The impact of systemic inflammation and bacterial infection on the blood-brain barrier transport of colistin

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ERRATA

Page vii. line 9: "LPS-INDEUCED" should read "LPS-INDUCED".

Page xi. paragraph 1, line 8: "BB" should read "BBB".

Page xiii. paragraph 1, line 6: "mice colistin" should read "mice treated with colistin".

Page 11. paragraph 1, line 1: "have been performed since long time ago" should read "have been performed since 1970".

Page 105. paragraph 1, line 7: "This assay was then utilized to determine the amount of colistin that permeated the blood-brain over a 2 hour..." should read "This assay was then utilized to determine the brain concentration of colistin over a 2 hour...".

Page 243. paragraph 1, line 1: "we observed increased BBB permeability was observed" should read "increased BBB permeability was observed".

ADDENDUM

Page 146. Figure 3-3B: the fill of the bars representing saline-treated mice (the left bar at each time point) should be black.

This thesis is dedicated to my parents, Jian Wen Jin and Yun Jie Du;

and to my loving wife, Zhao Hua Zheng.

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Abstract

The spread of multidrug-resistant (MDR) infections caused by Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae*, has led to increasing use of colistin, an old antibiotic from the polymyxin family. Colistin (also known as polymyxin E) is a cationic polypeptide antibiotic whose clinical use waned in the 1970s due to concerns related to its neurotoxicity. Though this toxicity may have been exaggerated in the past due to a lack of understanding of colistin pharmacology and the use of inappropriate doses, it has nonetheless attracted the attention of clinicians and scientists. Whether this neurotoxicity is centrally or peripherally mediated remains unclear, however, if colistin were to exert any centrally mediated toxicity following systemic administration, it would require transport across the blood-brain barrier (BBB). Therefore, this thesis investigates the brain uptake of colistin under healthy conditions, in addition to assessing the impact of systemic inflammation and bacterial infection on the brain uptake of this polypeptide antibiotic.

In order to undertake this project, it was necessary to develop a liquid chromatography method to quantify colistin concentrations in mouse brain homogenate. Our method consisted of protein precipitation of mouse brain homogenates with trichloroacetic acid (TCA), solid-phase extraction (SPE) of colistin, and derivatization with fluorenylmethyloxycarbonyl chloride (FMOC-Cl), followed by liquid chromatography with fluorescence detection. A linear correlation between peak area and colistin concentration was observed over the concentration range from 93.8 to 3,000 ng/g in brain tissue ($R^2 > 0.994$). Intra- and inter-day coefficients of variation were 5.1 to 8.3% and 5.8 to 8.5%, respectively,

suggesting the method was reliable and sensitive enough to quantify the amount of colistin that had penetrated the BBB following systemic administration to mice.

Following a single intravenous dose of colistin sulfate at 5 mg/kg in healthy Swiss outbred mice, brain-to-plasma (B:P) ratios of colistin were very low ranging from 0.03 to 0.06. To determine whether higher plasma concentrations would lead to higher brain uptake of colistin, colistin sulfate (40 mg/kg) was administered subcutaneously to Swiss outbred mice as single and multiple doses. The brain uptake of colistin with these dosing regimens was still low with average B:P ratios of 0.03 and 0.02. To assess whether P-glycoprotein (P-gp) at the BBB was responsible for the limited brain uptake of colistin, colistin sulfate (5 mg/kg) was intravenously co-administered with P-gp inhibitors (i.e. PSC833 or GF120918) (10 mg/kg). However, the BBB transport of colistin was not significantly enhanced in the presence of these inhibitors, suggesting that the negligible BBB transport of colistin in healthy mice was not a result of the P-gp transporter function at the BBB.

Given colistin is not administered to healthy patients, but rather to patients with infections, it was decided to investigate whether the brain uptake of colistin altered during disease state. Systemic inflammation was induced in mice by three intraperitoneal injections of lipopolysaccharide (LPS; *Salmonella enterica* 3 mg/kg). Colistin concentration in brain homogenate was measured following subcutaneous administration of colistin sulfate and following transcardiac perfusion of colistin sulfate using a modified *in situ* brain perfusion technique. Significantly increased brain uptake of colistin was observed in LPS-treated animals compared with that in saline-treated animals. Following subcutaneous administration of colistin sulfate, the value of the area under the brain colistin concentration versus time

curve (AUC_{brain}) from 0 to 4 h was 11.7 ± 2.7 μg·h/g and 4.0 ± 0.3 μg·h/g in LPS- and saline-treated mice, even though plasma exposure of colistin was no different between these two treatments. Similarly, *in situ* perfusion of colistin led to higher colistin brain concentrations in LPS-treated animals than in saline-treated animals, with colistin brain-to-perfusate concentration ratios of 0.019 ± 0.001 and 0.014 ± 0.001, respectively. The B:P ratio of a BBB integrity marker (i.e. ^{14}C-sucrose) was also 2-fold higher in LPS-treated mice following a single intravenous dose (2 μCi), suggesting the enhanced brain uptake of colistin during systemic inflammation was likely due to disruption of the BBB paracellular route.

To further assess the impact of systemic bacterial infection on BBB integrity and colistin brain uptake, bacteremia was established 8 h after intramuscular administration of *Pseudomonas aeruginosa* to mice, at which time a single intravenous dose of ^{14}C-sucrose (2 μCi) or subcutaneous dose of colistin (40 mg/kg) was administered. Plasma levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) were also measured to assess whether there was any relationship between the degree of BBB disruption and plasma cytokine levels. Despite a substantial elevation of these three cytokines in plasma, the brain uptake of colistin was low with average B:P ratios ranging from 0.012 to 0.044 and the B:P ratio of ^{14}C-sucrose was no different between infected and non-infected mice, suggesting a lack of effect of bacteremia on BBB integrity. The results demonstrated that the brain uptake of colistin was not increased during *P. aeruginosa*-induced systemic bacteremia and there did not appear to be a direct relationship between BBB disruption and the plasma levels of three pro-inflammatory cytokines.

The above findings suggested that there might be some LPS-specific effects on the BBB, given that LPS from *S. enterica* induced BBB disruption but bacterial infection with *P. aeruginosa* had no effect. To address this, the brain uptake of ^{14}C-sucrose or colistin was assessed in mice treated with LPS from *P. aeruginosa* using the same dosing regimen as LPS from *S. enterica*. No difference was found between *P. aeruginosa* LPS- or saline-treated mice regarding to the B:P ratios of colistin or ^{14}C-sucrose ($p < 0.05$), confirming the possibility of LPS-dependent effects on BBB disruption. To further clarify this, immortalized human brain capillary endothelial cells (hCMEC/D3) were treated with LPS from *P. aeruginosa* or *S. enterica* at pathologically-relevant concentrations of 3.75 to 30 µg/mL. The claudin-5 or β-actin bands were visualized following western blotting and the ratios of claudin-5: β-actin were then quantified using Image J analysis software. LPS (*S. enterica*) was found to significantly decrease the claudin-5 expression by approximately 33.3% at a relative low concentration (i.e. 7.5 µg/mL) ($p < 0.05$) while LPS (*P. aeruginosa*) was found to affect the claudin-5 expression only at the highest experimental concentration (i.e. 30 µg/mL). These results indicated that BBB disruption induced by LPS is species and dose-dependent, suggesting that different bacterial infections may induce varying disruptive effects on the BBB.

In addition to its bactericidal effect, colistin has also been shown to bind to bacterial LPS and prevent the pathological effects of bacterial endotoxin in the circulation. Hence, it was hypothesized that colistin could have the potential to reverse the LPS-induced BBB disruption observed in earlier studies from this thesis. In hCMEC/D3 cells treated with LPS (*S. enterica*) and colistin simultaneously, the claudin-5:β-actin ratio was 1.09 ± 0.07, which was not different from that of medium-treated cells ($p > 0.05$). The results suggested that

colistin was able to ameliorate the disrupting effect of LPS on claudin-5 expression, potentially the BBB integrity. To confirm whether any reversal effect of colistin observed *in vitro* was reflective of the *in vivo* setting, the brain uptake of ^{14}C-sucrose was measured in mice treated with LPS (*S. enterica*) in the presence and absence of colistin sulfate. While LPS caused an 1.3-fold increase in brain uptake of 14C-sucrose, the B:P ratio of ^{14}C-sucrose in mice treated with colistin and *S. enterica* LPS simultaneously was 0.026 $\pm$ 0.001. These results suggested that the disruptive effect of LPS on the BBB can be rescued by co-administration of colistin.

Overall, this body of work demonstrates that (1) the brain uptake of colistin following systemic administration is low in healthy conditions regardless of the plasma concentration and the functional activity of P-gp; (2) the brain uptake of colistin following systemic administration is significantly increased during systemic inflammation due to disruption of the BBB paracellular route, however, but not in the presence of *P. aeruginosa* infection likely due to bacterial species-dependent effects on the BBB; (3) the BBB-disrupting effect of LPS is dose- and species-dependent; (4) colistin is able to ameliorate BBB disruption induced by LPS from *S. enterica.*

This thesis provided significant insight into the mechanisms associated with the BBB transport of colistin. Important findings about BBB transport of colistin under disease state and potential mechanisms of BBB disruption during systemic inflammation induced by different bacterial species have been provided.

Statement of originality

In accordance with Monash University Doctorate Regulation 17/Doctor of Philosophy and Master of Philosophy (MPhil) regulations, the following declarations are made:

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 3 original papers published in peer reviewed journals and 1 unpublished paper. The core theme of the thesis is "The impact of systemic inflammation and bacterial infection on the blood-brain barrier transport of colistin". The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences under the supervision of Joseph A. Nicolazzo, Roger L. Nation and Jian Li.

Thesis chapter	Publication title	Publication status*	Nature and extent of candidate's contribution
2	Brain penetration of colistin in mice assessed by a novel high-performance liquid chromatographic technique	Published	70%
3	The impact of P-glycoprotein inhibition and lipopolysaccharide administration on the blood-brain barrier transport of colistin in mice	Published	70%
4	The Effect of Systemic Infection Induced by Pseudomonas aeruginosa on the Brain Uptake of Colistin in Mice	Published	70%
5	Species-dependent lipopolysaccharide-induced blood-brain barrier disruption: amelioration by colistin *in vitro* and *in vivo*	In preparation	70%

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

Liang Jin [redacted] 06/08/12

August, 2012

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Last but not least, I would like to express my endless gratitude to my parents, Jianwen Jin and Yunjie Du. The love and encouragement they give day after day are sincerely appreciated and treasured.

Publications

This thesis is a completion of the following manuscripts:

Chapter 2: Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. Brain Penetration of Colistin in Mice Assessed by a Novel High-Performance Liquid Chromatographic Technique. Antimicrob. Agents Chemother. 2009, 53: 4247-4251.

Chapter 3: Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. Impact of P-glycoprotein inhibition and lipopolysaccharide administration on blood-brain barrier transport of colistin in mice. Antimicrob. Agents Chemother. 2011, 55: 502-507.

Chapter 4: Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. The Effect of Systemic Infection Induced by Pseudomonas aeruginosa on the Brain Uptake of Colistin in Mice. Antimicrob. Agents Chemother. 2012 Jul 30.

Chapter 5: Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. Species-dependent Lipopolysaccharide-induced Blood-brain Barrier Disruption: Amelioration by Colistin *in vitro* and *in vivo*. (manuscript in preparation)

Conference abstracts

Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. 2009. Brain penetration of colistin in mice assessed by a novel HPLC technique. Australasian Pharmaceutical Science Association conference, Hobart, Tasmania, Australia (Poster presentation).

Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. 2010. Transport of colistin across the healthy and inflamed mouse blood-brain barrier. American Association of Pharmaceutical Scientists conference, New Orleans, Louisiana, USA (Poster presentation).

Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. 2010. Transport of colistin across the healthy and inflamed mouse blood-brain barrier. 8th Globalization of Pharmaceutics Education conference, North Carolina, USA (Oral presentation). (selected as faculty representative)

Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. 2011. Transport of colistin across the blood-brain barrier during systemic inflammation and bacterial infection. The Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists conference, Perth, western Australia, Australia (Poster presentation).

Glossary of abbreviations

ABC	ATP binding cassette
AJ	adherens junctions
ANOVA	analysis of variance
ATP	adenosine-5’-triphosphate
ATCC	American Type Culture Collection
AUC	area under the concentration-time curve
BBB	blood-brain barrier
BCEC	brain capillary endothelial cells
BCRP	breast cancer resistance protein
BCSFB	blood-cerebrospinal fluid barrier
BSA	bovine serum albumin
B:P	brain-to-plasma ratio
CaMHB	cation-adjusted Mueller-Hinton broth
cfu	colony forming units
CMS	sodium colistin methanesulfonate
CNS	central nervous system
CP	choroid plexus
CSF	cerebrospinal fluid
Dab	diaminobutyric
EAE	experimental autoimmune encephalomyelitis

ELISA	enzyme-linked immunosorbent assay
FMOC	fluorenylmethyloxycarbonyl chloride
g	gram
×g	relative centrifugal force
GLUT	glucose transporter
h	hour
H_2O_2	hydrogen peroxide
HBD	hydrogen bond donor
hCMEC/D3	human brain capillary endothelial cell line
HIV	human immunodeficiency virus
HPLC	high-performance liquid chromatography
IFN	interferon
IL-1β	interleukin-1β
IL-6	interleukin-6
i.p.	intraperitoneal
i.v.	intravenous
JAM	junctional adhesion molecules
kg	kilogram
L	litre
LC-MS	liquid chromatography–mass spectrometry
Leu	leucine
LogP	logarithm of the octanol-water partition co-efficient

LPS	lipopolysaccharide
MDR	multidrug-resistance
mg	milligram
min	minute
mL	millilitre
MLCK	myosin light chain kinase
MMP	matrix metalloproteinase
MRP	multidrug resistance-associated protein
MS	multiple sclerosis
M_w	molecular weight
µg	microgram
µL	microlitre
NF-kB	nuclear factor-kappa B
OCTN	organic cation/carnitine transporter
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
PBS	phosphate buffered saline
PD	pharmacodynamics
P-gp	P-glycoprotein
Phe	phenylalanine
PK	pharmacokinetics
pKa	acid dissociation constant

PSA	polar surface area
ROS	reactive oxygen species
rpm	revolutions per minute
s.c.	subcutaneous
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
SLC	solute carrier
TEER	transendothelial electrical resistance
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
Thr	threonine
TJ	tight junction
TLR4	toll-like receptor 4
TNF	tumor necrosis factor
VAP	ventilator-associated pneumonia
VE-cadherin	vascular endothelium cadherin
v/v	volume in volume
w/v	weight in volume
ZO	zonula occludens

Chapter One

General Introduction

General Introduction

1.1 Background

The spread of multidrug-resistant (MDR) Gram-negative bacteria within the last decade presents a critical problem to physicians attempting to treat infections, in particular those caused by *Pseudomonas aeruginosa, Acinetobacter baumannii,* and *Klebsiella pneumoniae* (166). A marked decline in the discovery and development of novel antimicrobials for such infections has dramatically narrowed the available therapeutic options and has led to the reappraisal of the old polymyxin antibiotics – colistin (also known as polymyxin E) and polymyxin B (178). For the bacterial strains mentioned above, colistin is often times the only active antibiotic currently available and is increasingly used as a last-line therapy (150).

Colistin, produced by a species of bacteria known as *Bacillus polymyxa* subspecies colistinus, is a cationic polypeptide antibiotic from the polymyxin family (149). Introduced in the 1950s, two different forms of this antibiotic are commercially available, namely colistin sulfate and sodium colistin methanesulfonate (CMS, also known as colistimethate sodium), which is the inactive pro-drug of colistin (147). The usage of colistin has waned since the 1970s due to concerns related to its adverse effects and was replaced by other antibiotics such as aminoglycosides (244). Since the ongoing increase in MDR amongst Gram-negative bacteria has rekindled the use of colistin, it has become necessary to obtain a better understanding of its pharmacokinetics (PK) and pharmacodynamics (PD) as well as the toxicities. One of

the adverse events associated with colistin treatment is neurotoxicity (216); however, it is unclear whether this side effect is centrally or peripherally mediated. If the neurotoxicity induced by colistin is indeed centrally mediated, colistin would be required to cross the blood-brain barrier (BBB) following administration of this antibiotic or its inactive pro-drug CMS.

The BBB is formed by specialized endothelial cells of cerebral microvessels (1). Unlike those in other organs, the endothelial cells in the central nervous system (CNS) exhibit unique properties such as tight junctions to seal the paracelluar route, preventing the movement of molecules between the cells under normal conditions. However, modified BBB permeability and functions are observed during disease states including inflammation and infection (110). Alterations in the BBB may therefore affect the subsequent brain uptake of systemically administered agents like colistin, which is used to treat infections, potentially leading to the neurotoxicity observed.

The objective of the following review is to provide a general understanding of colistin and the BBB. An overview of colistin chemical structure, physicochemical properties, mechanism of action, pharmacokinetic properties and adverse effects and the BBB structure under normal and disease states will be provided, with particular emphasis on alterations to the BBB during systemic inflammation and infection.

1.2. Colistin

1.2.1 Chemical structure

The general chemical structure of colistin consists of a tri-peptide side chain attached to a cyclic hepta-peptide ring (Fig. 1-1) (66). The polypeptide structure is composed of a mixture of D- and L-amino acids with the tri-peptide side chain at the N-terminus acylated to a fatty acid side chain. Due to the difference in the structure of the amino acids and fatty acids, at least thirty colistin components have been discovered. The two major components that have been identified are colistin A (polymyxin E_1) and colistin B (polymyxin E_2), which account for more than 85% of the total composition of colistin (146). The only difference between colistin A and colistin B lies in the composition of the fatty acid side chain (i.e. 6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B). The proportion of colistin A and colistin B present in commercially available products varies between pharmaceutical companies and batches (149), which can often time complicate dosing regimens and therapeutic outcome.

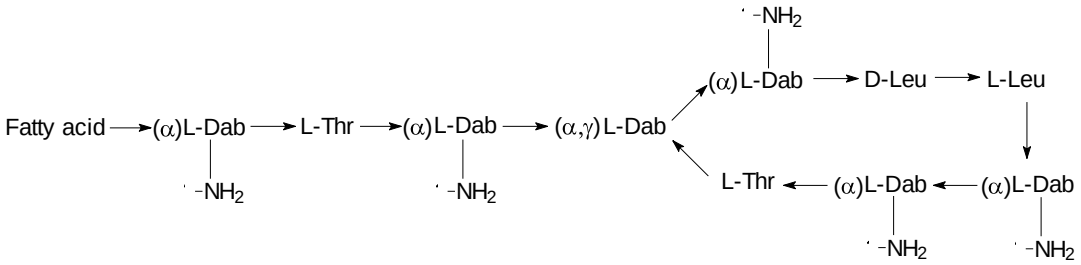


Figure 1-1. Structure of colistin A and B. Fatty acid: 6-methyloctanoic acid for colistin A and 6-methylheptanoic acid for colistin B. Amino acids: Leu: leucine, Dab: α,γ-diaminobutyric acid, Thr: threonine.

1.2.2 Physicochemical properties

The molecular weight (M_W) of colistin A and colistin B are 1,169 g/mol and 1,155 g/mol, respectively (146). Colistin exhibits amphipathic characteristics (149). The hydrophobicity of colistin results from the fatty acid chain and the hydrophobic D-Leu-L-Leu amino-acid region of the hepta-peptide ring, whereas the five free γ-amino groups bound onto the diaminobutyric acid residues give colistin its basic properties generating a pKa of approximately 10 (135). As a result of this characteristic, colistin can distribute efficiently into both polar and non-polar environments in the body such as water and lipid membranes (264). The octanol-water partition co-efficient LogP (a measure of drug hydrophobicity) values for colistin A and B, predicted using the program ACD/PhysChem Suite Version 9 (Toronto, Ontario, Canada), are -3.15 and -3.68, respectively. This physicochemical property demonstrates that colistin is predominately hydrophilic in nature even though it has some hydrophobic characteristics. The primary γ-amino groups in the structure are protonated at physiological pH conveying the cationic character to colistin, which is important for interaction with not only Gram-negative bacteria but also biological membranes (32). The polar surface area (PSA) of colistin is 490 $Å^2$ predicted by the program MolConverter Version 5.10.0 (ChemAxon, Budapest, Hungary). The PSA is defined as the area of a molecule associated with oxygen and nitrogen atoms and the attached hydrogen atoms (191) and a higher PSA value generally means the more hydrophilic nature of the compound. Therefore, the high PSA value suggests that colistin exhibits a hydrophilic character. Moreover, number of hydrogen bonding

donors (HBD) and acceptors (HBA) are another two crucial physicochemical parameters and the calculated values of HBD and HBA for colistin are both 18.

Generally, passive diffusion of large Mw hydrophilic compounds across the BBB is very poor (194). By comparison of physicochemical properties between colistin and other compounds with high BBB permeability in Section 1.3.2.4, it can be predicted that the transport of colistin at the intact BBB will be minimal. Plasma protein binding, which is another property that can affect the BBB transport of colistin, will be described in Section 1.2.5.

Colistin sulfate, one of the commercial forms of colistin, is a white powder and freely soluble in water, slightly soluble in alcohol and almost insoluble in acetone and ether. The stability of colistin sulfate at a concentration of 100 μg/mL at 4 and 37°C is for up to 60 days and 120 h, respectively (145). Degradation was observed when colistin was stored in isotonic phosphate buffer (0.067 M, pH 7.4) and human plasma at 37°C. However, when stored at -80°C, colistin sulfate showed a substantially better stability in plasma and urine for 6 to 8 months (59, 83). The stability studies of colistin sulfate had important implications for its clinical formulation.

