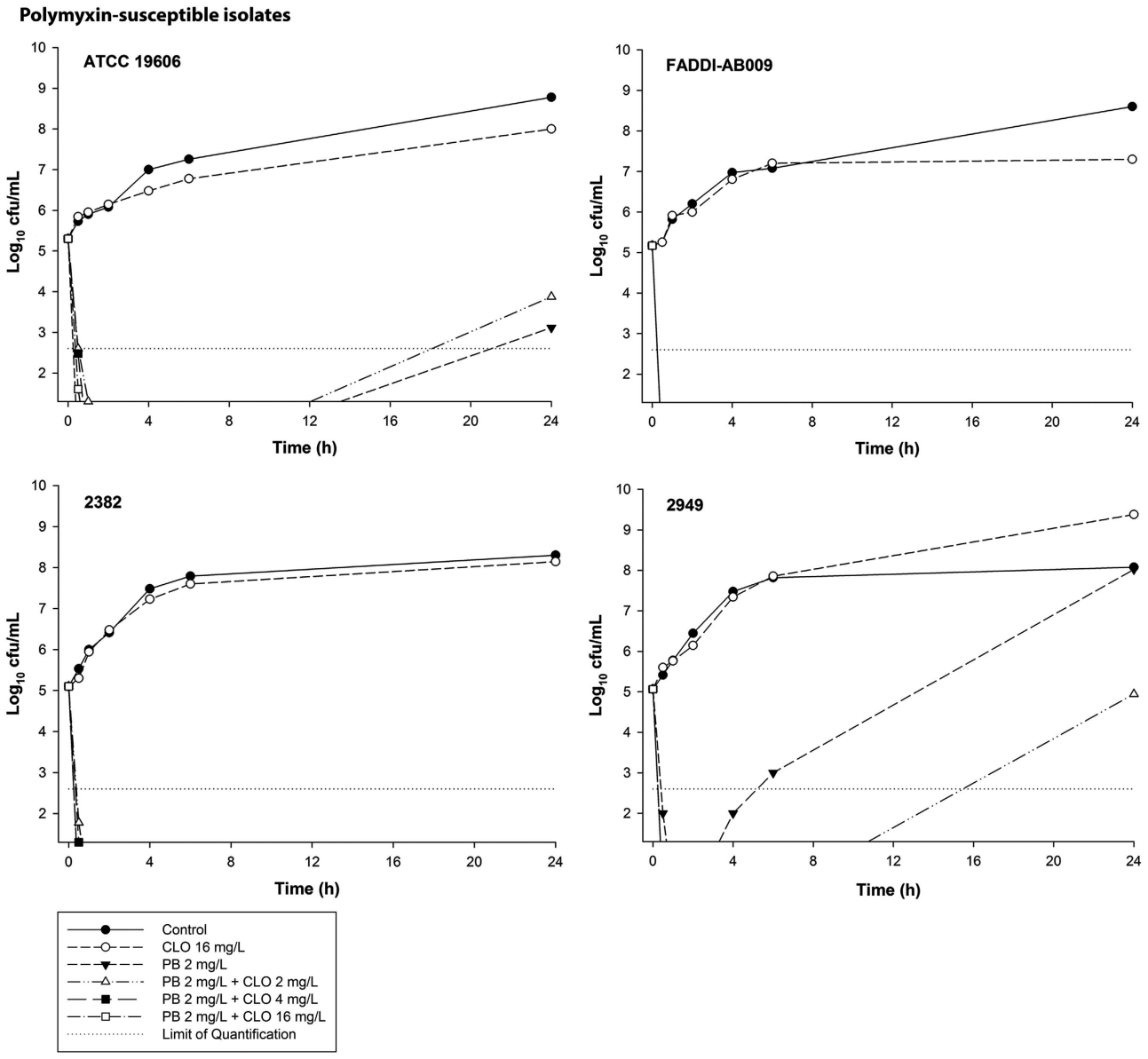

Figure 2 Time-kill curves for polymyxin B (PB) and closantel (CLO) monotherapy and combination therapy against polymyxin-susceptible *A. baumannii* isolates ATCC 19606, FADDI-AB009, 2382 and 2949. The *y* axis starts from the limit of detection and the limit of quantification is indicated by the horizontal dotted line. All isolates start at a similar initial inoculum. For combinations with CLO 2 mg l^{-1} (FADDI-AB009 and 2382) and 4 and 16 mg l^{-1} (all isolates), regrowth (if present) is below the limit of detection.