CMS is another commercial form of colistin and is produced by the reaction of colistin with formaldehyde and sodium bisulfate (27). CMS is the form available for parenteral administration. When parenterally administered to animals and patients,

CMS undergoes hydrolysis to form colistin as well as other partially sulfomethylated derivatives (147, 151). It was demonstrated in *in vitro* time-kill studies that bacterial killing with CMS was not evident until the concentration of formed colistin was approximately 0.5 to 1 times the minimum inhibitory concentration (MIC) for colistin, indicating that CMS is an inactive prodrug of colistin (29). To avoid the potential confounding factors such as conversion of CMS to colistin in the circulation, this PhD project mainly focuses on the BBB transport of colistin after administration of colistin sulfate. Therefore, this introduction does not review the details for CMS; however, more information on CMS can be found in other reviews (22, 66).

1.2.3 Mechanism of antibacterial action

It is thought that, in order to exert its antibacterial action, colistin directly interacts with its primary target, the lipid A component of the lipopolysaccharide (LPS) (details in Section 1.3.2.7.1) at the Gram-negative bacterial outer membrane (OM) (169). As mentioned in Section 1.2.2, colistin exhibits cationic characteristics at physiological pH, which enables it to interact with the anionic LPS molecules of the Gram-negative bacterial outer membrane via electrostatic interactions (281). This interaction results in the competitive displacement of divalent cations magnesium (Mg^{2+}) and calcium (Ca^{2+}) from the negatively charged phosphate groups of the OM (88). The displacement of the cations brings the hydrophobic domains of the colistin molecule into proximity with the OM, which then leads to the insertion of the fatty acyl chain and D-Leu-L-Leu amino-acid region to weaken the packing of adjacent lipid A fatty

acyl chains and cause the expansion of the OM monolayer (208, 257). It should be noted that both the initial electrostatic interaction and the later insertion of hydrophobic regions are important for the antibacterial action of colistin (257). Following insertion and disruption of the OM, it has been proposed that colistin interacts with the inner membrane (IM) of the Gram-negative bacteria and cause transient pores and lead to disruption of the phospholipid bilayer of the IM (42). The permeabilizing actions may consequently result in cell content leakage and cell death; however, the ultimate bactericidal effect of colistin still requires further investigation. Nevertheless, it is very clear that amphipathic character (hydrophilicity generated by the cationic charge together with hydrophobicity via the fatty acid tail and the D-Leu-L-Leu amino-acid region) is a key requirement for interaction with the OM and ultimately antimicrobial activity (208).

In addition to antibacterial action, colistin has a potent anti-endotoxin activity as it has a high affinity for the endotoxin of Gram-negative bacteria, which is the lipid A portion of LPS molecules (172). Through the tight binding to lipid A, colistin inactivates the endotoxic action of LPS and prevents the release of cytokines which may lead to shock (87). A recent study has shown that co-incubation with colistin significantly reduces LPS-induced cellular cytotoxicity as well as the production of inflammatory cytokines *in vivo* (15). A protective effect of polymyxin B, which is another antibiotic from the polymyxin family, on brain endothelial cell monolayer permeability has been observed following LPS treatment (200). Protection against

LPS-induced BBB disruption will be discussed in Section 1.3.2.7.3.

1.2.4 Clinical uses

Given the ability of colistin to induce severe toxicity including nephrotoxicity and neurotoxicity after intravenous administration, the use of colistin sulfate is limited to oral and topical formulations, whereas CMS, the inactive pro-drug of colistin, is administered parenterally (68). CMS undergoes hydrolysis to form colistin in aqueous solution (276). Both colistin sulfate and CMS have been used in an aerosol formulation for respiratory tract infections (167).

Recently, most clinical reports have focused on the treatment of infections induced by Gram-negative bacteria resistant to other antibiotics including *P. aeruginosa* and *A. baumannii* (149), the most common of which was ventilator-associated pneumonia (VAP) (276). In a recent retrospective cohort study of 258 patients who had pneumonia and treated with CMS (69), cure of infection occurred in 79.1% of patients, nephrotoxicity in 10% and hospital survival in 65.1%. A clinical study in Spain showed that when treated with CMS, 12 out of 21 patients with VAP due to *A. baumannii* were cured (81). In another prospective cohort study comparing colistin with carbapenem treatment, similar outcomes were observed in cure rates and the adverse effect profile (217).

CMS was also used as salvage therapy to treat sepsis caused by MDR *P. aeruginosa*

in critically ill patients, which showed a good outcome (160). Another study reported an approximate clinical response rate of 70% when CMS was intravenously administered to critically ill patients with sepsis caused by Gram-negative bacilli resistant to all other antibiotics (152).

In addition to being used for the treatment of systemic infections, colistin (in the form of CMS) has been used to treat infections within the brain. Intraventricular administration of CMS has been shown to successfully treat ventriculitis induced by carbapenem-resistant *A. baumannii* (74). Another clinical case showed that the patient's recurrent meningitis due to MDR Gram-negative rods was eradicated only when combined administration of CMS (both intraventricular and intravenous) was administered (117). In a clinical study to treat patients with MDR *A. baumannii* CNS infection, CMS was intrathecally administered and a high cure rate of 83% was observed (120). There are also several successful cases using CMS through intrathecal administration to treat meningitis with no apparent adverse effect (19, 35). The reason why CMS is given intraventricularly or intrathecally is due to its poor penetration across the BBB, albeit this transport process has not been well characterized (221).

It should be mentioned that the current dosing regimen of colistin (in the form of CMS) for treating infections is derived from experience with its use several decades ago. Therefore, a better understanding of colistin pharmacokinetics has been urgently required to maximize its effect and avoid potential development of resistance.

1.2.5 Pharmacokinetic properties

The pharmacokinetic (PK) studies of colistin have been performed since 1970. Utilising microbiological assays to measure drug concentrations in biological fluids and tissues, previous studies in the 1970s to 1980s revealed controversial results regarding the tissue disposition of colistin. One study suggested that colistin accumulated at the injection site (i.e. muscle) and was eliminated through kidney (141), while several other studies showed that colistin bound to tissue components including liver, kidney, lung, muscle and heart after a single intramuscular injection (46, 131, 132). However, such microbiological assays lack specificity for colistin, especially when samples contain any other co-administered antibiotics (149). Therefore, the accuracy of these studies is always a concern. Thin layer chromatographic (246) and immunological methods (123) have also been developed to quantify colistin or CMS *in vivo*, however, such methods have their own limitations as they are unable to differentiate between colistin and CMS.

With the recent development of HPLC and LC-MS/MS assays (83, 148), differentiation between colistin or CMS can be achieved to accurately determine the PK properties of colistin following the administration of colistin or CMS. Using such techniques, it has been demonstrated that CMS is eliminated predominantly by the kidneys (149). Following intravenous administration to rats, approximately 60% of the CMS dose was excreted in urine (~50% of which was in the form of colistin formed by hydrolysis of CMS in the urinary tract and collection vessel). In patients

with cystic fibrosis, similar urinary recovery (62.5%) following intravenous administration of CMS was reported (216). The elimination of the remaining CMS remains unclear and biliary excretion has been excluded (133). Colistin, on the contrary, is eliminated predominantly by the non-renal route by means of mechanisms not yet fully understood (149). Following an intravenous colistin sulfate dose of 1 mg/kg into rats, only 0.18 ± 0.14% of the dose was recovered as unchanged colistin in the urine in the first 12 hours suggesting colistin exhibits extensive net re-absorption from the renal tubules into the systemic circulation(147, 168). In rats received a single intravenous bolus of 15 mg/kg CMS, substantial concentrations of colistin were observed within 5 min after administration of CMS (123). Approximately 60% of the dose was eliminated via the urine in 24 h and presented as a mixture of CMS and colistin, suggesting an intra-renal conversion of CMS into colistin within tubular cells of the kidney along with hydrolysis of CMS in the bladder. In addition, colistin is not absorbed from the gastrointestinal tract and is poorly distributed to the pleural cavity, lung parenchyma, bones, pericardial fluid and cerebrospinal fluid (CSF) (168).

Preclinical PK studies have indicated that following administration of CMS, only a small portion of the dose (about 7-16%) is converted to colistin in the systemic circulation (147, 158). The limited formation of colistin is because the formation clearance of colistin is substantially less than the renal clearance of CMS. In rats receiving a single intravenous bolus of 15 mg/kg CMS, total body clearance, volume of distribution at steady state and terminal half-life of CMS averaged 11.7 mL/min/kg,

299 mL/kg and 23.6 min, respectively (147). Linear relationships were observed in rats between the PK of CMS or formed colistin and the intravenous doses of CMS (158). In rats administered 1 mg/kg of colistin sulfate as an intravenous bolus the total body clearance, volume of distribution at steady state and terminal half-life of colistin were 5.2 mL/min/kg, 496 mL/kg and 74.6 min, respectively (148). In neutropenic mouse thigh and lung infection models, single-dose PK studies were performed after subcutaneous administration of colistin sulfate at 5, 10, 20, or 40 mg/kg. Nonlinearity in the unbound PK of colistin was observed (60). Following the highest dose (i.e. 40 mg/kg), the total body clearance, volume of distribution at steady state and the terminal half-life of colistin were 16.4 mL/min/kg, 3270 mL/kg and 83.6 min, respectively. Preclinical PK studies have also been performed in other animals including dogs and calves (6, 284).

Clinical PK of colistin was assessed in healthy young volunteers after a 1 h infusion of 80 mg of CMS, and these studies suggested the apparent distributions of CMS and formed colistin (14.0 and 12.4 L, respectively) were restricted to extracellular fluid of plasma (45). While CMS had a higher renal clearance of 103 mL/min, the renal clearance of colistin was only 1.9 mL/min, which was consistent with the results from animals suggesting a non-renal elimination route of colistin (45). In addition to the evaluation of PK in healthy subjects, studies have been performed to assess the impact of disease state on colistin PK. One clinical study showed that at steady-state following administration of CMS in critically ill patients with moderate to good renal

function, the plasma concentration at steady state, the elimination half-life and apparent volume of distribution of colistin were 0.7–4.7 mg/L, 5.9 ± 2.6 h and 1.5 ± 1.1 L/kg, respectively (105). Compared with healthy subjects (45), the half-life and the apparent volume of distribution of colistin in patients have increased. In septic patients with stable renal function following an intravenous administration of CMS, the colistin plasma concentration at steady state was 1.2–5.1 mg/L, apparent volume of distribution was 20 L/kg, and half-life was 7.4 h (161). In patients with competent renal function receiving CMS administration to treat infections caused by Gram-negative bacteria, the predicted maximum concentrations of colistin in plasma were 0.60 mg/L and 2.3 mg/L for the first dose and at steady state, respectively, which are below or close to the MIC breakpoint (2 mg/L) signifying a delay in appropriate treatment (206). A loading dose is recommended to maximize efficacy and avoid the development of resistance. In critically ill patients receiving a 480 mg loading dose of CMS, it is predicted that the time to 3-log-unit bacterial kill was reduced to half compared to the dose of 240 mg with 8 hourly dosing (173). In another large clinical PK study, patients with diverse renal function were treated with a physician-selected CMS dosage regimen (82). The range of the plasma colistin concentrations at the steady state across all patients was 0.48 to 9.38 mg/L. A greater fraction of the administered dose of CMS was converted to colistin as the renal clearance of CMS declined in these patients with damaged renal function. It is suggested that colistin may best be used as part of a highly active combination, especially for patients with moderate to good renal function.

Plasma protein binding of colistin can also affect its PK and studies have been performed using plasma from various species. Colistin protein binding in rat spiked plasma at concentrations from 4 to 12 μg/mL was found to be 55 to 57% (148), which is similar to values determined in plasma from dogs (7). In neutropenic infected mice, it is suggested that colistin plasma protein binding was highly concentration-dependent and involves multiple proteins including albumin and α_1-acid glycoprotein (AAG) (61). As AAG is an acute-phase reactant induced by pathophysiological stresses including infection (176), its concentration will increase depending on the severity and stage of infection. Therefore, the plasma unbound fraction of colistin may be altered in infection, which not only affects plasma pharmacokinetics but also the fraction available for disposition into other tissues, including the brain. The unbound fraction of colistin in human plasma is ~50% (99), however, it is not surprising that this value decreases in critically ill patients as the clinical study demonstrated that the unbound fractions of colistin in the infected patients were 26-41% at clinical concentrations (173).

Overall, the clearance of colistin seems to decrease in the presence of critical illness, which leads to the increased total plasma colistin concentrations in patients (144, 151, 213). These altered PK properties such as relatively high plasma concentration over a long period can potentially affect brain uptake of colistin.

1.2.6 Adverse effects

With the increasing use to treat infections caused by MDR Gram-negative bacteria, a few adverse effects of colistin have been observed (244). Nephrotoxicity and neurotoxicity are two common adverse effects and both of them appear to be dose-dependent (67).

While the exact mechanism of colistin-induced nephrotoxicity remains unclear, it is proposed that the toxicity starts with the interaction between the D-Leu-L-Leu amino-acid region and fatty acid side chain of colistin and the cell membrane. Once anchoring into the cell membrane, colistin is thought to increase membrane permeability and cause edema and lysis, which similar to how colistin disrupts the OM of Gram-negative bacteria described in Section 1.2.3 (143). It is also suggested that oxidative stress may have a key role in colistin-induced toxicity (279). In more recent reports, the incidence of nephrotoxicity (which is approximately 45-55%) have been decreasing, and this is likely to be due to the more advanced supportive therapy, renal function monitoring and the avoidance of administering other nephrotoxic drugs with colistin (67, 114, 125). Recent studies used the RIFLE (Risk- Injury- Failure- Loss- End stage renal disease) classification to determine CMS-associated nephrotoxicity (93). It is suggested that the incidence of nephrotoxicity could be decreased by adjusting the dosage of colistin together with shortening the duration of treatment for specific infections (e.g., pneumonia). The best approach to treat this adverse effect is to immediately discontinue the antibiotic. Recent studies also

suggested co-administration with ascorbic acid or melatonin could protect the nephrotoxicity caused by colistin suggesting that nephrotoxicity is mediated through oxidative stress (278, 279).

Compared with the number of patients suffering nephrotoxicity, patients experiencing neurotoxicity are fewer (67). The proposed mechanism of colistin neurotoxicity involves a short phase of competitive blockade between acetylcholine and polymyxins at receptor sites followed by a prolonged phase of depolarization associated with calcium depletion (67). Neurotoxicity is characterized by dizziness, muscle weakness, paraesthesia, partial deafness, visual disturbance, vertigo, seizures, ataxia, respiratory failure and confusion. Concomitant administration of muscle relaxant drugs, sedatives, corticosteroids, impaired renal function and being a female patient increases the risk of neurotoxicity (51, 126). The most frequently experienced neurological adverse effects before 1970 were paresthesias that occurred in approximately 27% and 7% of patients receiving intravenous and intramuscular CMS, respectively, with a higher risk in patients with cystic fibrosis (72, 75). Recently, Falagas et al. described four patients who had polymyoneuropathy during colistin treatment (70). Another study also reported four patients with varying neurological complaints (228). In a cohort of 115 patients, four cases of potential colistin-induced neurotoxicity were identified, including three patients with focal seizures and one patient with altered mental activity, which could be mediated centrally (39). Interestingly, the incidence of neurotoxicity in patients receiving intrathecal or intraventricular CMS is less than

patients receiving intravenous and intramuscular CMS (120). One of the potential reasons could result from the lower dose of CMS that were administered centrally as intraventricular CMS was given 5-10 mg per 12 h (74), whereas intravenous CMS was normally given 300 mg per day (142). Therefore, during infections, the brain exposure to colistin following systemic administration may be higher than following central administration. Similar to the treatment of nephrotoxicity, discontinuation of colistin and reducing any risk factor is first line treatment.

As the clinical use of colistin is associated with neurotoxicity, whether it is peripherally or centrally mediated remains unclear as some of the symptoms such as numbness and weakness could be potentially mediated through either mechanism. However, if the neurotoxicity was to be induced by a central mechanism, it is expected that colistin or CMS would have to permeate into the brain parenchyma across CNS barriers following systemic administration.

1.2.7 Penetration at the central nervous system barriers

The BBB and blood-cerebrospinal fluid barrier (BCSFB) are two major barriers separating the systemic circulation from the CNS (64). Although both barriers play pivotal roles in maintaining the CNS homeostasis, the locations and the characteristics of the BBB and the BCSFB are quite different from each other. The BBB is the endothelial lining of the cerebral blood vessels and is characterized by the presence of tight junctions and various transporters (197). Due to its unique structure, the

paracellular route of the BBB is sealed and the movement of molecules through the transcellular route between the blood and the brain is precisely regulated to maintain homeostasis in the CNS. The BCSFB, on the other hand, is established by the epithelium between blood and ventricular or subarachnoid CSF (214). As formed by the choroid plexus epithelial cells, the BCSFB has less restriction than the BBB to the paracellular entry of polar substances (247). The details of the BBB and the BCSFB will be described in Section 1.3.

Based on the physicochemical properties of colistin (large molecular weight, hydrophilicity) described in Section 1.2.2, it is not surprising that the penetration of colistin at the BBB and the BSCFB has been reported to be poor under normal conditions (159). However, after being administered to patients with CNS infection, colistin has been detected in the CSF. In a study using a microbiological assay, the colistin CSF concentration was about 25% of that in serum of patients who received CMS intravenously (5 mg/kg of colistin base activity per day) (108). Nevertheless, the study failed to provide information on the relative concentrations of CMS and colistin in serum and CSF due to the non-specific nature of the microbiological assay. Combination therapy with intravenous and intraventricular CMS also resulted in a high concentration of colistin in CSF and successful clinical and microbiological outcome (49). Another clinical study measured the colistin concentration in CSF under normal condition and CNS infection following intravenous CMS (14). While the colistin concentration in the CSF appeared to be low (<0.2 μg/ml) in the absence

of meningeal inflammation, enhanced colistin penetration in CSF (0.5 μg/ml) was observed in pediatric patients in the presence of meningitis with CSF levels reaching 34 to 67% of those in serum using a LC-MS method. Although it was concluded in some of these reports that the BBB was disrupted, all these studies actually measured blood-CSF barrier penetration, and it is likely that this barrier, and not necessarily the BBB, was compromised during these infections.

However, there is scarce information on whether colistin has the ability to cross an intact BBB. In addition, BBB dynamics can differ in various disorders and subsequently affect the drug transport at the BBB (251). Therefore, before assessing the brain uptake of colistin, a comprehensive understanding of the structures and functions of CNS barriers, especially the BBB, under healthy and disease-state condition (infection) is required.

1.3 Central nervous system barriers

The brain is one of the most important organs in the body and a perfectly regulated and maintained CNS environment is essential for controlling physiological processes such as respiration, vision and motor coordination (182). With the CNS evolution, barriers were developed to protect the brain parenchyma from toxic chemicals and to facilitate the uptake of nutrients at the same time. Two barriers at three main barrier sites have been recognized so far. Firstly, the BCSFB is located at the choroid plexus epithelium between blood and ventricular CSF and at the arachnoid epithelium

between blood and subarachnoid CSF. The second barrier is the BBB formed by the cerebrovascular endothelial cells between blood and the brain tissue. These barriers are not only anatomical barriers but also dynamic tissues expressing multiple transporters, receptors and enzymes. At all the barrier sites, the paracellular transport of substances within the blood into the brain tissue is limited by the tight junctions between the adjacent cells, and the transcellular influx or efflux of nutrients or metabolites is regulated by the transporter proteins on the barrier membranes (137). The brain barriers are summarized in Fig. 1-2.

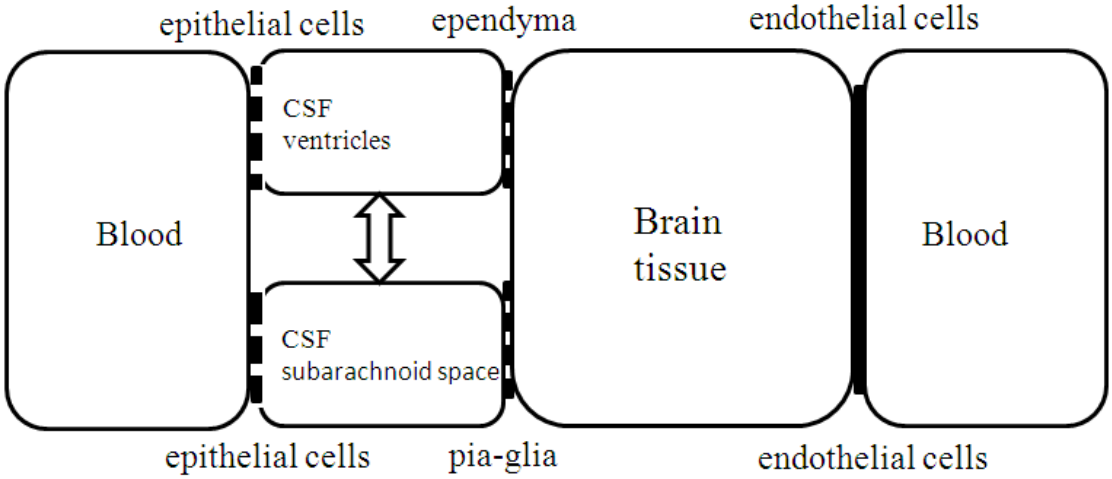


Figure 1-2. Two major barriers between the blood and the brain. The BCSFB is formed by the choroidal plexus epithelial cells. Brain tissue is separated from the ventricular CSF by the ependymal lining and from subarachnoid CSF by pia-glia. The BBB is formed by the capillary endothelial cells.

1.3.1 The blood-cerebrospinal fluid barrier (BCSFB)

CSF is mainly secreted by choroid plexus (CP) epithelial cells which are located at lateral, third and fourth ventricles and flows through all the ventricles, and out to subarachnoid cavity before draining back into the vein called the superior sagital sinus. Under normal conditions, the CP-CSF nexus furnishes micronutrients, growth factors and neurotrophins to neuronal networks. Thus, blood-borne substances and pharmacological agents may enter the brain along similar pathways (109).

Two prominent areas where the blood is near CSF are the CP of the ventricles and the arachnoid membrane over the subarachnoid space, and the BCSFB at those two regions are formed by single or multiple epithelial cell layers, respectively. Regional transport distinctions are associated with the BCSFB at different sites. However, compared with the greater attention on the CP which is believed to be mainly responsible for fluid formation, there is a lack of insight on the function of arachnoid membrane, and only the BCSFB at the CP epithelium has drawn serious attention regarding drug transport and metabolism (109).

Evidence has suggested the BCSFB plays a crucial role in maintaining the CNS homeostasis due to its several unique anatomical and physiological features (215). First of all, it is generally accepted that CPs of all the ventricles possess a considerable surface area for transport due to the extensive basolateral infoldings and microvilli at the apical membrane, which could be the same order of magnitude as the

entire BBB (118). Secondly, the epithelial cells at CPs are fenestrated and leaky, which lead to less restriction to paracellular movement of large polar substances than the BBB (179). Trans-endothelial/epithelial electric resistance (TEER) is used to estimate the tightness of a barrier and the TEER value of an established model of endothelial cells is usually 10 times higher than that of an established model of epithelial cells (230). Thirdly, the CP epithelial cells have high blood flow compared to other brain regions and high fluid-producing capacity. It has been shown that high blood flow to the CP produces an efficient influx or efflux of blood-borne substances at the BCSFB and also increases the chance to be insulted by the toxic chemicals (156). Last but not least, numerous transporters including solute carrier transporters and ATP-binding cassette (ABC) transporters and ion transporters are found at the apical and basolateral membrane of the BCSFB (214). Together with the transporters at the BBB, transport of nutrients and metabolic wastes between blood and brain extracellular fluids is well regulated.

1.3.2 The blood-brain barrier (BBB)

1.3.2.1 Anatomy of brain capillaries

The BBB is formed by the brain capillary endothelial cells (BCECs) separating the blood and brain interstitial fluid (Fig. 1-3). It acts as a physical barrier as the endothelial cells are absent of fenestrations and have extensive tight junctions and sparse pinocytic vesicular transport forcing most molecules to cross the BBB through the transcelluar route rather than moving paracellularly through the junctions (24). In

addition to the endothelial cells, the neurovascular unit is composed of the capillary basement membrane, pericytes embedded within the basement membrane and astrocyte end-feet ensheathing the vessels (1). Pericytes and astrocytes are believed to be involved in the formation of the functional neurovascular unit and regulate BBB functions. It is estimated that there are approximately 100 billion capillaries with a total surface area of 20 m^2 in human brain and individual neurons are normally no farther than 8-20 μm from a brain capillary (199, 236). With all of these characteristics, the BBB is considered the most important barrier for the exchange of substances between the CNS and the peripheral circulation.

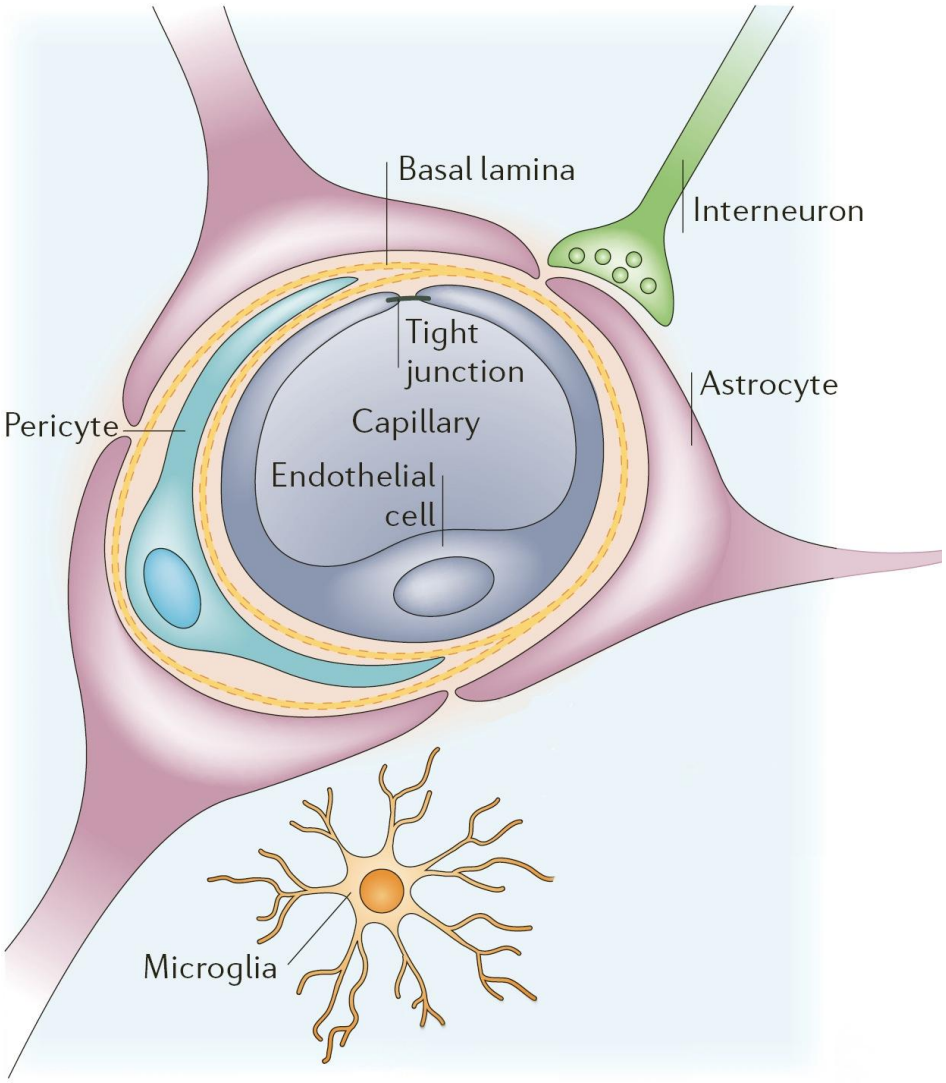


Figure 1-3. Cellular structure of the BBB. The barrier is formed by brain capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular endfeet, pericytes and microglia (taken from (Abbott et al. 2006) (2)).

1.3.2.2 Tight junctions (TJs)

BCECs have junctional complexes existing between comprising TJs and adherens junctions (AJs) which together, help to seal the paracellular route for most large and hydrophilic substances. The TJs, shown in Fig. 1-4, consist of a complex of major

transmembrane proteins spanning the intercellular cleft including occludin, claudins and junctional adhesion molecules (JAM 1, 2 and 3) (85, 162), and several cytoplasmic proteins including zonula occludens (ZO 1, 2 and 3) and cingulin (270). The cytoplasmic proteins link membrane proteins to the actin cytoskeleton to maintain the structural and functional integrity of the endothelium.

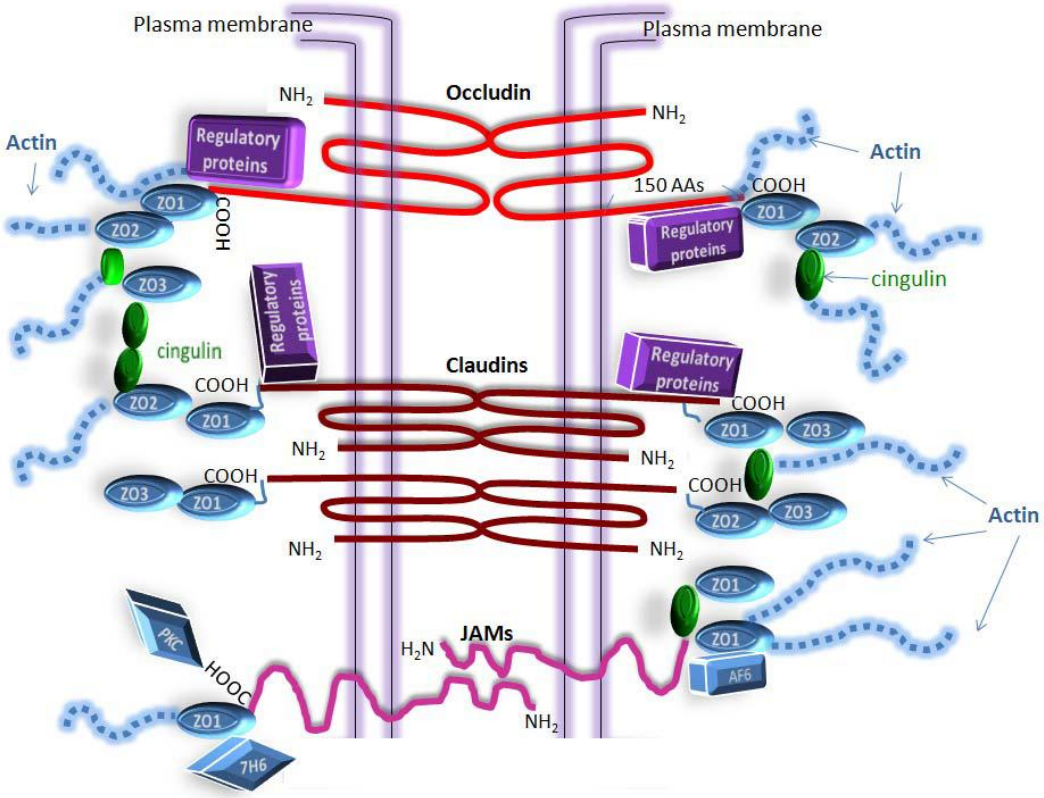


Figure 1-4. Cellular structure of the TJs at the BBB. The TJs consist of a complex of major transmembrane proteins including occludin, claudins and junctional adhesion molecules (JAMs) and several cytoplasmic proteins including Zonula Occludens (ZOs), and cingulin. (taken from (Redzic et al. 2011) (214)).

Occludin was the first integral protein identified in adherens junction fractions from chicken liver and its expression is much higher in BCECs compared to non-neural tissues (78). The structure of occludin appears to be essential for occludin function of TJs in the BBB. Occludin has four transmembrane domains, a long COOH-terminal cytoplasmic domain, and a short NH_2-terminal cytoplasmic domain. The COOH-terminal associates with ZO proteins and the transmembrane and the COOH-terminal cytoplasmic domains of the occludin as well as the extracellular loops are important for regulating the paracellular permeability (102), and it is believed that its extracellular loops determine the TEER of the BBB (73). However, studies have shown that other TJ proteins are able to compensate for the lack of occludin and the TJs appear to function normally in occludin-deficient mice (231, 232) suggesting occludin is not irreplaceable for mature BCECs to establish their barrier properties.

Claudins were first isolated from chicken liver junction fractions and with time, a larger family of TJ proteins was identified. To date, 24 members of the claudin family have been identified in mammals (136, 252). However, studies have demonstrated that only claudin 1, 3, 5, 11 and 12 are present at the BBB endothelial cells (94, 183) and claudin 1, 2, 3 and 11 are expressed in the CP epithelium (271). It should be noted that the difference in molecular composition of TJs between the BCSFB and the BBB is with respect to claudin 2, 5 and 12, which could be the potential reason for the disparity in the two barriers regarding paracellular permeability. Claudins are 22 kDa

phosphoproteins and share the same structural pattern which includes four transmembrane domains, two extracellular loops and two cytoplasmic domains, a long COOH-terminal sequence, and a short NH_2-terminal sequence (97). No amino acid sequence of claudin is similar with that of occludin. Claudins are the primary barrier forming proteins which cannot be compensated by other TJ proteins. For example, in claudin 5 deficient mice, the BBB showed severe dysfunction and increased permeability (183). Claudins from adjacent endothelial cells homotypically bind to each other and exhibit stronger adhesion than occludin (130, 205). It has been found that occludin only localizes to TJs in cells that have been already transfected with claudin, which may indicate occludin is only a protein to support the claudins to seal the TJs (130). Other studies suggested claudin 3 and 5 work together in BBB genesis and controlling paracellular transport of molecules (23, 175, 252).

The third major transmembrane protein at the BBB is JAM which was identified in 1998 (162). Three JAM-related proteins, JAM 1, 2, 3 have been discovered and they belong to the immunoglobulin superfamily. JAMs have a single transmembrane domain and their extracellular portion has two immunoglobulin-like loops. JAM 1 and 3 but not JAM 2 were found in rodent brain blood vessels (18), and JAM 2 and 3 are widely expressed in the human brain (16). *In vitro* studies have demonstrated that JAM 1 increased cellular resistance and promoted occludin localization at intercellular junctions (52, 162). However, there is a paucity of knowledge on the function of JAMs and more investigations are required to elucidate their functional

role at the BBB.

In addition to TJs present between the BCECs, AJs have been identified in the endothelium of brain microvessels (239). AJs are formed by cadherins which are a large family of transmembrane proteins and associated proteins including catenins, β-catenin, and γ-catenin. The cadherin complex is anchored to the actin cytoskeleton by p120 catenin, which helps to regulate BBB vascular permeability (11, 258). One of the cadherins, vascular endothelium cadherin (VE-cadherin) which forms the complex between cells, was thought to be a marker of microvascular integrity both *in vitro* and *in vivo* (180). On the contrary, recent studies have shown that barrier-forming endothelium or epithelium mainly expressed cadherin-10, that the expression of VE-cadherin is scarce and that VE-cadherin is not specifically associated with BBB function in human brain microvessels (226, 268). Thus, these findings suggested that cadherin-10 in addition to VE-cadherin may have an important role in the maintenance of the BBB. However, one should bear in mind that though disruption of AJs at the BBB can lead to increased permeability, it is primarily the TJs that confer the low paracellular permeability and high electrical resistance (222).

In addition, the cerebral vascular endothelial cells have a close physical and functional contact with surrounding cells including astrocytes, pericytes and microglia. Isolated brain endothelial cells lose the barrier characteristics if separated from these neighbouring cells (220).

1.3.2.3 Neighbouring cells of BCECs

Astrocytes lie abluminally to BCECs and establish direct interaction with endothelial cells through their endfeet. Astrocytes cover more than 99% of the BBB endothelium and help to maintain BBB morphology and function (17, 28). *In vitro* studies have shown that co-culture of brain endothelial cells with astrocytes produces a higher endothelial electrical resistance (38). Astrocytes also serve as scaffolds, guiding neurons to their proper place during development of the BBB. In addition, astrocytes increase the functional expression of transporters at the BBB including P-glycoprotein (P-gp) (80).

Pericytes have received intensive attention in recent years because they are present when the BBB is formed in the embryo state at a stage when astrocytes have not arisen yet (47). Located closer to the endothelium than astrocytes, pericytes appear to play a key role in angiogenesis, structural integrity and differentiation of the vessel and formation of the endothelial TJs (9, 21, 25). Lack of pericytes during the development of the BBB will lead to endothelial hyperplasia (98). It was suggested that the BBB permeability inversely correlates with pericyte coverage *in vivo* and junctional abnormalities and increased transendothelial transport was also associated with pericyte loss (47). Therefore, pericytes have been considered crucial for the development and regulation of endothelial TJs.

Neurons are also found close to the endothelial cells and contribute to the CNS

vascular permeability (91). Through communication with astrocytes, neurons have been demonstrated to promote vasodilation (37). Moreover, neurons express high levels of nitric oxide and cyclooxygenase-2 suggesting a potential mechanism to regulate endothelial cell function in a process independent of astrocytes (262).

Microglia are major glial cells and are considered the resident macrophages of the CNS and they infiltrate the CNS early in development (202). Microglia serve as scavenger cells and will be activated in response to infection, inflammation, neurodegeneration and other CNS disorders (165, 248). Due to their expression of proinflammatory cytokines that induce BBB disruption *in vitro*, microglia have been recognized as mediators of CNS microvascular permeability (283). Another study also showed that *ex vivo* isolated microglia could activate T cells to express cytokines to cause enhanced BBB permeability (154).

With the presence of these neighbouring cells of BCECs, the BBB characteristics such as TJs and transporters are well maintained.

1.3.2.4 Physicochemical properties of drugs affecting BBB transport

Due to the unique TJs structure of the BBB, the movement of drugs across the BBB requires the involvement of the transcellular route through the BCECs. It is generally accepted that the more lipophilic a drug is, the higher propensity for it to cross the BBB (41). Molecular weight, HBDs and HBAs are also two significant features

affecting BBB permeability (194). Compounds with a molecular weight less than 450 have a greater ability to permeate the BBB. Because hydrogen bonding is mainly associated with oxygen and nitrogen moieties in a molecule, if the sum of the oxygen and nitrogen atoms in the molecule is 3 or less, then there is a greater ability for the molecule to cross the BBB. Additionally, a drug with a pKa value between 7.5 and 10.5 (weak basic compounds) tends to have higher BBB permeation (116). Other physicochemical properties including polar surface area (PSA) and LogP will also affect the penetration of molecules across the BBB. The suggested maximum value for PSA is less than 70 $Å^2$ and the suggested range for LogP is between 2 to 5 (195). The physicochemical properties that favor BBB penetration are illustrated in Table 1-1. Comparing these values with the corresponding physicochemical properties of colistin described in Section 1.2.2, it is likely that the colistin penetration across the intact BBB will be minimal.

Table 1-1. Physicochemical properties of compounds favoring BBB penetration (194)

Properties	Suggested limits
M_W	<450
HBD	<3
HBA	<8
LogP	2-5
PSA ($Å^2$)	<70
pKa	7.5-10.5

To date, researchers have utilized chemistry-based strategies to design the drugs to target the brain for the treatment of CNS diseases, so that such compounds exhibit

appropriate physicochemical properties to passively diffuse across the BBB (110). However, the brain uptake of drugs does not always correlate with their physicochemical properties (153) as the BBB is not only a static barrier but also a dynamic barrier consisting of diverse transport systems which are expressed at the luminal and/or abluminal membranes of the BCECs.

1.3.2.5 Transport systems

Transport of nutrients, waste products, and drugs at the brain are actively regulated by the BBB by means of transport systems. Drug transport is a result of the concerted action of efflux and influx pumps, in addition to passive diffusion processes. The influx transporters at the BBB provide the brain with hydrophilic nutrients and essential large substances such as glucose, amino acids, vitamins, most of which are unable to passively diffuse across the BBB. Carrier-mediated transporters and receptor-mediated transporters are two main influx transport systems at the BBB. To protect the CNS from the toxic chemicals and substances from the blood, there are also some efflux transporters located at the BBB such as P-gp and breast cancer resistance protein (BCRP). A summary of transporters of different transport systems are listed in Table 1-2.

Table 1-2. A summary of the different transporters across the BBB

Transport systems	Transporters	
Carrier-mediated transporters (CMT)	Glucose transporter (GLUT1)	(71)
	Large neutral amino acid transporter	(31)
	Cationic amino acid transporter	(186)
	Monocarboxylic acid transporter	(121)
Receptor-mediated transporters (RMT)	Insulin receptor	(10)
	Transferrin receptor	(76)
	Insulin-like growth factor receptor	(128)
	low density lipoprotein receptor-related proteins	(272)
Active efflux transporters (AET)	P-gp (ABCB1)	(48)
	ABC transporter, subfamily C (ABCC1-6)	(57)
	BCRP (ABCG2)	(44)
	Organic anion transporter (OAT or SLC22)	(134)
	Organic anion-transporting polypeptide (OATP or SLC21)	(124)
	Glutamic acid amino acid transporter (SLC1A)	(101)
	γ-aminobutyric acid transporter 2 (SLC6A12)	(245)

1.3.2.5.1 Influx transporters

There is a high expression of carrier-mediated influx transporters at the BBB, including carriers for glucose (GLUT1) (71), monocarboxylic acids (121), large neutral amino acids (31), cationic amino acids (186), and organic anion-transporting polypeptide (189). Several polar therapeutic drugs, including gabapentin and melphalan, gain entry into the brain via carrier-mediated transport (243). Through incorporating substrate modifications which enhance carrier binding and transport, the brain delivery of drugs can be improved >10 fold (243). Carrier-mediated transport relies on molecular carriers present at both the apical (blood) and basolateral (brain) membranes of the BCECs. These carriers tend to be highly stereospecific and function

in the selective transport of small molecules such as energy sources and amino acids (198). Therefore, they are not particularly amenable to the transport of large-molecule therapeutics.

For certain large peptides such as leptin, insulin and transferrin, transport across the BBB occurs via receptor-mediated transport (111). This process is comprised of three steps: initiation of endocytosis as a circulating ligand interacts with a specific receptor at the luminal plasma membrane of the endothelial cells, transcytosis of the generated transporting vesicles, and release of the drugs into brain interstitial space (197). Researchers have delivered therapeutics of interest conjugated to a molecule that has the capability of targeting the transferrin receptor, insulin receptor, insulin-like growth factor receptor and low density lipoprotein receptor-related proteins 1 and 2 to improve brain delivery (10, 76, 128, 272). Thus, the receptor-mediated transport system provides an alternative for drug delivery to the brain.

1.3.2.5.2 Efflux transporters

The efflux transporters include one class of energy-dependent transporters which are derived from the ATP-binding cassette (ABC) gene family and another class of energy-independent transporters which are produced by the solute carrier (SLC) gene family.