with closantel (2, 4 and 16 mg l^{-1}) compared with polymyxin B alone (T3LR: >22 h vs 6.08 h).

DISCUSSION

Infections caused by MDR *A. baumannii* are increasing globally and are already a major burden on the public health-care system.[17–19] Although polymyxins are increasingly used as a last-line therapy against this very problematic Gram-negative pathogen,[20,21] reports of polymyxin-resistant MDR *A. baumannii* are increasing.[22] In addition, emerging pharmacokinetic and pharmacodynamic data for polymyxins suggest caution with polymyxin monotherapy due to the presence of polymyxin heteroresistant isolates.[23,24] Consequently, novel treatment strategies that optimize bacterial killing and minimize the emergence of polymyxin resistance are urgently required.[25]

In the present study, we evaluated the *in vitro* efficacy of the combination of polymyxin B with the non-antibiotic closantel against a range of clinical isolates (including MDR isolates) of *A. baumannii* with various susceptibilities to polymyxin B (Table 1). Closantel is a veterinary anthelmintic drug with activity against multiple nematode species.[26] The anthelmintic activity of closantel involves the uncoupling of oxidative phosphorylation and inhibition of chitinase.[27,28] Our study is the first to demonstrate the synergistic antibacterial activity between polymyxins and closantel against MDR *A. baumannii*. The repositioning of veterinary drugs has been successful for drug discoveries for humans. An example is ivermectin,[29] a drug that is currently being used to treat river blindness in human but was initially developed for veterinary use. Currently, the pharmacokinetics of closantel is unavailable in humans; hence, multiple concentrations of closantel (2, 4 and 16 mg l^{-1}) were employed based on its

pharmacokinetics in animals[30,31] and to ensure an appropriate concentration range was covered. The concentration of polymyxin B ($2\,mg\,l^{-1}$) employed in this study is clinically achievable as demonstrated by pharmacokinetic studies in critically ill patients.[32,33]

For *A. baumannii*, regrowth with polymyxin monotherapy (polymyxin B or colistin) is driven in part by the amplification of polymyxin-resistant subpopulations.[23,24] Such regrowth was similarly observed here in two of four polymyxin-susceptible isolates (Figure 2). This finding again illustrates that caution is required for treatment of *A. baumannii* infections with polymyxin monotherapy. For the polymyxin-resistant isolates, rapid and marked improvements in bacterial killing were observed with all three combinations against isolates 2384, and with the combination of polymyxin B/closantel $16\,mg\,l^{-1}$ against 2949A. These improvements occurred despite the virtual absence of bacterial killing with each monotherapy. For example, against isolate 2384 improvements in bacterial killing of >5 Log_{10} c.f.u. ml^{-1} compared with each monotherapy were observed within 1 h of the commencement of treatment with the combination containing $4\,mg\,l^{-1}$ closantel. Despite subsequent regrowth, such rapid and extensive initial killing by an antibiotic/non-antibiotic combination against isolates highly resistant to each drug is an important finding. The rapid and extensive reduction in the bacterial load at the commencement of therapy may facilitate clearance of bacteria by the immune system of the host. Interestingly, closantel showed antibacterial activity as monotherapy against FADDI-AB065 and FADDI-AB085, but even then the combinations with all concentrations of closantel (2, 4 and $16\,mg\,l^{-1}$) demonstrated superiority through better regrowth suppression after 24 h. The addition of closantel to polymyxin B had no effect on initial bacterial killing of polymyxin-susceptible isolates due to extensive bacterial killing by polymyxin B alone (Figure 2). However, the additional closantel at 4 or $16\,mg\,l^{-1}$ did suppress the regrowth observed with polymyxin B monotherapy against ATCC 19606 and 2949 (Figure 2). These findings merit further research given increasing reports of polymyxin resistance[34–38] and a diminishing arsenal of effective antibiotics.[39–41]

Similar to previous reports,[42–44] our current study shows that the MIC results did not completely mirror that of the results from the time-kill studies (Table 1; and Figure 1). For isolates 2384 and 2949A, closantel MICs were 1 and $2\,mg\,l^{-1}$, respectively, in the presence of $2\,mg\,l^{-1}$ of polymyxin B (Table 1). However, in the time-kill studies, regrowth was observed for both isolates with $16\,mg\,l^{-1}$ of closantel in the presence of $2\,mg\,l^{-1}$ of polymyxin B (Figure 1). As MICs are obtained after 20-h incubation via visual observation for turbidity and viable counting using agar plates is not part of the MIC measurement, the MIC results do not necessarily indicate lack of viable cells (for example, in the 24-h time-kill studies).

The antibacterial mechanism of closantel is unclear. However, closantel has been shown to exhibit antimicrobial activity against Gram-positive bacteria *in vitro*[45,46] and against *Staphylococcus aureus* in a *Caenorhabditis elegans* infection model.[47] For Gram-negative bacteria, the unique structure of the cell envelope creates a permeability barrier to hydrophobic compounds such as closantel (logP 7.2). LPS, the principal component of the external leaflet of the Gram-negative outer membrane, is the initial binding target of polymyxins via electrostatic interaction of the cationic L-α,γ-diaminobutyric acid (Dab) side chains present on polymyxins with the negatively charged phosphate groups of the lipid A component of LPS.[48] Binding displaces the divalent cations (Ca^{2+} and Mg^{2+}) that bridge adjacent LPS molecules, disorganizing the outer membrane and increasing its permeability.[49] Although it was originally proposed that bacterial killing by the polymyxins resulted from permeabilisation of the bacterial outer membrane and subsequent leakage of cell contents, the precise mechanism(s) by which polymyxins ultimately kill bacterial cells is still unknown and several alternative mechanisms of action have been reported.[50–53] A previous study has demonstrated polymyxin resistance in isolates 2384 and 2949A is conferred by the modifications of lipid A with cationic galactosamine.[11] It is apparent that this outer membrane modification on its own did not lead to enhanced penetration of closantel as the MIC for both isolates was $>128\,mg\,l^{-1}$ and closantel monotherapy produced no bacterial killing. However, the enhanced bacterial killing observed when combined with polymyxin B suggests sufficient permeabilisation of the outer membrane by the polymyxin to allow closantel to enter into the cell and exert an antibacterial effect. Complete loss of LPS in *A. baumannii* is also known to confer polymyxin resistance, although such resistance comes at the cost of rendering the outer membrane more permeable to hydrophobic compounds that would otherwise be unable to enter the bacterial cell.[10] This may explain the antibacterial activity of closantel in its own right (closantel MICs of $0.5\,mg\,l^{-1}$) against strains FADDI-AB065 (which is LPS deficient) and FADDI-AB085. This would also be consistent with the previously reported antibacterial activity of closantel against Gram-positive species that do not possess LPS.[45,46]

CONCLUSIONS

In an era of declining antibiotic discovery and rapidly emerging antibiotic resistance, novel treatment strategies for MDR Gram-negative organisms such as *A. baumannii* are urgently needed. The off-label use of non-antibiotic drugs for antibacterial purposes in combination with existing antibiotics is a currently underexplored area with significant potential to expedite discovery of new treatment options for infections caused by MDR pathogens. The findings from the present study demonstrate that the 'unexpected' combination of polymyxin B with an anthelmintic, closantel, may substantially increase the antibacterial activity against MDR, including polymyxin-resistant, *A. baumannii*. Further investigations in animal infection models are required for translation into the clinic.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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