P-glycoprotein (P-gp, ABCB1) is a well known ABC transporter located at the

luminal membrane of the brain capillary endothelial cell. It has a molecular mass of 170 kDa with a broad substrate specificity including a variety of structurally diverse drugs (48). Drugs which are substrates for P-gp are transported from the brain (basolateral) side to the blood (apical or luminal) side of the endothelial cells. In P-gp deficient mice, the penetration of P-gp substrates can increase up to 10- to 100-fold, sometimes leading to toxic reactions (235). In addition to P-gp, the multidrug resistance-associated protein (MRP, ABCC) family and breast cancer resistance protein (BCRP, ABCG2) are members of the ABC family and have been reported to act as efflux transporters at the BBB. MRPs mainly transport anionic compounds (139), whereas BCRP transports compounds with a negative or positive charge (275). The MRP family has 12 members and so far 6 members including MRP1-6 have been recognized at the BBB (57, 129). It is reported that MRP1, MRP3 and MRP5 are located at luminal membranes and almost equal distributions of MRP2 and MRP4 are found on the luminal and abluminal plasma membrane of the BBB (282). Due to the lack of specific antibodies, the exact subcellular localization of other MRPs in brain capillary endothelial cells remains to be determined. MRPs and P-gp have overlapping substrate specificity (34, 240). The role of BCRP in protecting brain tissue from potentially harmful xenobiotics has more recently become recognized. BCRP is expressed in capillary endothelial cells of pigs, mice and humans at the luminal surface (44) and it also has an extensive overlap of tissue distribution with that of P-gp. Moreover, BCRP is reported to be more strongly expressed than P-gp at the BBB on mRNA analysis in porcine capillary endothelial cells (62). It has also

been demonstrated that BCRP could compensate for the lack of P-gp, with a three-fold increase in the expression of BCRP observed in P-gp knockout mice (40), though others have shown no change to BCRP expression in P-gp knockout mice (5). There is a considerable overlap between substrates for these efflux transporters suggesting a synergistic protective effect on BBB protection.

The superfamily of SLC can be divided into 43 families coding transport proteins (96), of which SLC1A, SLC6A, SLCO/SLC21A and SLC22 family are reported to be involved in efflux processes across the BBB. Excitatory amino acids such as L-glutamic acid and L-aspartic acid undergo efflux from the brain to blood through excitatory amino acid transporters (SLC1A) (101). γ-aminobutyric acid transporter 2 (SLC6A12) is reported to mediate the efflux transport of the neurotransmitter, γ-aminobutyric acid (245). Several organic anion transporting polypeptide (Oatps) including Oatp1a4 (Slco1a4), Oatp1a5 (Slco1a5) and Oatp1c1 (Slc1c1) are expressed at the luminal and abluminal brain capillary membranes of rats and they have been shown to be involved in the efflux of amphipathic organic anions such as taurocholate (124) and steroid conjugates such as estrone sulphate (100). Among the members of the SLC22 family, organic anion transporters 3 (OAT3, SLC22A8), organic cation/carnitine transporter 2 (OCTN2, SLC22A5) and renal specific transporter (RST, SLC22A12) have been shown to be expressed at the human BBB (134). Substrates of OAT3 include para-aminohippuric acid, benzylpenicillin and homovanillic acid (122, 188). OCTN2 has been characterized as a sodium-dependent carnitine transporter

(106) and RST is involved in the efflux of benzylpenicillin and urate (104).

Together with the TJs between the BCECs, these efflux transporters at the BBB assist to eliminate the metabolic wastes and protect the CNS from toxic blood-borne substances.

1.3.2.6 The impact of disease on BBB function

In a variety of neurological and systemic inflammatory or infectious diseases, BBB dysfunctions have been reported. Two related alterations, including disruption of TJs and altered function of transporters, will be discussed below.

1.3.2.6.1 BBB alteration during viral infection

About 60% of patients with acquired immune deficiency syndrome suffer from neurological disorders such as dementia or human immunodeficiency virus (HIV)-1-associated encephalitis (12). Increased BBB permeability has been detected when brain endothelial cells are exposed to viral proteins. *In vitro* studies demonstrate a down-regulation of TJ proteins, claudin-5 and occludin, in human brain endothelial cells after being treated with HIV-infected human lymphocytes (203). The results were confirmed by another study using the same human brain endothelial cell line demonstrating that the BBB breakdown by the HIV-1-infected lymphocytes was associated with disorganisation and altered expression of TJ proteins such as ZO 1 (4). From the brain tissue of HIV encephalitis patients, reduced expression of claudin-5

and occludin at the brain endothelium was found by immunohistological studies (203). Similar observations were made in the brains of patients with HIV-1-associated dementia, in which disrupted immunoreactivity for occludin and ZO 1 was detected (115). The reduced expression of TJ proteins could be due to the inflammatory mediators including cytokines, reactive oxygen species (ROS) and matrix metalloproteinases (MMPs) produced by the infected brain endothelial cells. Another mechanism of altered expression of TJ proteins involved protein kinase C pathways and receptor mediated Ca^{2+} release which lead to cytoskeletal alterations and increased monocyte migration across human brain microvascular endothelial cells (115). Studies also demonstrate that Tat, the principal transactivator for HIV-1 replication actively secreted by infected cells, plays an import role in decreasing expression of claudin-5 at the BBB in mice (13).

A few studies have also been performed to evaluate alterations to efflux transporters at the BBB during CNS viral infection. The expression of P-gp and MRP1 were found to be up-regulated by HIV-Tat protein which could reduce the penetration and efficacy of the HIV protease inhibitors (95). However, in the astrocytes treated with viral protein HIV gp120 secreted during infection, P-gp expression and functional activity were decreased significantly (223). To date, a few studies have described the change of uptake transporters during CNS viral infection. GLUT1 expression was found decreased in cortical, grey matter, caudate nucleus, and cerebellum during infection (157).

1.3.2.6.2 BBB alteration during multiple sclerosis

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the CNS with an assumed autoimmune cause. Experimental autoimmune encephalomyelitis (EAE) is an animal model for the inflammatory phase of MS and reflects various aspects of MS including inflammatory CNS lesion and BBB disruption. BBB breakdown is the major hallmark of disease in this model and the alteration of the BBB is correlated with the severity of the disease (192). Studies on animal EAE models have shown that BBB permeability to sodium fluorescein was markedly increased with striking dephosphorylation of the TJ occludin just prior to the apparent changes in BBB permeability (65, 174). Ultrastructural studies performed on brain tissues from EAE mice demonstrated dramatic alterations in TJ morphology with an extended upfolding of membranes at the level of cell to cell contact and these up-folded junctional membranes lead to the interrupted immunostaining for junctional proteins (161). In MS patients, increased BBB permeability has also been observed. Immunostaining of brain tissue biopsies from patients with various forms of MS showed 20 to 40% of CNS microvessel abnormalities in immunostaining for ZO 1 and occludin (43). In addition, decreased expression of JAM 1 has been detected in CNS microvessels in brain biopsy tissues from MS patients (193). It was also concluded that serum from MS patients significantly reduced the expression of occludin and VE-cadherin in endothelial cells compared with normal controls (171). Using magnetic resonance imaging techniques, the diffusion of gadolinium through brain endothelial TJs during MS was also detected indicating the paracellular route at the BBB was opened, given

the presence of gadolinium enhancement is quite sensitive to reflect BBB disruption (267). CNS inflammation associated with MS and EAE has been described to involve a wide range of cytokines, chemokines, MMPs and ROS produced by infiltrating immune cells and CNS resident cells. Proinflammatory cytokines and chemokines such as interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and α-chemokines are elevated in MS and *in vitro* studies have demonstrated that they could decrease the expression of occludin at the BBB (190). Growing evidence also suggests that MMPs are involved in various phases of pathogenesis of MS and can be released by astrocytes, microglia, T cells and macrophages which are close proximity to the BBB (140). MMPs are known to disrupt the BBB causing an enhanced brain uptake of ^{14}C-dextran and ^{3}H-sucrose and the increased BBB permeability can be protected by the MMP inhibitors (225). In addition, levels of ROS are elevated in blood, urine, and CSF of MS patients, which could contribute to the increased BBB permeability by reorganizing TJs architecture involving ZO 1 and occludin pairing (90, 119). The reduced brain function could be attenuated by pretreatment of brain endothelial cells with the ROS scavenger such as α-lipoic acid and nitronyl nitroxides (30).

Only a few studies have been performed to evaluate the function of transporter systems at the BBB during MS or EAE. One recent report demonstrated that the expression and function of brain endothelial P-gp in EAE was significantly impaired via a Nuclear Factor-KappaB (NF-kB) signaling pathway (127). Nevertheless, due to the elevation of the cytokines and chemokines induced during MS or EAE, potential

alteration of the efflux transporter systems may also occur (253).

1.3.2.6.3 BBB alteration during bacterial meningitis

Bacterial meningitis starts with nasopharyngeal colonization and invasion of bacteria, followed by CNS invasion (254). Subsequently, a generic intense subarachnoid space inflammation process develops, leading to the release of various cytokines that induce inflammatory reactions predominantly interleukin-1β (IL-1β) and TNF-α (107). In rats with experimental meningitis (induced by *E. coli* K1+, *Streptococcus pneumoniae* type III, *Haemophilus influenzae* type b), increased pinocytotic vesicles and complete separation of the TJs between the endothelial cells is detected ultrastructurally (210). The BBB was disrupted in neonatal rats with experimental meningitis and sepsis induced by *S. pneumonia*, and increased brain levels of cytokines and chemokines may be responsible for the BBB breakdown (26). The precise localization of the opened intercellular TJs during meningitis is the meningeal venules (211). After intracisternal administration of bacterial endotoxin from *H. influenzae* (as a model for bacterial meningitis), a progressive opening of TJs was observed from 4 to 18 h after administration, which resulted in enhanced transport of systemically-administered radiolabelled albumin into the CSF (269). TNF-α, nitric oxide and MMPs in cells and animals treated with *Neisseria meningitidis* were found to be responsible for the BBB disruption causing proteolytic cleavage of the TJ protein occludin and use of the MMP inhibitor batimastat (BB-94) could significantly reduce the BBB disruption (201, 238). However, it should be noted that when an increasing BBB

permeability was claimed in most of the studies mentioned above, it was usually the CSF concentrations of paracellular marker that were detected which theoretically indicates a disruption at the BCSFB. Although it is likely the BBB is also perturbed, studies needs to be performed to clarify these suggestions.

As for the transporters at the BBB, *in vitro* studies demonstrate that TNF-α and IL-1 released during bacterial meningitis infection regulate the expression of brain transporters in primary cultures of porcine brain capillary endothelial cells (263). IL-1 causes a continuous decrease in protein expression of P-gp and BCRP. BCRP mRNA expression was rapidly decreased by TNF-α but returned to normal value after 24 hours. On the contrary, P-gp protein expression was transiently increased and then reduced after 24 hours. In another study, bacterial meningitis induced by *Escherichia coli* LPS produced a loss in the expression of P-gp mRNA in the brain and an increase in the brain uptake of systemically-administered digoxin (P-gp substrate), suggesting that P-gp function was modified under this pathological condition (86). The delivery of morphine, a substrate for P-gp, is significantly increased during *E. coli* LPS–induced meningitis, which is interpreted as being a combination in alterations in passive diffusion and decreased active efflux at the BBB (253).

Along with the alterations at the BBB under CNS pathological conditions, a big concern has risen on the potential increased brain uptake of therapeutics and their associated neurotoxicity during such pathologies (234). Therefore, a number of

studies and reviews have focused on the BBB integrity and functions in health and CNS disease (50, 63). On the contrary, less is known about the impact of systemic inflammation and systemic infection on the BBB and the subsequent impact on drug delivery across the BBB.

1.3.2.7 BBB function during systemic inflammation and infection

1.3.2.7.1 Increased BBB permeability

BBB integrity and TJs could be disrupted during systemic inflammation and infection as a result of many different stimuli. Studies have shown that BBB integrity is disturbed in mice after a systemic challenge with *S. enterica* LPS, with a significantly higher brain to serum ratio of systemically-administered ^{125}I-albumin (185, 273). The expressions of TJs were also found to be decreased in rats with a significantly increased brain uptake of ^{14}C-sucrose after systemic inflammation was established by intraplantar injection of lambda-carrageenan (103). Another study further demonstrated that lambda-carrageenan-induced peripheral inflammatory pain (i.e., hyperalgesia) elicits a biphasic increase in BBB permeability, with the first phase occurring from 1-6 h and the second phase occurring at 48 h (163). Cytokines such as TNF-α, IL-1β and IL-6 are capable of inducing systemic inflammation and then cause enhanced BBB permeability. In experimental stroke, mice with systemic inflammation induced by peripheral IL-1β, demonstrated altered kinetics of BBB disruption, which was caused through conversion of a transient to a sustained disruption of the TJ protein, claudin 5 (164). Another study demonstrated that

following intracarotid administration in newborn pigs, TNF-α induced a time- and dose-dependent increase in BBB permeability, leading to an increase in the brain uptake of sodium fluorescein and Evan's blue-albumin (3). Other studies demonstrated controversial results with the injection of IL-2 and IL-6 (but not of IL-1β) inducing a significant enhancement in the brain uptake of ^{14}C-α-aminoisobutyric acid within several brain areas in rats and when given TNF-α, a striking decrease in BBB permeability was observed (229). It should be noted that the BBB-disrupting effects of these cytokines has been shown to be concentration-dependent and high concentrations of cytokines (over 10 ng/mL) are required to increase BBB permeability (3, 53, 251). Overall, it appears that systemic inflammation (mainly induced by LPS and cytokines) leads to altered BBB integrity and altered brain uptake of blood-borne substances. In order to better understand the disrupting-effect of LPS on the BBB, and also the interaction between colistin and LPS, a general review of LPS is required.

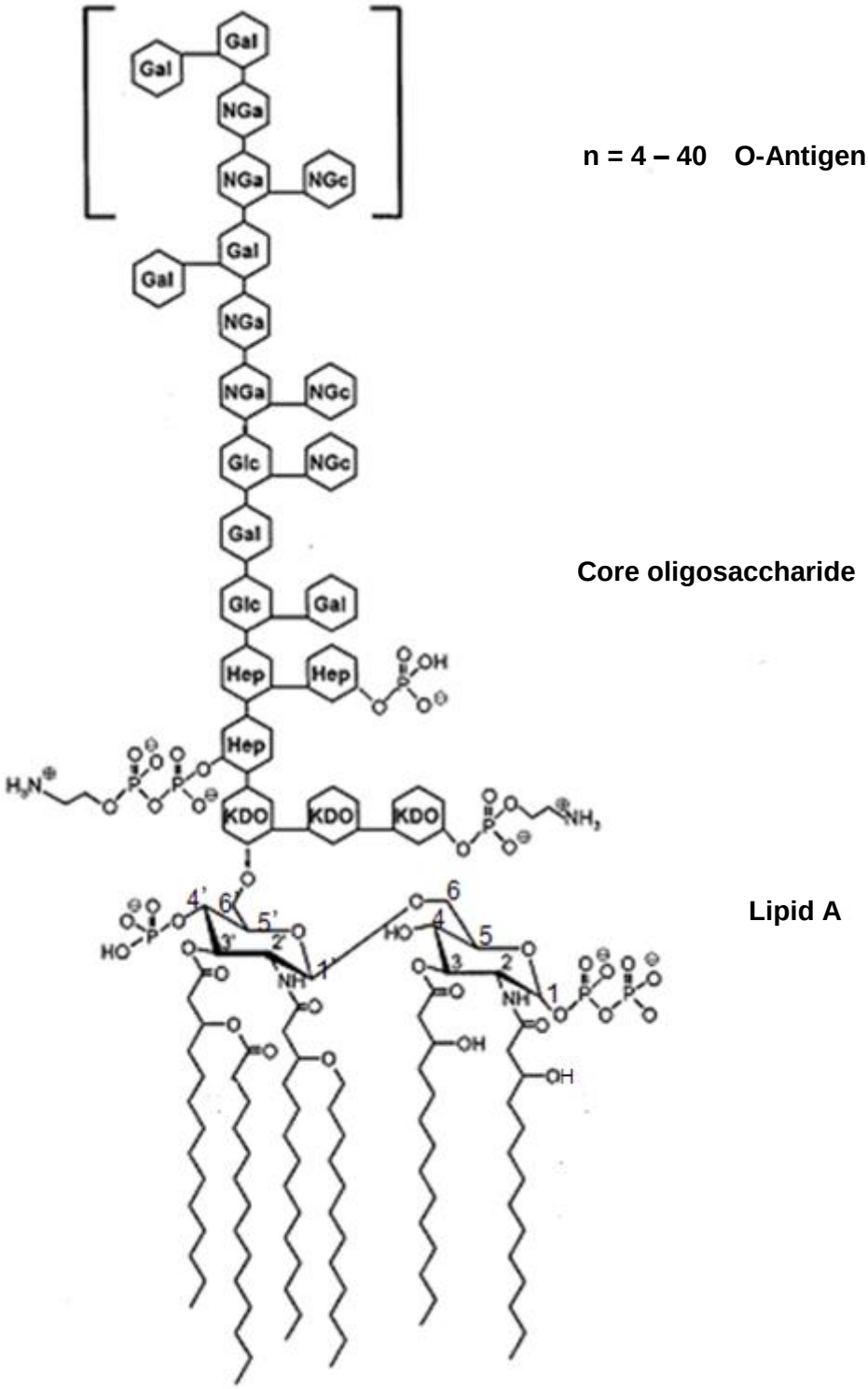


Figure 1-5. Chemical structure of *E. coli* LPS. (Hep) L-glycerol-D-manno-heptose; (Gal) galactose; (Glc) glucose; (KDO) 2-keto-3-deoxyoctonic acid; (NGa) N-acetyl-galactosamine; (NGc) N-acetyl-glucosamine. (reproduced from (Magalhães et al. 2007)) (155).

Gram-negative bacteria have two distinct membranes: an inner membrane and an outer membrane. LPS, also known as endotoxins, is the prominent constituent of the outer leaflet of the outer membrane (265). The LPS molecule consists of three parts: lipid A, core oligosaccharide and O-antigen repeating units (Fig. 1-5). Lipid A is covalently linked to the KDO (2-keto-3-deoxy-D-*manno*-oct-2-ulopyranosonic acid) monosaccharide residues of the core oligosaccharide, which has a conserved structure of the inner KDO-heptose region and the outer hexose region. The core oligosaccharide is directly connected to an outer O-antigen side chain through the attachment site at the outer region. The O-antigen side chain is generally composed of a sequence of identical oligosaccharides (with three to eight monosaccharides each), the structures of which are considerably diverse among different bacterial strains (204). The numbers of O-antigen repeating units can be up to 40 (242). The molecular mass of a LPS molecule is approximately 20 kDa, depending on the variability of the oligosaccharide side chain (155). LPS exhibits an amphipathic characteristic as lipid A represents the hydrophobic component locating in the outer leaflet of the OM, whereas hydrophilic core polysaccharides and O-antigen repeating units are displayed on the surface of the bacterial cells which serve a protective function for the bacterium (212). The core region close to lipid A and lipid A itself are partially phosphorylated and may carry phosphoethanolamine substituents, thus the LPS molecules are normally negatively charged (112). Divalent cations (Mg^{2+} and Ca^{2+}) associated with anionic lipid A serve to bridge adjacent LPS molecules and constitute a stable OM of Gram-negative bacteria (242). Cationic colistin molecules can bind

with the negatively charged lipid A and replace the divalent cations to initialize its antibacterial effect as mentioned in Section 1.2.3.

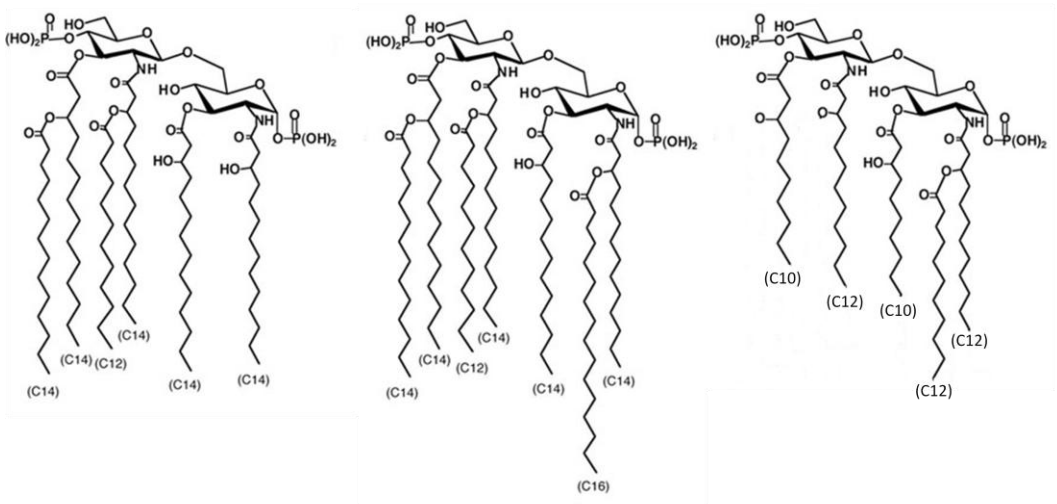
Lipid A is the highly conserved portion of LPS and is responsible for most of the biological activities of LPS (218, 256). Studies on Gram-negative bacteria including *Escherichia coli*, *P. aeruginosa* and *Salmonella enterica* have revealed a general structure of lipid A (219). Lipid A has a hydrophilic backbone, which is a β-1-6 linked disaccharide of glucosamine, and phosphorylated at positions 1- and 4'-. In addition, four 3-hydroxymyristate chains linked to both glucosamines at positions 2-, 3-, 2'- and 3'- are the hydrophobic moiety of lipid A. The hydroxyl groups of the 2-, 2'- and 3'- can be further esterified with laurate and myristate. Lipid A is linked to KDO through a hydroxyl group at the O-6'- position (280).

LPS is responsible for activation of the innate immune system and it has been demonstrated that lipid A is the biologically active component of LPS (8). The response from the host immune system depends on both the severity of infection and the particular structure of lipid A of the invading bacteria (170). After being introduced into the circulation, the lipid A component of LPS will be recognized by the LPS-binding protein and soluble CD14 to form a complex (255). This complex is subsequently bound to CD14 at the surface of the host cells, which then presents LPS to Toll-like receptor 4 (TLR4) (241, 249). The TLR4 is located on the membrane of many cells types including macrophages, dendritic cells and cerebral endothelial cells

(266). Through binding with TLR4, LPS may potentially disrupt the BBB integrity directly by activating various signaling molecules including nuclear factor-kappaB (NF-kB) and mitogen-activated protein kinase (MAPK) and/or indirectly through inducing the release of pro-inflammatory mediators from systemic cells.

Studies have shown that different LPS strains may impact on inflammatory mediator production (209), for example with *E. coli* LPS having a TNF-α-inducing activity eight-fold greater than *P. aeruginosa* LPS (285). The different abilities of LPS to induce immune response and promote TLR4 activation are largely arising from the lipid A structure including phosphate substituents at positions 1- and 4'- and in the number and composition of fatty acid chains (84, 219) (shown in Fig. 1-6). Lipid A with a hexa-acylated structure is able to activate human TLR4 response at a lower concentration than lipid A with a penta-acylated structure (89). Evidence also suggests that lipid A that adopts a conical conformation is more active than lipid A that adopts a cylindrical shape in binding to receptors (77, 237); and furthermore, lipid A with side chains of 12-14 carbons in length has been shown to stimulate immunological responses more efficiently than that with less or more carbons (170, 181). In addition, it has been demonstrated that the disruptive effect on the BBB induced by LPS is dose-dependent and the effective dose of each LPS may vary from strain to strain (55, 79, 259). Significantly increased sucrose permeability across the brain capillary endothelial cells was observed following treatment with *E. coli* LPS at the highest experimental concentration (i.e. 5 ng/mL) (54). Another study has showed that TEER

value in rat brain endothelial cell monolayers treated with *S. enterica* LPS was not changed until the concentration of LPS reached 0.1 μg/mL (259). All the studies mentioned above implied that different LPS may exert different effects on the BBB integrity and subsequent penetration of drugs to enter the CNS.



(a) *E. coli* (b) *S. enterica* (c) *P. aeruginosa*

Figure 1-6. The structural diversity of lipid A in Gram-negative bacteria. (a) hexa-acylated *Escherichia. coli* (b) hexa-acylated *Salmonella enterica* (c) penta-acylated *Pseudomonas aeruginosa*. (reproduced from (Ogawa et al. 2007)) (187).

Only a few studies have been performed to assess the impact of systemic infection (bacteremia) on BBB integrity. A high level of bacteraemia was achieved following inoculation of mice with *E. coli* and *S. pneumonia* in one study (251). The increased BBB permeability in mice was confirmed by transmission electron microscopic examination of brain tissues stained with horseradish peroxidase and it is suggested

that TNF-α generated during sepsis was the main reason for the induced leakage of brain microvascular vessels. Another study demonstrated an increase in the number of horseradish peroxidase-labelled cerebral vessels in rats infected with a peritoneal implant of *E. coli*, suggesting that peritonitis and bacteremia are associated with increased permeability of the BBB (58). However, whether this leads to altered brain uptake of drugs remains to be investigated.

Several reasons have been proposed to contribute to the opening of TJs at the BBB during systemic inflammation and infection. Firstly, elevated cytokines levels such as IFN-γ, TNF-α, IL-1β and IL-6 in the systemic circulation may affect BBB integrity through different mechanisms (36, 277) including increased myosin light chain kinase (MLCK) protein expression, NF-kB signaling activation and actin cytoskeletal contraction. NF-kB is a protein complex that controls the transcription of DNA. After activation by cytokines such as TNF-α or IL-1β, NF-kB is then translocated from cytoplasm to the nucleus to bind to the MLCK promoter region. As the MLCK promoter activity is up-regulated, MLCK mRNA transcription and MLCK protein expression are increased, which leads to an increase in MLCK activity. MLCK then activates the contraction of junctional actin-myosin filaments and alters the TJ proteins localization and expression. Other inflammatory mediators (e.g., chemokines, prostaglandins, and nitric oxide) could also be responsible for the opening of TJs (56). Secondly, *in vitro* studies also show that through binding between the lipid A component of LPS and LPS receptors, such as TLR-2 and TLR-4 at the BBB (196,

227), LPS could directly affect the BBB to increase the permeability through TLR-NF-kB or TLR-RhoA signaling pathway. RhoA is a protein known to regulate the actin cytoskeleton and the integrity of intercellular junctions. Activated RhoA induced by LPS causes the formation of actin stress fibres, which effectively retract the actin cytoskeleton of the cells and lead to disrupted TJs. Thirdly, given that increased BBB permeability has been observed in mice inoculated with *S. pneumonia*, a Gram-positive bacteria, it may suggest another non-LPS-related signaling pathway involving cell wall constituents of Gram-positive bacteria such as lipoteichoic acid and muramyl dipeptide (33). One study has shown that lipoteichoic acid from *S. pneumonia* has led to increased permeability in a brain endothelial cells monolayer in a concentration- and time-dependent manner, in which nitric oxide (NO) is potentially involved (20).

1.3.2.7.2 Altered expression and function of transporters

Acute inflammation induced by pro-inflammatory cytokines has been suggested to alter the expression and activity of BCRP and P-gp at the BBB. BCRP mRNA levels were significantly reduced by IL-1β, IL-6 and TNF-α in the human hCMEC/D3 cells (207). P-gp mRNA levels were slightly reduced by IL-6, but significantly increased after TNF-α treatment (207). Another *in vitro* study also found that TNF-α and IL-1β rapidly decreased BCRP mRNA expression within 6 h and this returned to control values at 24 and 48 h (263). P-gp protein expression was transiently increased after TNF-α addition within 6 h of incubation followed by a reduction at 24 and 48 h. LPS

treatment can also directly inhibit the P-gp function. An increased transport of P-gp ligands across cultured brain endothelial cells was observed (260). The observation was supported by another *in vitro* study demonstrating that *E. coli* LPS reduced P-gp activity in freshly isolated rat brain microvessels (92). Similar results were also found in animal studies. Down-regulated P-gp mRNA expression was observed in mice brain during inflammation induced by *E. coli* LPS and an increased brain penetration of digoxin, a well-know P-gp substrate, was detected (86). Another study also showed that after systemic challenge with *S. enterica* LPS in mice, P-gp function was first impaired as measured by P-gp protein expression and then stimulated through post-translational mechanisms (233). These results indicate that the penetration of BCRP and P-gp substrates into the brain could be affected during systemic inflammation. In addition, it is possible that other efflux transporters or drug metabolizing systems at the BBB could be affected by *E. coli* LPS as P-gp, MRP2 and cytochrome P450 expression has been shown to be reduced in endotoxin-treated rats (113).

Other than the active efflux transporters, a peptide transport system at the BBB responsible for delivering pituitary adenylate cyclase activating polypeptide was found altered during systemic inflammation suggesting a potential change of peptide supply from blood (185). In addition, the receptor-mediated transport of leptin was decreased as examined by brain uptake of radioactive leptin following a single intravenous injection during systemic inflammation with *S. enterica* LPS pretreatment

(184). The saturable transport of insulin across the BBB was found regulated by *S. enterica* LPS as well (274). So far, the mechanism behind the altered transporter systems during systemic inflammation and infection still remains unclear and will require further investigation.

1.3.2.7.3 Protection against LPS-mediated BBB disruption

Given that the disruption at the BBB induced by LPS is observed during systemic inflammation and infection, there has been interest in neutralizing the cytotoxic effect of LPS. Different strategies and molecules have been studied to protect the BBB from disrupting by LPS. Polymyxin B, which is another antibiotic from polymyxin family, has been shown to completely block the disrupting effect of *H. influenza* LPS on bovine brain endothelial cells (200). It is suggested that polymyxin B can bind to the lipid A component of LPS to shield its interaction with receptors (i.e. TLR4), similar as what colistin does, and block the downstream signaling (177). Human serum amyloid P component, which is a LPS-binding protein, alleviated the symptoms of *S. enterica* LPS-induced septic shock, and significantly inhibited the enhanced BBB permeability through binding to LPS as well (261). A negatively charged polysaccharide, pentosan polysulfate, has also been demonstrated to reduce the deleterious effects of LPS on the permeability in brain endothelial cells (259). The authors proposed that pentosan polysulfate may exert its protective effect either directly through blocking the interaction of LPS with TLRs or indirectly through protecting the negative luminal charge contributing to the barrier permeability. As

inflammatory mediators including cytokines and MMPs induced by LPS are possibly involved in BBB-disrupting process, studies have been performed to assess the BBB protective effect of inhibitors of inflammatory mediators. LPS-induced disruption of the BBB is reported to be reversed in the presence of MMP inhibitors such as GM6001 and quercetin in rats (138, 224). Similarly, minocycline treatment could prevent BBB from systemically-challenged LPS through decreasing the release of cytokines including TNF-α (250). It is also interesting to note that co-culture of brain capillary endothelial cells and glial cells caused no change on the endothelial cell monolayer permeability suggesting a protective effect of glial effect on the LPS-mediated injury (54).

Taken together, there is evidence to suggest that BBB integrity and various BBB transport systems appear to be affected during systemic inflammation and infection and it can be reasonably predicted that the brain exposure to substances which normally do not cross the BBB would be significantly higher during systemic inflammation or infection. This may lead to potential neurotoxicity especially for drugs such as colistin which is used to treat systemic infections.

1.4 Hypothesis and aims

The principal hypothesis of this project is that, given its physicochemical properties described in Section 1.2.2, the BBB transport of colistin will be minimal following systemic administration; however, during systemic inflammation and/or infection, the

brain uptake of colistin may be significantly enhanced due to alterations at the BBB. Furthermore, due to the LPS-binding effects of colistin, it is hypothesized that colistin can amoliate the BBB-disturbing effects of LPS.

Therefore, the aims of this project were:

1. To develop and validate a sensitive and reliable liquid chromatographic method for the determination of colistin concentrations in mouse brain homogenate
2. To assess the ability of colistin to permeate the BBB following systemic administration to healthy mice
3. To examine the impact of systemic inflammation induced via administration of LPS on the BBB transport of colistin in mice
4. To assess the effect of systemic infection on the BBB transport of colistin in mice
5. To investigate the mechanisms responsible for the alterations in BBB transport of colistin following systemic inflammation and infection
6. To investigate the impact of colistin co-administration on the BBB dysfunction induced by LPS

1.5 Structure of this thesis

The structure of this thesis can be summarized as:

1. A liquid chromatography method was developed and validated to quantify colistin concentrations in mouse brain homogenate. The recovery, accuracy and precision of the method have been assessed. The method was reliable and sensitive and was used to detect the brain uptake of colistin after a single intravenous dose. (Chapter 2, adapted from the manuscript published – Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. Brain Penetration of Colistin in Mice Assessed by a Novel High-Performance Liquid Chromatographic Technique. Antimicrob. Agents Chemother. 2009, 53: 4247-4251).

2. BBB transport of colistin was first assessed in healthy mice following subcutaneous dosing and *in situ* perfusion. The effect of P-gp inhibitors on the brain uptake of colistin was examined. To compare the results with colistin brain uptake during systemic inflammation, brain penetration of colistin was assessed in mice following *S. enterica* LPS administration. (Chapter 3, adapted from the manuscript published – Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. Impact of P-glycoprotein inhibition and lipopolysaccharide administration on blood-brain barrier transport of colistin in mice. Antimicrob. Agents Chemother. 2011, 55: 502-507).

3. The impact of systemic bacterial infection on BBB integrity and colistin brain uptake was investigated following inoculation of *P. aeruginosa*. The relationship between plasma levels of the pro-inflammatory cytokines and the degree of BBB

disruption was determined. (Chapter 4, adapted from the manuscript accepted –Liang Jin, Jian Li, Roger Nation and Joseph Nicolazzo. The Effect of Systemic Infection Induced by Pseudomonas aeruginosa on the Brain Uptake of Colistin in Mice. Antimicrob. Agents Chemother. 2012 Jul 30).

4. The dose- and species-dependent effects of two LPS species on BBB disruption were determined both *in vitro* and *in vivo*. The potential for colistin to ameliorate the BBB disrupting effect of *S. enterica* LPS (Chapter 5, is the subject of a manuscript which is currently in preparation).

1.6 Significance

As only a few studies have been performed to assess the impact of systemic inflammation and infection on the BBB, the results obtained in this project will provide a better indication of how BBB integrity and the brain uptake of a clinically-utilised antibiotic, is altered during these disease conditions. Secondly, given the potential for colistin to induce neurotoxicity, a better understanding of whether brain uptake differs during disease may provide some insight into the potential for colistin to induce centrally-mediated neurotoxicity. Thirdly, this project will provide insight into the BBB transport of drugs, which normally do not cross the BBB, during systemic inflammation and infection so that the potential risk of high brain exposure can be taken into account. Finally, the potential to ameliorate LPS-induced BBB disruption using a clinically-used antibiotic suggest another potential therapeutic application of colistin in Gram-negative bacteria-caused sepsis.

1.7 References

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Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

- Design of the study
- All laboratory experiments
- Data analysis and interpretation
- Preparation of the initial draft of the manuscript and subsequent revisions; and
- Formulation of conclusions and hypotheses arising from the results of the study

The extent of my contribution was 70%. The following co-authors also contributed to the work.

Name	Nature of contribution
Joseph A. Nicolazzo	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study
Roger L. Nation	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study
Jian Li	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study

Candidate's Signature [redacted] **Date** 06/08/12

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences

	Signature	Date
Signature 1	[redacted]	06/08/2012
Signature 2		6/8/2012
Signature 3		06/8/2012

Chapter Two

A novel high-performance liquid chromatographic assay for quantitating colistin in mouse brain homogenate

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2.1 Abstract

A sensitive and reliable liquid chromatographic method was developed and validated for the determination of colistin concentrations in mouse brain homogenate. With a mobile phase consisting of acetonitrile-tetrahydrofuran-water (50:25:25, v/v) at a flow rate of 1 mL/min, a linear correlation between peak area and colistin concentration was observed over the concentration range of 93.8 to 3000 ng/g in brain tissue (R^2>0.994). Intra- and inter-day coefficients of variation were 5.1-8.3% and 5.8-8.5%, respectively, and the recovery ranged from 85% to 94%. This assay was then utilized to determine the brain concentration of colistin over a 2 hour period following bolus intravenous administration of colistin sulfate to mice. After a single dose of 5 mg/kg to mice, brain homogenate concentrations of colistin were very low, relative to colistin plasma concentrations, suggesting that colistin permeability across the healthy blood-brain barrier is negligible during this experimental period.

2.2 Introduction

Colistin (also known as polymyxin E) is a cationic polypeptide antibiotic and is bactericidal against multidrug-resistant Gram-negative pathogens (8). Like other members of the polymyxin family, colistin consists of two major components (colistin A and B), with colistin A and B differing only in the fatty acid side chain. In studies conducted several decades ago, colistin was shown to result in adverse effects after intramuscular or intravenous administration to patients (6, 14); nephrotoxicity and neurotoxicity were the most commonly-observed adverse effects. It is possible that the prevalence of such toxicity may have been exaggerated due to a lack of understanding of colistin pharmacology and the use of inappropriate doses (22). Over the last few years, however, resistance to many other antibiotics and limited development of new antibiotics has resulted in an increasing use of colistin, administered in the form of its inactive prodrug colistin methanesulfonate (CMS) (1) for the treatment of infections caused by Gram-negative pathogens, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (5). With its increased use, interest in the pharmacology of this old antibiotic has been rekindled and optimization of dosage regimens is therefore urgently required in order to maximize its efficacy while minimizing potential toxicity.

Gram-negative bacterial species are increasingly reported to cause severe central nervous system (CNS) infections, such as meningitis (11, 24, 26) and ventriculitis (7, 30), which have a high mortality rate if untreated or treated inappropriately. There

have been several clinical case reports of successful treatment of meningitis and ventriculitis by parenteral administration of CMS, either alone or in combination with other antibacterials (11, 12, 18), resulting in eradication of the Gram-negative pathogens from the cerebrospinal fluid (CSF). These studies suggest that CMS, or colistin that is generated from CMS *in vivo*, has the potential to permeate the blood-cerebrospinal fluid barrier (BCSFB). Indeed, using a microbiological assay, Jiménez-Mejías et al. were able to detect 'colistin' in human CSF following intravenous administration of CMS (12). However, less is known about the ability of colistin to penetrate the blood-brain barrier (BBB), the endothelial lining of the cerebral blood vessels - a process which would be required in order to treat infections of the brain parenchyma, such as meningoencephalitis (27). A more thorough understanding of colistin penetration into the brain parenchyma, therefore, would be useful for optimizing treatment of such infections. In addition, the neurotoxicity associated with colistin (2) warrants a better understanding of colistin penetration across the BBB so that more rational dosing regimens can be designed with minimal potential for CNS-mediated toxicity. However, in order to accurately determine brain penetration of colistin, a robust and reliable analytical technique is required.

Numerous methods based on microbiological (15, 16, 19), thin-layer chromatographic (TLC) (32), immunological (13) and LC/MS (10, 25) techniques have been developed to quantitate the polypeptide antibiotics in plasma and tissues. While LC/MS methods are quite sensitive, they are often not very accessible, and the other techniques listed

above often lack sensitivity or selectivity required for accurate quantitation. With respect to colistin, several methods for assaying this polypeptide in rat or human plasma have been developed utilizing derivatizing reagents **s**uch as orthophthalaldehyde (OPA) (4, 17) and 9-fluorenylmethyl chloroformate (FMOC-Cl) (20, 21). While these assays have been successfully used to measure concentrations of colistin in human and rodent plasma and some tissue samples (34), quantitative assays for measuring the concentration of this antibiotic in animal and human brain tissues are not available.

The purpose of this study, therefore, was to develop a reliable and sensitive method to quantify colistin in mouse brain homogenate. Our method consists of protein precipitation with trichloroacetic acid (TCA), solid-phase extraction of colistin from brain tissue, derivatization with FMOC-Cl, followed by liquid chromatography with fluorescence detection. This method was then employed in our laboratory to determine the concentration of colistin in mouse brain homogenate following intravenous administration of colistin sulfate, to determine the potential of colistin to penetrate the BBB in healthy mice.

2.3 Materials and methods

2.3.1 Chemicals and reagents

Colistin sulfate was purchased from Zhejiang Shenghua Biok Biology Co., Ltd (EP5 grade, Zhejiang, China). FMOC-Cl, TCA, boric acid and sodium hydroxide were all

of analytical grade and obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia), and sodium hydrogen carbonate (analytical grade) was from Merck Chemicals (Kilsyth, Victoria, Australia). Acetonitrile (ACN), methanol and acetone of HPLC grade were all obtained from Merck KGaA (Darmstadt, Germany) and tetrahydrofuran (THF) was purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Carbonate buffer (1%, w/w, pH 10) was prepared as described previously (20). Solid-phase extraction (SPE) cartridges (C_{18} Sep-Pak®, 100 mg) were purchased from Waters (Waters Corporation, Massachusetts, USA).

2.3.2 Preparation of stock solutions, working standards and quality control (QC) solutions

A stock solution (1.00 mg/mL) of colistin sulfate was prepared in water and stored at 4°C. Working standard solutions with concentrations of 0.47, 0.94, 1.88, 3.75, 7.50 and 15.0 µg/mL were prepared by serial dilution of the stock solution with water. Quality control (QC) solutions were prepared separately at 0.47, 1.88 and 15.0 µg/mL, using an independently-prepared stock solution (1.00 mg/mL).

2.3.3 Choice of protein precipitant (ACN vs TCA)

While ACN is often used to precipitate brain homogenate proteins, extraction recovery of the compound of interest may be low, and so we compared the efficiency of both ACN and TCA as brain homogenate protein precipitants for this assay. Blank

mouse brains were homogenized in a volume of Milli-Q water (in mL) equal to twice the weight (in g) of the tissue using a Kinematica Polytron PT-DA 3007/2EC homogenizer (Luzernerstrasse, Switzerland). A 3000 ng/g colistin-spiked homogenate was prepared by adding 20 µL of a 15.0 µg/mL working standard into 280 µL of blank brain homogenate. Aliquots of this homogenate sample were subjected to either an ACN or TCA/ACN pre-treatment to precipitate proteins, as follows: To 300 µL of 3000 ng/g colistin-spiked brain homogenate was added ACN at varying volumes (300, 600, 900 or 1200 µL) or various TCA solutions (750 µL of 2% (w/v) TCA, 300 µL of 5% TCA, 150 µL of 10% TCA or 75 µL of 20% TCA). To TCA-treated homogenates, ACN was added at the same volume of TCA. Both ACN and TCA/ACN-treated brain homogenates underwent tissue processing as detailed below.

2.3.4 Preparation of calibration and QC samples

Calibration and QC samples were prepared by adding a 20 µL aliquot of each working standard or QC solution into 280 µL of blank brain homogenate to achieve concentrations of 93.8, 188, 375, 750, 1500 and 3000 ng/g. To each mixture was added 300 µL of 5% TCA, given that this was found to be the most appropriate tissue precipitant (see Results). This mixture was vortex-mixed for 5 min and another 300 µL of ACN was added prior to further vortex-mixing and centrifugation at 16,100 x*g* for 15 min. The entire supernatant was transferred to a SPE cartridge which had been conditioned by washing with 1 mL of acetone and 1 mL of methanol, followed by 1 mL of carbonate buffer. An aliquot (30 µL) of 100 mM FMOC-Cl and 80 µL of

methanol were then added to the SPE cartridge and after 10 min, colistin-FMOC derivatives were eluted with 900 µL of acetone. The eluent was vortex-mixed for 1 min and 40 µL of this solution was injected onto the HPLC column. The peak areas of the FMOC derivatives of colistin A and B were summed and employed for calibration (20). The low, medium and high QC samples of 93.8, 375 and 3000 ng/g were prepared in the same manner, and intra-day accuracy and precision were assessed. Intra-day assay performance was determined by comparative analyses of each of the six QC samples at each concentration, and inter-day assay parameters were determined by analysis of the QC samples on three separate days.

2.3.5 Chromatographic conditions

All chromatographic analyses were performed on a Shimadzu HPLC system consisting of an LC-10AT*vp* pump, a SIL-10ADvp autoinjector, a DGU-14A degasser and a RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan) connected to a data acquisition and process system (Shimadzu CLASS VP 6.14 SP1). The analyses were performed at 25°C. Samples were injected onto a Phenomenex Onyx Monolithic® C_{18} column (5×4.6 mm) using a mobile phase of ACN-tetrahydrofuran-water (50:25:25, v/v). The flow rate was set at 1 mL/min and colistin-FMOC was detected at an excitation wavelength of 265 nm and an emission wavelength 315 nm. For the linear least-squares regression analysis of the calibration curve, data were weighted according to 1/response.

2.3.6 Determination of recovery

Duplicate sets of colistin-spiked samples were prepared in either 300 µL of blank brain homogenate or the same volume of water. The concentrations used for determining recovery were 93.8, 375 and 3000 ng/g. The recovery was calculated by comparing the peak areas of colistin derivatives extracted from brain homogenate-spiked samples to those obtained from water-spiked samples.

To mimic the extraction scenario that would be encountered with samples obtained from *in vivo* mouse brain uptake studies, another set of colistin-spiked brain homogenate samples were prepared and the recovery of colistin measured after a 4 h incubation at 37°C. The reason for incubating colistin for 4 h in brain homogenate is that this would provide an opportunity for colistin to diffuse into the brain tissue and more closely resemble what would be expected in an *in vivo* brain uptake study. Brain homogenate (300 µL) at 93.8, 375 and 3000 ng/g was incubated in a 37°C shaking water bath for 4 h (120 rpm). After this period, water-spiked and brain homogenate-spiked samples underwent the extraction procedure described above and recovery was calculated in the same manner.

2.3.7 Mouse brain uptake study

Animal experiments were approved by the Faculty of Pharmacy and Pharmaceutical Sciences Animal Ethics Committee and performed in accordance with the National Health and Medical Research Council (NHMRC) guidelines for the care and use of

animals for scientific purposes. On the day of the study, a colistin sulfate dosing solution was prepared by dissolving colistin sulfate in saline, which was filtered with a 0.2 μm syringe filter (Minisart, Sartorius). The brain uptake study was carried out with 16 Swiss Outbred male mice (6-8 weeks of age, 22-28 g). A 50 μL bolus dose of colistin was administered to each mouse by tail vein injection at a dose of 5 mg/kg. Approximately 4 min prior to tissue harvesting, mice were anaesthetized with an intraperitoneal dose of 133 mg/kg ketamine and 10 mg/kg xylazine. At 5, 30, 60 or 120 min (n = 4 mice at each time point), blood was collected by cardiac puncture and centrifuged immediately to obtain plasma (~200 μL per sample). Animals were humanely killed by cervical dislocation, and then the whole brain was removed. Plasma and whole brain were stored at -20°C until analysis.

On the day of analysis, the brain was thawed and homogenized as described above. A 280 μL sample was taken and 20 μL of water was added, to match the homogenate standards. These brain homogenate samples were then processed as detailed above. Concentrations of colistin in mouse plasma were determined using the reported HPLC method (21), which was validated for mouse plasma in the current study. Precision and accuracy results from this assay validation demonstrate that the method for assaying colistin in rat plasma is transferable to mouse plasma.

2.4 Results and Discussion

2.4.1 Choice of precipitant (ACN vs TCA) and chromatography

Previously, HPLC methods for the measurement of colistin in rat and human plasma have been developed and validated using ACN to precipitate the proteins in plasma (20, 21). However, we were unable to use ACN for brain homogenate protein precipitation due to poor recovery of colistin, even at the highest concentration of colistin (3000 ng/g) and the largest volume of ACN (1200 µL). TCA, a widely used precipitating reagent in brain homogenate assays (3, 35), was consequently assessed for its ability to extract colistin at different concentrations and volumes, and the extent of recovery of colistin from each of the volumes and concentrations of TCA are shown in Table 2-1. An aliquot of 75 µL of 20% w/v TCA provided the lowest extraction of colistin, whereas 750 µL of 2% w/v TCA provided the highest extraction. However, to minimize the liquid handling on the SPE cartridge, it was deemed most appropriate to use 300 µL of 5% w/v TCA combined with the same volume of ACN, and this protocol was applied to carry out the assay validation. This extraction method provided satisfactory recovery at low, medium and high concentrations of colistin.

Table 2-1 Average HPLC peak areas of colistin obtained following addition of various combinations of TCA and ACN to 300 µL of 3000 ng/g colistin-spiked brain homogenate

Concentration of TCA (%, w/v)	**Volume of TCA (µL)**	**Volume of ACN (µL)**	**Peak area of colistin (n = 3, mean ± SD x 10^7)**
2	750	750	3.2 ± 0.3
5	300	300	3.1 ± 0.1
10	150	150	3.0 ± 0.4
20	75	75	1.9 ± 0.1

Under the current chromatographic conditions, well-resolved and symmetric peaks of FMOC derivatives of colistin A and colistin B were observed and there was no interference from components of the brain homogenate. The average retention times of FMOC derivatives of colistin B and colistin A were 5.6 and 6.5 min, respectively, while the run time was 9 min. Fig. 2-1 shows representative chromatograms of blank brain homogenate, a colistin-spiked brain sample and a brain homogenate sample obtained following intravenous administration of colistin (5 mg/kg) to a mouse.

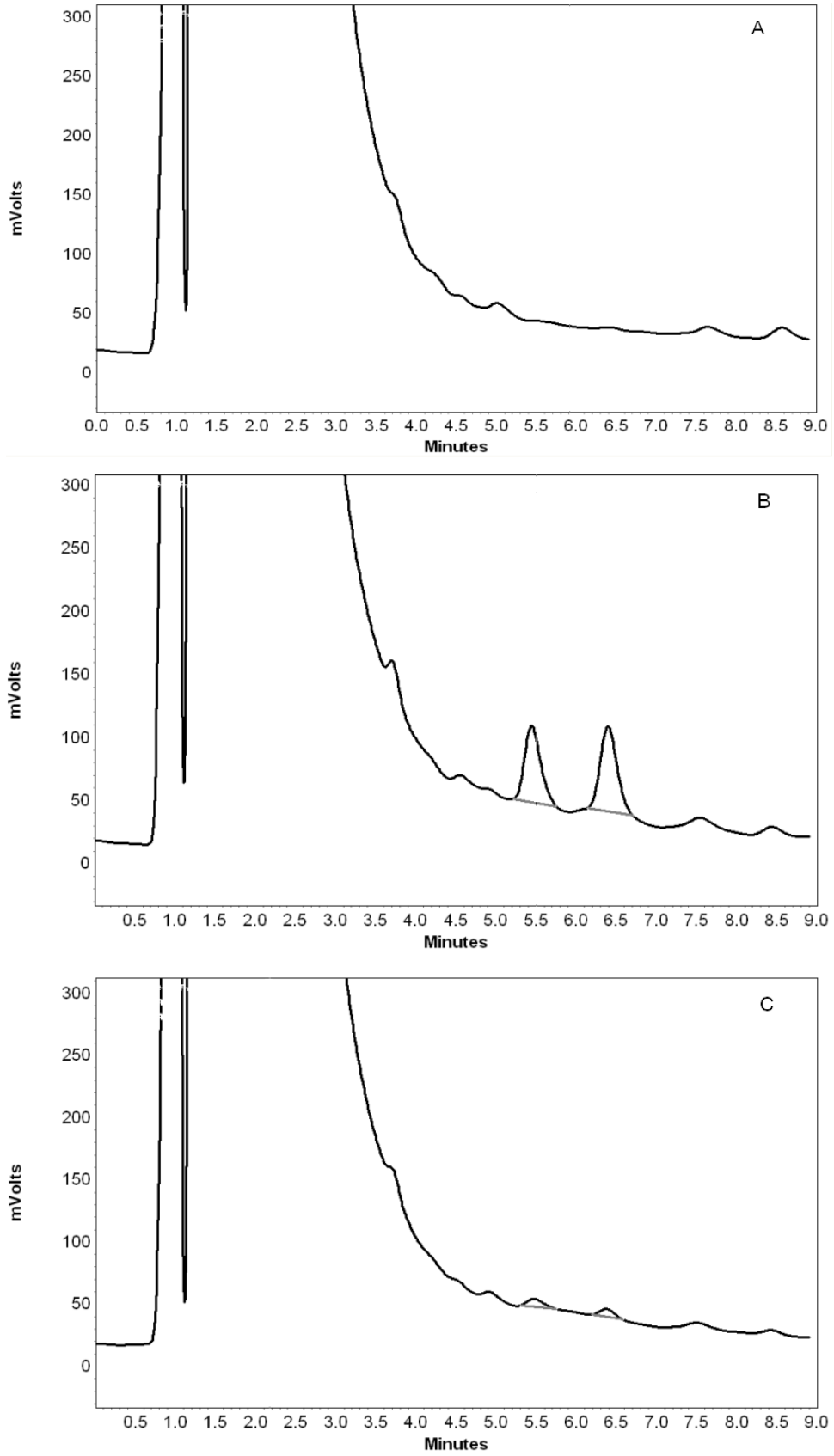

Figure 2-1 Chromatograms of (A) blank brain homogenate, (B) brain homogenate spiked with 3000 ng/g colistin and (C) brain homogenate at 5 min after intravenous

administration of colistin sulfate to a mouse at a dose of 5 mg/kg; the colistin concentration in brain homogenate in this mouse was 220 ng/g.

2.4.2 Linearity, precision and accuracy

The calibration curve relating colistin response to brain homogenate concentration exhibited good linearity (R^2>0.994) over the range of 93.8 to 3000 ng/g. The lower limit of quantitation was 93.8 ng/g, as demonstrated by the satisfactory precision and accuracy values presented in Table 2-2. Intra-day and inter-day precision for QCs at three different concentrations was less than 8.3% and 8.5%, respectively, and the accuracy ranged from 92 to 109%. These performance characteristics demonstrate that this novel method was robust and reliable for the determination of colistin concentration in brain homogenate following *in vivo* administration.

Table 2-2 Precision and accuracy values of the assay quantitating colistin in mouse brain homogenate

Parameter	**Target Concentration (ng/g)**	**Measured Concentration (ng/g) (mean ± SD)**	**Precision (%)**	**Accuracy (%)**
Intra-day	93.8	91.6 ± 7.2	7.9	97.7
(n = 6)	375	384 ± 31.8	8.3	102
	3000	3264 ± 167	5.1	108
Inter-day	93.8	97.5 ± 8.3	8.5	103.9
(n = 3)	375	345 ± 27.6	8.0	92
	3000	3210 ± 186	5.8	107

2.4.3 Recovery

The values of recovery for colistin at concentrations of 93.8, 375 and 3000 ng/g ranged from 85% to 94%. After a 4 h incubation of colistin sulfate in brain homogenate at 37^{o}C, the values of recovery for the three corresponding concentrations were 87%, 86% and 96%, respectively, indicating that colistin could be adequately recovered from brain tissue obtained from *in vivo* studies. While several HPLC methods have been reported for measuring colistin concentrations in human and rodent plasma (20, 21) and some tissue samples (34), this current method allows for excellent recovery and quantitative analysis of colistin in brain tissue. With some

minor modification, this method may also be utilized to quantify the concentration of colistin (and other polymyxins) in other tissues from rodents.

2.4.4 Brain uptake of colistin following intravenous administration to mice

The brain and plasma concentrations of colistin at various times after intravenous administration of colistin sulfate to mice at a nominal dose of 5 mg/kg are shown in Fig. 2-2. Mice appeared to tolerate this dose with no observed toxicity during the whole experimental period, which is consistent with earlier findings (31). While the concentration of colistin in plasma declined rapidly over the 2 h experiment, the concentration of colistin in the brain homogenate remained relatively constant over a 90 min period, albeit the concentrations were relatively low. This resulted in an increasing brain-to-plasma ratio with time (ranging from 0.02 to 0.06), suggestive of potential accumulation of colistin within the brain. However, further studies with multiple dosing or intravenous infusions would be required to more clearly identify the potential of colistin to accumulate within the brain, as there are limited data in the current study to conclusively confirm this hypothesis.

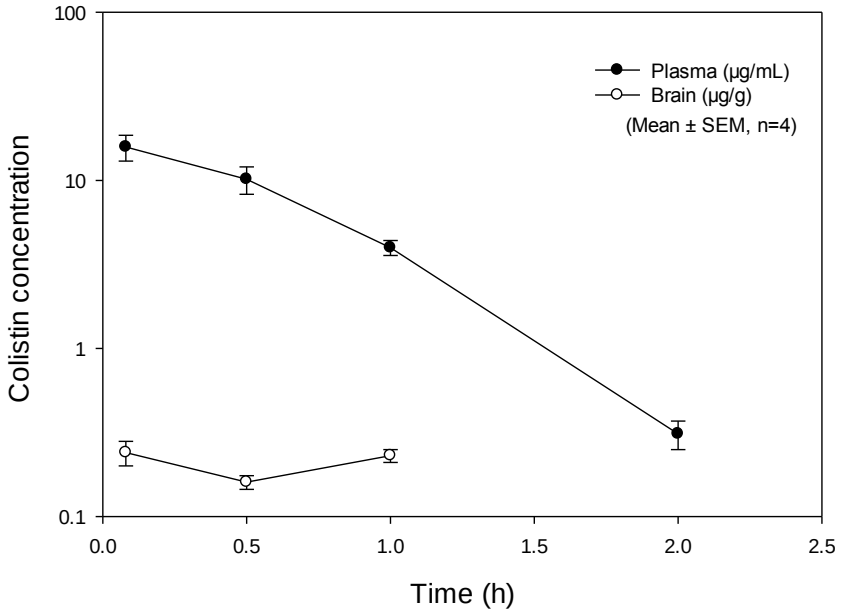

Figure 2-2 Plasma (●, μg/mL) and brain homogenate (○, μg/g) concentrations of colistin after intravenous administration of colistin sulfate to Swiss Outbred mice at a dose of 5 mg/kg. At 120 min, the concentration of colistin in brain homogenate was below the lower limit of quantitation. Data are presented as mean ± SD (n = 4).

To accurately determine if a compound has permeated the BBB using brain homogenate studies, it is common to account for the concentration of compound remaining within the brain microvasculature (33). Our group has determined the brain microvascular volume in Swiss Outbred mice to be 0.026 mL/g using ^{14}C-inulin as a vascular marker (unpublished data), and when this volume is taken into account, the concentrations of colistin within the brain parenchyma are close to zero. This indicates that any colistin detected in the mouse brain homogenate following a single intravenous dose of colistin sulfate is likely to be restricted within the brain microvessels, suggesting poor penetration across the healthy BBB. However, as

mentioned above, multiple doses of colistin (as prescribed clinically) may result in appreciable accumulation of this compound within the brain parenchyma, and future studies may be directed at addressing this issue.

The limited penetration of colistin into brain parenchyma following a single intravenous dose could be due to several factors. Firstly, the average molecular weight of colistin (1163) far exceeds the 'threshold' for optimum BBB permeability (450) (9). Secondly, the LogP values (where P is the octanol-to-water partition coefficient) of the two main components of colistin (i.e. colistin A and colistin B) are -3.15 and -3.68 (28), respectively, which are below the range of the LogP values recommended for passive permeation of the BBB (9). Thirdly, like many large cationic compounds, colistin may be actively excluded from the brain parenchyma by active efflux systems, such as P-glycoprotein (23). The mechanisms limiting the BBB penetration of colistin warrant further investigation so that appropriate measures can be taken to not only enhance colistin exposure for the treatment of life-threatening CNS infections, but to minimize the potential for CNS-induced neurotoxicity when colistin is being used for the treatment of systemic infections.

While colistin has been reported to penetrate the BCSFB (12), the current study demonstrates that colistin has minimal penetration into the brain parenchyma of healthy mice. The reason for this observed difference could be due to differences between the BBB and BCSFB transport of colistin, species differences associated

with CNS exposure, or the fact that the BBB and BCSFB are intact in the healthy mice, which during infection may become compromised to allow greater CNS exposure of colistin (29). Therefore, further studies may be performed to determine whether the penetration of colistin into the brain parenchyma is enhanced during meningitis and/or meningoencephalitis, which may be considered beneficial when treating meningoencephalitis and other infections of the brain parenchyma.

The use of the protein precipitation with TCA and derivatization with FMOC-Cl has allowed for the accurate and reproducible quantitation of colistin in mouse brain homogenate. This HPLC method is reliable and has been successfully applied to evaluate colistin penetration across the BBB in healthy mice. This method can be conveniently applied to determine whether the brain uptake of colistin is altered during meningitis and meningoencephalitis, which more closely mirrors the scenario encountered in patients, and may assist in the understanding of colistin efficacy and/or neurotoxicity in these infections.

2.5 Conclusion

A HPLC method involving protein precipitation with TCA and derivatization with FMOC-Cl was developed to measure colistin concentration in mice brain homogenate. This sensitive and robust HPLC method has been successfully applied to evaluate the colistin penetration across the BBB in healthy mice following a single intravenous injection which was minimal.

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Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

- Design of the study
- All laboratory experiments
- Data analysis and interpretation
- Preparation of the initial draft of the manuscript and subsequent revisions; and
- Formulation of conclusions and hypotheses arising from the results of the study

The extent of my contribution was 70%. The following co-authors also contributed to the work.

Name	Nature of contribution
Joseph A. Nicolazzo	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study
Roger L. Nation	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study
Jian Li	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study

Candidate's Signature [redacted] **Date** 06/08/12

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences

	Signature	Date
Signature 1	[redacted]	06/08/2012
Signature 2		6/8/2012
Signature 3		06/8/2012

Chapter Three

The impact of P-glycoprotein inhibition and lipopolysaccharide administration on the blood-brain barrier transport of colistin in mice

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3.1 Abstract

The aim of this study was to investigate the factors limiting the blood-brain barrier (BBB) transport of colistin under physiological conditions and to assess the impact of systemic inflammation on the transport of this antibiotic across the BBB. Colistin sulfate (40 mg/kg) was administered subcutaneously to Swiss Outbred mice to determine any relationship between brain uptake of colistin and plasma concentrations. To assess the effect of P-glycoprotein (P-gp) on BBB transport, colistin sulfate (5 mg/kg) was concomitantly administered intravenously with PSC833 or GF120918 (10 mg/kg). Systemic inflammation was induced by three intraperitoneal injections of lipopolysaccharide (LPS, 3 mg/kg) or saline, and colistin brain uptake was subsequently measured either following subcutaneous administration or by *in situ* brain perfusion. Brain-to-plasma (B:P) ratios of colistin were low (between 0.02 and 0.04) following single subcutaneous administration of colistin sulfate while multiple subcutaneous dosing exhibited the similar result (0.024 ± 0.002), and these ratios were not increased when P-gp inhibitors were co-administered. With pretreatment of LPS, the brain uptake was significantly increased following subcutaneous administration with AUC_{brain} values of 11.7 ± 2.7 μg·h/g and 4.0 ± 0.3 μg·h/g for LPS- and saline-treated mice, respectively (z=2.81). The findings were also supported by the results from *in situ* perfusion. This study, for the first time, showed that the BBB transport of colistin is significantly enhanced following systemic inflammation due to increased paracellular permeability, although the brain uptake of colistin in healthy mice is minimal and does not appear to be limited by P-gp mediated efflux.

3.2 Introduction

Colistin (polymyxin E) is one of two polymyxins clinically used to treat infections caused by Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumonia* (12). Although the clinical use of colistin waned in the 1970s due to concerns related to its adverse effects (10), colistin has re-emerged as a last-line therapy for Gram-negative bacterial infections, and is increasingly employed against multidrug-resistant Gram-negative bacteria (9). One of the adverse effects of colistin that has attracted the attention of clinicians and scientists is neurotoxicity (6, 8); however, it remains unknown as to whether this toxicity is centrally or peripherally mediated. If colistin were to exert any centrally-mediated toxicity, it would need to permeate the blood-brain barrier (BBB), the endothelial lining separating the brain parenchyma from the blood. It has been clinically demonstrated that intravenous administration of colistin methanesulfonate, an inactive prodrug of colistin (5), leads to detectable levels of colistin in cerebrospinal fluid (CSF) (1, 15, 21), however, this is an indicator of blood-CSF barrier penetration, and not BBB penetration. We have previously demonstrated that the brain uptake of colistin following a single intravenous dose to mice was negligible (16), suggesting minimal penetration across an intact BBB. However, the mechanisms governing the low BBB penetration of colistin remain unknown, and furthermore, it is unclear as to whether brain accumulation of colistin occurs with repeated doses (as is used clinically), given that our previous study only assessed colistin brain penetration after a single intravenous dose (16).

It is generally accepted that only small molecules with low molecular weight (<450 Da) and high lipid solubility permeate the healthy BBB by a passive transcellular process (13). The tight junctions of the inter-endothelial domains restrict the passage of large hydrophilic molecules through the paracellular route (14), and this is expected to be the main reason for the observed low BBB penetration of colistin (given a molecular weight of 1163 Da) (19). However, even for compounds that possess the appropriate physicochemical properties for transcellular permeation, efficient BBB transport is not always guaranteed due to the presence of active efflux proteins expressed at the luminal surface of brain endothelial cells (29). P-Glycoprotein (P-gp) is one such efflux transporter, and is responsible for restricting the brain penetration of a wide range of substrates, including anticancer drugs, analgesics and antibiotics (27). Many of these substrates share some common physicochemical properties such as a positive charge at physiological pH and a molecular weight >400 (7). Given that the primary amines of colistin possess a pK_a value of approximately 10 and colistin has a high molecular weight (19), it is plausible that the low brain uptake of colistin observed in our previous study (16) may also, in part, be due to P-gp mediated efflux.

While it is important to understand the mechanisms limiting colistin brain uptake under healthy conditions, it is crucial to identify whether colistin has a higher propensity to access the CNS during bacterial infections, where the integrity of the BBB may be compromised (26). Indeed, the clinical studies that demonstrated colistin

penetrates the blood-CSF barrier were undertaken in infected patients (1, 15, 21), and although those studies were representative of blood-CSF barrier penetration, a similar observation may also be made for colistin BBB penetration in the presence of a bacterial infection. Lipopolysaccharide (LPS), a key component of the outer membrane of Gram-negative bacteria, is often used to mimic the infective state as it results in the release of proinflammatory cytokines (25, 31). Several studies have shown that BBB disruption can be induced by LPS, which leads to increased brain uptake of compounds that normally exhibit limited access into the brain (3, 22). Whether a similar enhancement in BBB penetration would occur with colistin remains unknown, however, it is of importance to assess this phenomenon as this would better reflect the scenario encountered in infected patients. Therefore, the aims of the current study were to: (i) identify whether the brain uptake of colistin increases with repeated administration, under which conditions more sustained colistin plasma concentrations may be achieved; (ii) determine the impact of co-administration of a P-gp inhibitor on the brain uptake of colistin; and (iii) examine if the BBB penetration of colistin is enhanced following administration of LPS.

3.3 Materials and methods

3.3.1 Chemicals and reagents

Colistin sulfate was purchased from Zhejiang Shenghua Biok Biology Co., Ltd (EP5 grade, Zhejiang, China). Valspodar (PSC833) was a gift from Novartis (Basel, Switzerland) and elacridar (GF120918) was a gift from GlaxoSmithKline (Middlesex,

UK). ^{3}H-digoxin, Ultima Gold™ scintillation fluid and Solvable™ were purchased from Perkin Elmer (Boston, USA), and ^{14}C-inulin and ^{14}C-sucrose were obtained from American Radiolabeled Chemicals (St Louis, MO). Lipopolysaccharide from *Salmonella enterica* serotype typhimurium was obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Solid-phase extraction (SPE) cartridges (C_{18} Sep-Pak®, 100 mg) were purchased from Waters (Milford, MA). All other reagents were of analytical and/or HPLC grade and water was obtained from a Millipore purification system (Millipore Corporation, Billerica, MA).

3.3.2 Animal studies

Animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were performed in accordance with the Australian National Health and Medical Research Council (NHMRC) guidelines for the care and use of animals for scientific purposes. Male Swiss Outbred mice (6-8 weeks of age, 25-30 g) were used in all studies. Mice had free access to food and water during all experimental periods.

3.3.3 Brain uptake of colistin after subcutaneous administration

An aliquout (200 µL) of a sterile-filtered solution of colistin sulfate (equivalent to 40 mg/kg in saline) was administered to mice subcutaneously over the interscapular region. At 0.5, 1, 2 and 4 h, mice (n=4 at each time point) were anaesthetised with an intraperitoneal dose of 133 mg/kg ketamine and 10 mg/kg xylazine, blood was

collected by cardiac puncture and the whole brain was removed following cervical dislocation. Plasma and brain samples were stored at -20°C until the day of analysis. To determine whether the brain uptake of colistin increased with repeated administration, multiple subcutaneous injections of colistin sulfate were administered (at 0, 6 and 12 h) at the same dose and using the same method described above. Two hours after the last dose, plasma and brain samples were collected and stored at -20^{o}C until analysis. Concentrations of colistin in brain homogenate and plasma samples were measured using HPLC (16) and a brain-to-plasma concentration (B:P) ratio of colistin was calculated at each post-dose time point.

3.3.4 Impact of P-gp inhibitors on brain uptake of colistin

An aliquout (50 µL) of a colistin sulfate solution was administered to mice by tail vein injection (5 mg/kg) immediately after an intravenous injection of 50 µL of PSC833 (10 mg/kg), GF120918 (10 mg/kg) or blank vehicle (n=6 for each pretreatment). The blank vehicle consisted of 20% (v/v) ethanol, 60% (v/v) PEG400 and 20% (w/v) glucose. Mice were anaesthetised (as detailed above) and plasma and brain samples were harvested 5 min after administration of colistin sulfate. Samples were analysed for colistin (16) and B:P ratios of colistin, in the presence and absence of P-gp inhibitors, were generated.

To ensure that these doses of PSC833 and GF120918 were effective in inhibiting P-gp function at the BBB, the brain uptake of the P-gp substrate digoxin was assessed in

the presence and absence of the inhibitors. An aliquot (50 µL) of a ^{3}H-digoxin solution (40 µCi/mL) was administered to mice by tail vein injection in a vehicle consisting of 20% (v/v) ethanol, 60% (v/v) PEG400 and 20% (w/v) glucose with or without PSC833 or GF120918 (10 mg/kg) (n=6 mice for each group). Plasma and brain samples were collected 5 min post-dose, and radioactivity in plasma and brain determined using liquid scintillation counting. Briefly, 50 µL of plasma was placed into a 5-mL scintillation vial and 2 mL of Ultima Gold^{TM} scintillation fluid was added, followed by brief vortex mixing. Brain samples were placed into 20-mL scintillation vials containing 2 mL of Solvable^{TM} which were maintained at 50°C overnight. The next day, 200 µL of hydrogen peroxide (30% v/v) was added to the digested brains to bleach the samples, and allowed to sit for 30 min at 50°C. An aliquout (10 mL) of Ultima Gold^{TM} scintillation fluid was then added to each vial containing brain digest, followed by brief vortex mixing. Radioactivity in both brain digest and plasma samples was then measured by liquid scintillation counting (Tri-carb 2800 TR, Perkin Elmer, Boston, MA) and a B:P concentration ratio was calculated by the formula:

$$B:P = (\text{dpm/g of brain}) / (\text{dpm/mL of plasma})$$

3.3.5 Effect of LPS on brain uptake of colistin

Mice were administered intraperitoneal injections (200 µL) of 0.9% w/v saline (control) or LPS (3 mg/kg in saline) at 0, 6 and 24 h. An aliquout (200 µL) of a colistin sulfate solution (40 mg/kg in saline) was then administered subcutaneously to mice over the interscapular region 4 h after the third LPS or saline dose. At various

time points after administration of colistin (0.5, 1, 2 and 4 h), plasma and brain samples (n=4 mice at each time point) were harvested and concentrations of colistin in brain homogenate and plasma were determined by HPLC to obtain B:P concentration ratios at each time point. The area under the plasma concentration-time curve ($AUC_{plasma,\ 0\text{-}4h}$), the area under the brain concentration-time curve ($AUC_{brain,\ 0\text{-}4h}$), and their associated variances were determined by Bailer's approach (2).

It is possible that any enhancement in colistin brain uptake following LPS administration could have been due to a direct effect of LPS on BBB integrity or an indirect effect (e.g. an alteration to plasma protein binding and unbound fraction of colistin). To delineate these effects, the BBB transport of colistin was measured in saline- and LPS-treated animals using a modified *in situ* perfusion technique (4). The perfusion fluid used was carbogenated Krebs buffer consisting of the following components (mmol/L): 128.0 NaCl, 24.0 $NaHCO_3$, 4.2 KCl, 2.4 NaH_2PO_4, 1.5 $CaCl_2$, 0.9 $MgSO_4$ and 9.0 D-glucose. The buffer was carbogenated with 5% CO_2/95% O_2, the pH was adjusted to 7.4 and the buffer was warmed to 37°C before the experiment. Mice were pretreated with LPS or saline, as described above, and 4 h after the last administration, mice were anesthetised. After opening the thorax, the heart was exposed, and the descending thoracic aorta was clamped, followed by severing of both the left and right jugular veins. A 21-G butterfly needle was inserted into the left ventricle of the heart. To mimic the plasma concentrations obtained after multiple subcutaneous administration of colistin, 40 μg/mL of colistin was infused at a rate of

2 mL/min for 4 min. At the completion of the perfusion, brains were removed, and stored at -20^{o}C until the day of analysis. Three aliquots of the perfusion fluid eluting from the tubing were also collected and stored at -20^{o}C until the day of analysis. The concentrations of colistin in brain homogenate were then determined by the HPLC method described previously (16). The concentration of colistin in the perfusate solution was also determined using the above assay with minor modification. Briefly, 50 µL of acetonitrile was added to 50 µL of perfusion fluid, the mixture was vortex-mixed for 1 min and centrifuged at 16,100 × *g* for 10 min before being loaded onto a SPE cartridge. All other derivatisation and analytical parameters were identical to those reported previously (16). The calibration curve relating chromatographic peak area to perfusate colistin concentration exhibited good linearity (r^2>0.993) over the range of 0.313 to 10.0 µg/mL. The coefficient of variation for quality control samples (n=6) prepared at 0.625 µg/mL and 10.0 µg/mL was less than 4.4% and the accuracy values of these replicates ranged from 108.8% to 110.5% of the nominal concentrations.

To ensure that the LPS regimen used in the studies above was affecting the integrity of the BBB, the brain uptake and BBB transport of ^{14}C-sucrose and ^{14}C-inulin were determined following intravenous administration and *in situ* perfusion, respectively. At 4 h after the last saline or LPS administration, mice were intravenously administered a 50-µL solution of ^{14}C-inulin (2 µCi in saline) or ^{14}C-sucrose (2 µCi in saline) (n=6 per group). Plasma and brain samples were obtained 5 min following

intravenous administration, and samples were measured for radioactivity, as described previously. Similarly, 4 h after the last pre-treatment with saline or LPS, the BBB penetration of ^{14}C-sucrose or ^{14}C-inulin was determined following perfusion of each compound (0.25 μCi/mL) at a rate of 2 mL/min for 4 min (n=3 per group). Brain and perfusate were collected, and samples were prepared for liquid scintillation counting, as described previously. The brain-to-plasma or brain-to-perfusate ratio of ^{14}C-sucrose and ^{14}C-inulin (as a measure of BBB integrity) was calculated by comparing the concentration of compound in brain (dpm/g) to that in plasma or perfusate (dpm/mL).

3.3.6 Data analysis

All data are presented as mean ± SD, unless otherwise stated. When comparing the B:P or brain-to-perfusate ratios between saline- (control) and LPS-treated animals, a Student's *t*-test was used, whereas B:P ratios between vehicle-, PSC833- and GF120918-treated animals were compared using a one-way analysis of variance followed by a Newman-Keuls multiple comparisons test (PASW Statistics for Windows, version 17.0, Chicago, Illinois). A p value < 0.05 was considered to be a significant difference. When comparing AUC_{plasma} or AUC_{brain} between saline- and LPS-treated groups using Bailer's approach, a *z*-test was used to test significant differences. A z value > 1.96 was considered to be a significant difference between two groups.

3.4 Results

3.4.1 Brain uptake of colistin after single and repeated subcutaneous administration

Mice tolerated a subcutaneous dose of 40 mg/kg of colistin sulfate with no observed toxicity over the experimental period. Subcutaneous administration of colistin sulfate to mice at this dose resulted in sustained plasma concentrations of colistin over the 4-h sampling period. As shown in Fig 1, the maximum plasma concentration of colistin was 37.7 ± 5.0 μg/mL (at 1 h post-dose), and these concentrations were maintained over the 4 h dosing period. Despite the higher and sustained plasma concentrations with subcutaneous administration, the concentrations of colistin in brain were very low at all four post-dose time points (Fig. 3-1), resulting in B:P ratios of 0.021 ± 0.009, 0.037 ± 0.014, 0.029 ± 0.005, and 0.028 ± 0.007, respectively. Similar results were also obtained following multiple subcutaneous dosing to mice. Two hours after the last of 3 subcutaneous doses of colistin sulfate (40 mg/kg), the plasma concentration of colistin was 32.0 ± 3.5 μg/mL, yet the brain colistin concentration at this time was 0.8 ± 0.1 μg/g resulting in a mean B:P ratio of 0.024 ± 0.002 (n=4).

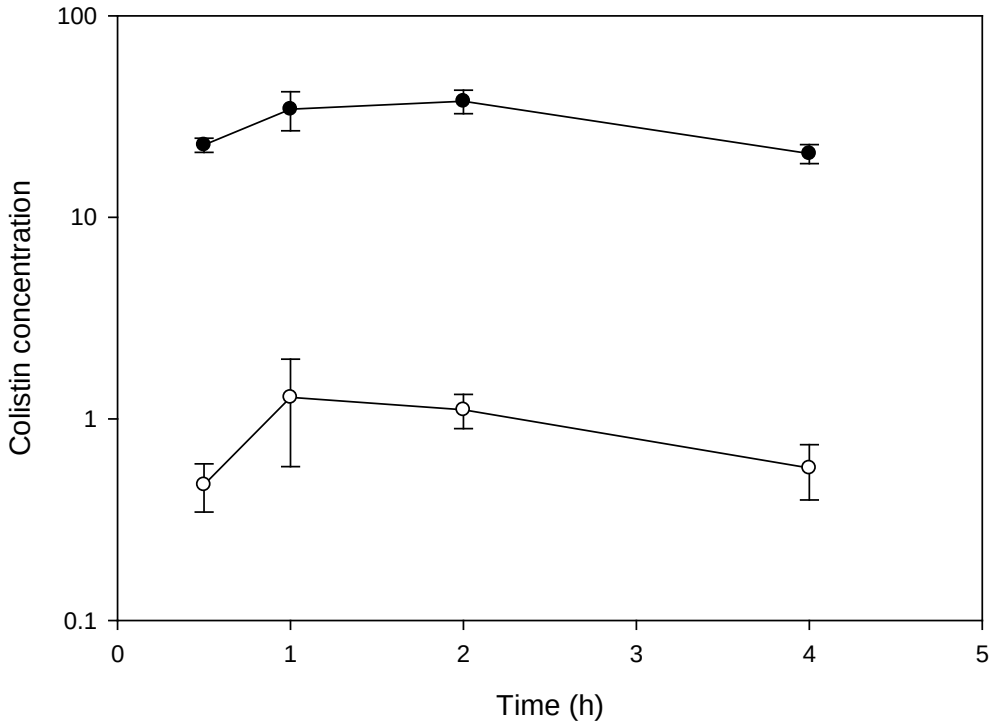

Figure 3-1 Plasma (●, μg/mL) and brain (○, μg/g) concentrations of colistin following single-dose subcutaneous administration of colistin sulfate (40 mg/kg) to Swiss Outbred mice. Data are presented as mean ± SEM (n=4).

3.4.2 Impact of P-gp inhibitors on brain uptake of colistin

The B:P ratios of ^{3}H-digoxin in the presence and absence of P-gp inhibitors are shown in Fig. 3-2A. Both of these inhibitors significantly (p<0.05) enhanced the brain uptake of ^{3}H-digoxin, demonstrating that the dose of inhibitors (10 mg/kg) was valid for assessing the involvement of P-gp in colistin brain uptake. However, the average B:P ratio of colistin following administration of the same dose of P-gp inhibitors was still extremely low (Fig. 3-2B); there was no significant difference in the B:P ratios of colistin between mice treated with vehicle, PSC833 or GF120918 (p > 0.05).

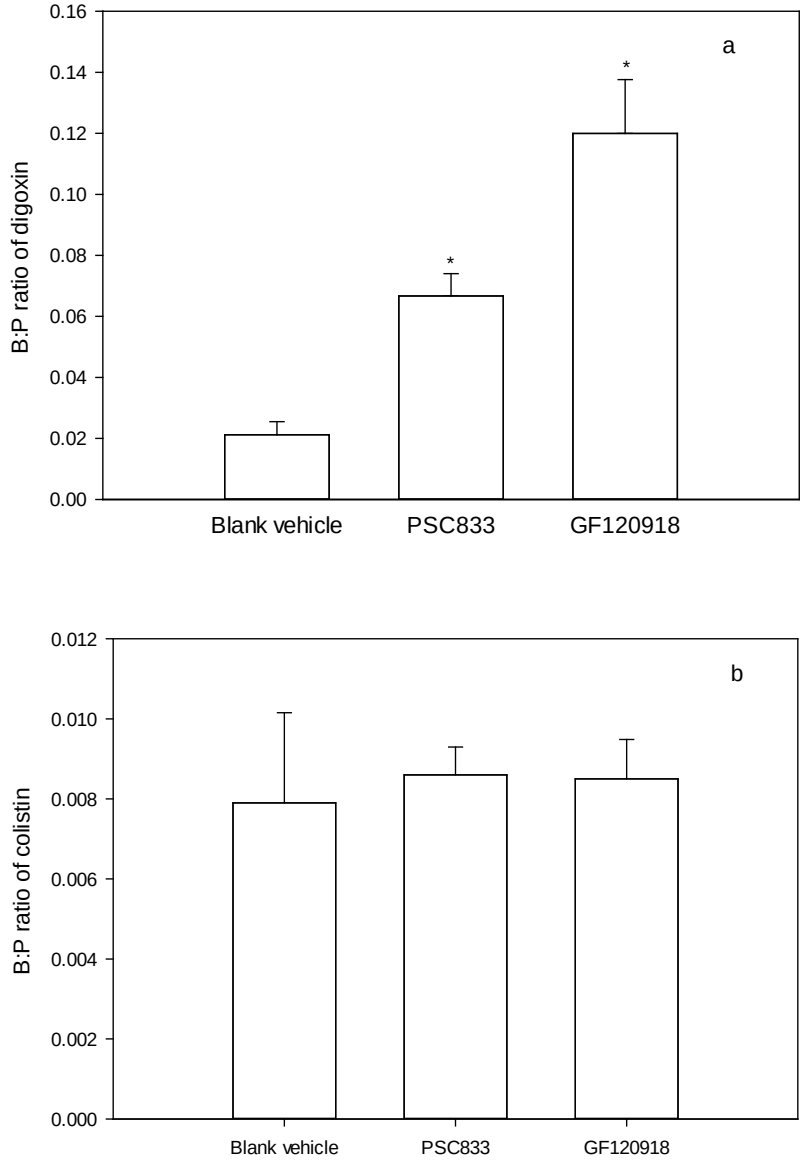

Figure 3-2 (a) B:P ratios of ^{3}H-digoxin 5 min after intravenous administration (2 µCi) to mice without (vehicle) or with co-administration of PSC833 or GF120918 (10 mg/kg). (**p* <0.05 between PSC833 or GF120918 and vehicle group using a one-way analysis of variance and Newman-Keuls multiple comparison test). (b) B:P ratios of colistin 5 min after intravenous administration of colistin sulfate (5 mg/kg) to mice without (vehicle) and with co-administration of PSC833 or GF120918 (10 mg/kg). Data are presented as mean ± SEM (n=6).

3.4.3 Brain uptake of colistin after pretreatment with LPS

The plasma and brain concentrations of colistin after subcutaneous administration of colistin sulfate to saline- and LPS-treated mice are shown in Fig. 3-3A. The plasma concentrations of colistin were not affected by administration of LPS at any post-dose time points, with AUC_{plasma} not significantly different between LPS- and saline-treated groups (values of 117.5 ± 9.2 μg·h/mL and 121.4 ± 8.2 μg·h/mL, respectively, z=0.33). Despite plasma concentrations being unaffected by LPS treatment, the brain concentrations of colistin in LPS-treated animals were significantly higher at all post-dose time points, as shown in Fig. 3-3A. The average B:P ratios of colistin at the designated time points in saline- and LPS-treated animals are shown in Fig. 3B. In addition, the overall brain exposure to colistin was significantly enhanced following LPS pre-treatment with AUC_{brain} values of 11.7 ± 2.7 μg·h/g and 4.0 ± 0.3 μg·h/g for LPS- and saline-treated mice, respectively (z=2.81). To ensure that LPS did not interfere with the HPLC analysis of colistin, and that the increased peak areas observed in brain homogenate were indeed due to increased brain concentration of colistin, the HPLC methods were also validated using brain and plasma from LPS-treated mice not exposed to colistin (see Appendix 1). No change in colistin chromatographic peak areas or retention times were observed in the presence of LPS, indicating that the increased peak areas detected in brain homogenates following LPS pretreatment were indeed reflective of increased brain concentrations of colistin.

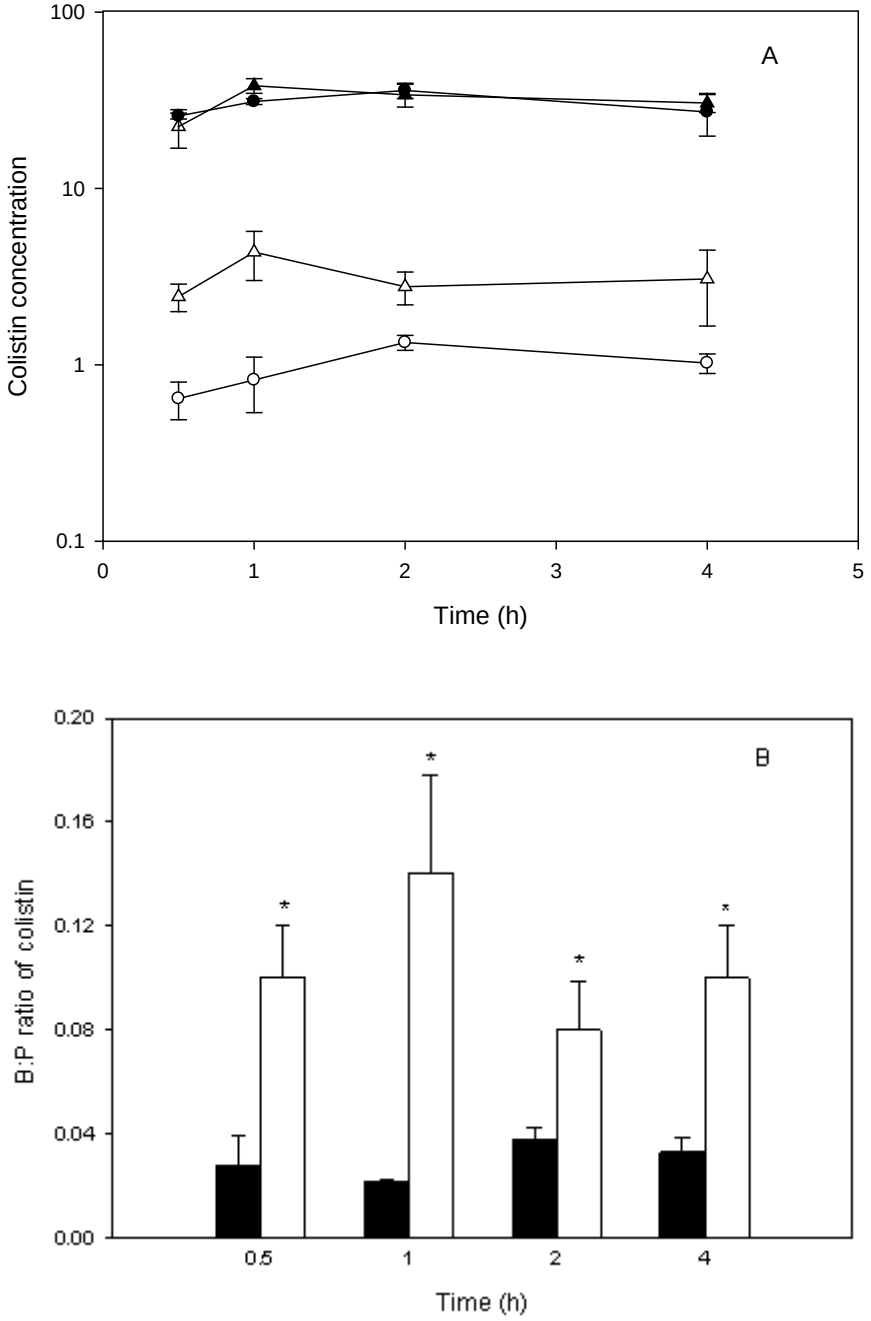

Figure 3-3 (A) Plasma (μg/mL) and brain (μg/g) concentrations of colistin after subcutaneous administration of colistin sulfate (40 mg/kg) to Swiss Outbred mice 4 h after the final dose of a pre-treatment regimen (comprising administration at 0, 6 and 24 h) with intraperitoneal LPS (3 mg/kg) (▲: plasma, Δ: brain) or saline (●: plasma, ○: brain). Data are presented as mean ± SEM (n=4). (B) Corresponding B:P ratios of

colistin from data depicted in (A) after intraperitoneal LPS (3 mg/kg) (□) or saline (■) administration. Data are presented as mean ± SEM (n=4, **p* <0.05 between saline- and LPS-treated animals using a Student's *t*-test).

Using the *in situ* perfusion technique, the BBB permeability of colistin was shown to be significantly ($p<0.01$) enhanced in LPS-treated mice (Fig. 3-4), with brain-to-perfusate ratios of 0.019 ± 0.001 and 0.014 ± 0.001, for LPS- and saline-treated mice, respectively. However, the increase in the brain uptake of colistin mediated by LPS as measured by the *in situ* perfusion (36% increase) was not as large as the effect measured following subcutaneous administration of colistin (average 286% increase). These findings suggested that either (i) the effect of LPS may have been due to an event imparted in the systemic circulation (e.g. altered plasma protein binding of colistin), or (ii) that the *in situ* perfusion method is a less sensitive technique than the *in vivo* brain uptake technique for detecting changes to BBB permeability for poorly-penetrating compounds. Therefore, the effect of LPS on the integrity of the BBB was compared following *in vivo* administration and *in situ* perfusion of the BBB integrity markers, ^{14}C-sucrose and ^{14}C-inulin. The B:P ratios of ^{14}C-sucrose and ^{14}C-inulin following intravenous administration in saline- and LPS-treated mice are shown in Table 3-1. The average B:P ratios of ^{14}C-sucrose and ^{14}C-inulin were 0.035-0.038 in saline-treated animals, whereas pretreatment with LPS resulted in average apparent B:P ratios of 0.065-0.069. This approximate doubling in the B:P ratios of ^{14}C-sucrose or ^{14}C-inulin, following administration of LPS, is

suggestive of a significant disturbance in the integrity of the BBB. However, when assessed using the *in situ* perfusion technique, the extent of BBB damage (as measured by the brain-to-perfusate ratio of ^{14}C-sucrose and ^{14}C-inulin) did not appear to be as marked as that observed following intravenous administration of these BBB markers (Table 3-2). Compared with the doubling in the apparent B:P ratio induced by LPS measured following intravenous administration of the BBB markers, the *in situ* perfusion technique demonstrated only a 20-32% increase in the brain-to-perfusate ratio, albeit this increase was still significant (p<0.05).

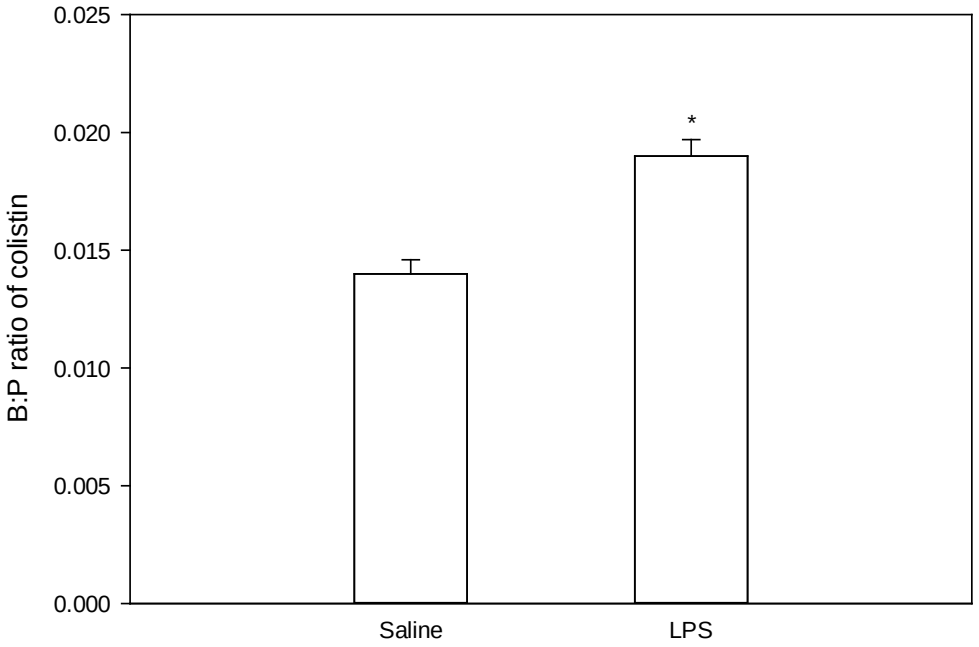

Figure 3-4 Brain-to-perfusate ratios following *in situ* perfusion of colistin (40 μg/mL) at a rate of 2 mL/min for 4 min in mice pre-treated with LPS (3 mg/kg) or saline (0, 6 and 24 h). Data are presented as mean ± SEM (n=4, *p <0.01 using a Student's t-test).

Table 3-1 B:P ratios of ^{14}C-inulin and ^{14}C-sucrose following intravenous administration (2 μCi) to saline- and LPS-pretreated Swiss Outbred mice. Data are presented as mean ± SD (n=6).

Pre-treatment	B:P ratio	
	^{14}C-inulin	^{14}C-sucrose
Saline	0.038 ± 0.004	0.035 ± 0.007
LPS	0.065 ± 0.016*	0.069 ± 0.007*

*p<0.05 compared to saline pre-treatment using a Student's *t*-test.

Table 3-2 Brain-to-perfusate ratios of ^{14}C-inulin and ^{14}C-sucrose following *in situ* perfusion at a rate of 0.25 μCi/mL/min for 4 min to saline- and LPS-pretreated Swiss Outbred mice. Data are presented as mean ± SD (n=3).

Pre-treatment	Brain-to-perfusate ratio	
	^{14}C-inulin	^{14}C-sucrose
Saline	0.019 ± 0.001	0.024 ± 0.002
LPS	0.025 ± 0.002*	0.029 ± 0.003*

*p<0.05 compared to saline pre-treatment using a Student's *t*-test.

3.5 Discussion

The relentless increase in resistance to almost all currently available antibiotics in Gram-negative bacteria, in particular *P. aeruginosa*, *A. baumannii* and *K. pneumoniae*,

together with the shortage of new antibiotics with activity against these pathogens reaching the clinic, has caused substantial worldwide concern (17). Consequently, the relatively old polymyxin antibiotic colistin is being increasingly used as a salvage therapy (20, 24). While there are a great number of clinical cases and reports on the use of colistin (1, 15, 18), there is little information available on the BBB transport of this antibiotic, which may assist in understanding the general disposition of this increasingly important antibiotic.

Consistent with its physicochemical properties, our previous study demonstrated that the brain penetration of colistin was negligible in mice after a single intravenous dose (16). It is possible that the low brain uptake of colistin observed following this dosage regimen in mice is not representative of that which may occur under circumstances where the colistin plasma concentrations are sustained at a relatively high level. Therefore, in the current studies, colistin sulfate was administered subcutaneously, and while plasma colistin concentrations were maintained in the range of 20.7 – 37.7 μg/mL over the 4 h experimental period which is higher than the initial plasma concentration obtained following intravenous administration of colistin sulfate at 5 mg/kg (15.8 ± 2.8 μg/mL), the mean B:P ratios of colistin were no higher than those observed from the previous intravenous study (16). Similar results were also obtained after administering colistin in a multiple dose regimen, more reflective of the scenario encountered clinically. Therefore, it is likely that in patients with an intact BBB, penetration of colistin into the brain would be minimal, even if this antibiotic was

dosed multiple times to allow for sustained plasma concentrations. This negligible entry of colistin across a healthy BBB is most likely to be impacted by the tight junctions sealing the paracellular route, hindering the penetration of this, and other, large molecular weight compounds.

While the inter-endothelial tight junctions minimize paracellular penetration of compounds, the transcellular transfer of many compounds can be retarded by P-gp (27). Given that colistin exhibits some of the characteristics possessed by known P-gp substrates (cationic charge at physiological pH and high molecular weight) (19), we investigated whether efflux by P-gp was also contributing to the low brain uptake of colistin. In this study, the brain uptake of ^{3}H-digoxin, a commonly used P-gp substrate (28) significantly increased when co-administered with PSC833 or GF120918, which indicated that the function of P-gp could be effectively attenuated with the dose of inhibitors used. However, when administered at the same dose, the inhibitors had no effect on the brain uptake of colistin. Since blank vehicle did not significantly change the colistin concentration in brain or plasma and showed no difference with previous intravenous study (16) with regard to the B:P ratio at 5 min post-dose (p>0.05), it is suggesting that P-gp does not contribute to the low brain uptake of colistin, and that it is likely that the existence of the tight junctions sealing the paracellular route is the main contributor to the low BBB penetration of colistin.

To further confirm that tight junctions were the major hindrance to colistin BBB

penetration, we intentionally disturbed the BBB paracellular integrity by systemic administration of LPS. The administration of LPS not only causes dysfunction of the tight junctions and decreased resistance of the paracellular route (32), but also has been shown to cause a down-regulation of efflux transporters, such as P-gp (11). Given that we have demonstrated that colistin appears not to be a substrate of P-gp, any increase in the brain uptake of colistin following LPS administration is therefore likely to be a result of increased paracellular diffusion. Adminstration of LPS also served another important purpose in this study in that it can reflect the infected state in which colistin is normally clinically used (25). The reduced BBB integrity during the infected state may be beneficial as it may lead to increased antibiotic concentrations in the brain. This may be an advantage if treating brain infections, or a disadvantage if the site of infection is outside the CNS and the antibiotic may cause centrally-mediated adverse effects such as neurotoxicity. The brain uptake of colistin was significantly enhanced after administration of LPS, as was the uptake of ^{14}C-sucrose and ^{14}C-inulin, suggesting that the enhanced colistin brain uptake was likely a result of increased BBB paracellular permeability, as might occur during the infected state (32). Moreover, the conclusion was supported by the results from the *in situ* brain perfusion technique showing a significant increase of brain uptake of colistin and the BBB markers following pretreatment with LPS, albeit the effect was not as marked *in situ* as *in vivo*, an effect which has also been observed in previous studies (23, 30). What remains unknown, however, is whether a similar increase in brain uptake of colistin will be observed in the presence of bacterial infection (i.e. live

bacterial cells), as the current studies only assessed the impact of free bacterial LPS on brain uptake. Such a study would provide better insight into the potential alterations to the BBB transport of colistin that may be observed in patients with infections, more closely resembling the clinical setting.

3.6 Conclusion

This study demonstrated that colistin exhibits minimal BBB penetration in healthy mice regardless of the plasma concentrations present and that this low brain uptake is not attributed to efflux by P-gp. Additionally, for the first time, this study has provided evidence that the brain uptake of colistin is significantly increased in mice when the BBB is perturbed by employing LPS, which may resemble the scenario encountered in infected patients.

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Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

- Design of the study
- All laboratory experiments
- Data analysis and interpretation
- Preparation of the initial draft of the manuscript and subsequent revisions; and
- Formulation of conclusions and hypotheses arising from the results of the study

The extent of my contribution was 70%. The following co-authors also contributed to the work.

Name	Nature of contribution
Joseph A. Nicolazzo	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study
Roger L. Nation	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study
Jian Li	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts and revisions; and • Formulation of conclusions and hypotheses arising from the results of the study

Candidate's Signature [redacted] **Date** 06/08/12

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences

	Signature	Date
Signature 1	[redacted]	06/08/2012
Signature 2		6/8/2012
Signature 3		06/8/2012

Chapter Four

The effect of systemic infection induced by *Pseudomonas aeruginosa* on the brain uptake of colistin in mice

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4.1 Abstract

In view of reports of colistin-induced neurotoxicity in infected patients, the aim of this study was to assess whether the integrity of the blood-brain barrier (BBB) and the brain uptake of colistin was altered in the presence of systemic *Pseudomonas aeruginosa* infection. Bacteremia was confirmed 8 h after intramuscular administration of *P. aeruginosa* ATCC27853 to Swiss Outbred mice, at which time a single subcutaneous dose of colistin sulfate (40 mg/kg) or intravenous dose of ^{14}C-sucrose (2 μCi) was administered. Despite a substantial elevation in plasma levels of the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) during bacterial infection, the brain uptake of colistin was low with average AUC_{brain}/AUC_{plasma} ratio of 0.023, which were not different ($p > 0.05$) from the AUC_{brain}/AUC_{plasma} ratio obtained in saline-treated mice. Similarly, the B:P ratio of ^{14}C-sucrose was no different between infected and non-infected mice, consistent with a lack of effect of bacteremia on BBB integrity. To further correlate any relationship between BBB disruption and plasma levels of pro-inflammatory cytokines, BBB integrity, colistin brain uptake and plasma pro-inflammatory cytokines were measured following administration of *Salmonella enterica* lipopolysaccharide (LPS), an agent known to induce BBB disruption. Despite LPS inducing a 4-fold increase in colistin brain uptake and a significant ($p < 0.05$) 1.2-fold increase in ^{14}C-sucrose BBB penetration, plasma cytokine levels were lower with LPS treatment relative to those obtained with bacterial infection with *P. aeruginosa*. This study demonstrates that the brain uptake of colistin is not increased in mice during *P.*

aeruginosa-induced systemic bacteremia despite a significant increase in plasma levels of three pro-inflammatory cytokines.

4.2 Introduction

The spread of multidrug resistance in Gram-negative bacteria presents a critical problem to physicians attempting to treat systemic infections, in particular those caused by *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae* (28). A decreased drive for the discovery of novel antibiotics has dramatically narrowed the available therapeutic options for such infections and has led to the reappraisal of colistin (polymyxin E). The use of colistin waned during the 1970s due to concerns related to its adverse effects (37) including neurotoxicity which manifests with dizziness, numbness, vertigo and lower limb weakness (28, 34). It is still unclear whether these side effects are centrally or peripherally mediated (6), however, if the neurotoxicity induced by colistin is indeed centrally mediated, colistin or its inactive pro-drug colistin methanesulfonate (CMS) would be required to cross the blood-brain barrier (BBB).

The BBB, formed by the endothelial cells lining the cerebral microvessels, is the interface between the blood and the cerebral tissue, and acts as a major hindrance to the movement of molecules from the bloodstream into the central nervous system (CNS) (1). The endothelial cells of these cerebral microvessels have minimal pinocytotic activity and a lack of membrane fenestrations (8). Under normal

conditions, the restrictive nature of the BBB is mediated by intercellular tight junctions preventing paracellular diffusion and various efflux transport systems limiting transcellular movement (20). In an attempt to understand the potential for colistin to traverse the BBB following systemic administration, we have previously assessed the brain uptake of this antibiotic following single and multiple injections to healthy mice, and these studies demonstrated minimal CNS penetration of this antibiotic (18, 19). This is not surprising given that, in addition to its relatively large molecular weight (1163), the free γ-amino groups of the five α,γ-diaminobutyric acid residues in the structure give colistin multiple positive charges at physiological pH, rendering it quite hydrophilic. These physicochemical properties would therefore limit the ability of colistin to traverse the BBB via the transcellular or paracellular routes (33). However, the integrity of the BBB paracellular route is known to be perturbed in a variety of diseases including acute bacterial infection (39), which may be a result of elevated plasma concentrations of pro-inflammatory cytokines (7). Indeed, previous studies have demonstrated that cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) can lead to decreased expression and re-organization of tight junction proteins, resulting in BBB disruption (2, 10).

Therefore, in the presence of a bacterial infection, it is likely that colistin may penetrate the BBB due to perturbation of the paracellular route. Indeed, colistin has been reported to penetrate the blood-cerebrospinal fluid (CSF) barrier in infected patients (3, 17, 25), however, whether enhanced BBB penetration of colistin would

occur during a bacterial infection has not been demonstrated. As a surrogate model of bacterial infection, we have shown that systemic administration of *Salmonella enterica* lipopolysaccharide (LPS) to mice leads to a significant enhancement in colistin BBB transport, and this is associated with increased brain uptake of the normally impenetrable ^{14}C-sucrose, a finding consistent with perturbation of the BBB paracellular route (19). This suggests that brain exposure to colistin could be substantially higher in infected patients during treatment and could potentially lead to centrally mediated neurotoxicity. However, whether a similar increase in brain uptake of colistin will be observed in the presence of a systemic bacterial infection (rather than following administration of LPS) remains unknown.

In order to assess the brain uptake of colistin during systemic infection, a mouse thigh infection model involving intramuscular administration of *Pseudomonas aeruginosa* was established. During the last decade, the opportunistic *P. aeruginosa* (40) has developed the multidrug resistance phenotype and sometimes is only susceptible to colistin (27), which has been shown to have a cure rate of approximately 70% to infections caused by this bacterium (26, 29). Therefore, *P. aeruginosa* was considered a clinically relevant bacterial species to develop the bacterial thigh infection model. The impact of systemic bacterial infection on the integrity of the BBB and colistin brain uptake was then determined, and related to the plasma levels of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6. Furthermore, we measured the plasma cytokine levels in mice pre-treated with LPS from *Salmonella enterica*, a

treatment we have shown increases paracellular permeability leading to increased brain uptake of colistin (19), to determine any relationship between plasma pro-inflammatory cytokine levels and BBB disruption.

4.3 Materials and methods

4.3.1 Bacterial strain and media

P. aeruginosa ATCC 27853 (American Type Culture Collection, Rockford, MD) was stored in tryptone soy broth (Oxoid, Thebarton, South Australia, Australia) with 20% v/v glycerol at -80^{o}C. The bacterial strain was subcultured onto nutrient agar plates (Media Preparation Unit, The University of Melbourne, Parkville, Australia) prior to each experiment and incubated overnight at 37^{o}C. A colony was then selected and grown overnight in 10 mL of cation-adjusted Mueller-Hinton broth (CAMHB, Oxoid, Hampshire, England) from which the logarithmic-phase growth was obtained.

4.3.2 Chemicals and reagents

Colistin sulfate was purchased from Zhejiang Shenghua Biok Biology Co., Ltd (EP5 grade, Zhejiang, China). Lipopolysaccharide from *Salmonella enterica* serotype Typhimurium was obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). ^{14}C-Sucrose was obtained from American Radiolabeled Chemicals (St Louis, MO). Solid-phase extraction (SPE) cartridges (C_{18} Sep-Pak®, 100 mg) were purchased from Waters (Milford, MA). Cyclophosphamide was obtained from Baxter Healthcare Pty Ltd (Old Toongabbie, New South Wales, Australia). Glycerol was

purchased from Ajax Finechem (Seven Hills, New South Wales, Australia). All other reagents were of analytical and/or HPLC grade and water was obtained from a Millipore purification system (Millipore Corporation, Billerica, MA).

4.3.3 Animal studies

Animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were performed in accordance with the Australian National Health and Medical Research Council guidelines for the care and use of animals for scientific purposes. Male Swiss Outbred mice (6-8 weeks of age, 25-30 g) were used in all studies. Mice had free access to food and water during all experimental periods.

4.3.4 Induction of systemic infection in neutropenic and non-neutropenic mice

Systemic bacterial infection was induced through the development of a thigh infection as described previously, with minor modification (13). The thigh infection was induced in both neutropenic and non-neutropenic mice to elucidate whether the presence of neutrophils (and the associated inflammatory cascade) was essential for any bacterial-induced BBB disturbance. To induce neutropenia in mice, cyclophosphamide was injected intraperitoneally 4 days (at a dose of 150 mg/kg) and 1 day (at a dose of 100 mg/kg) prior to the bacterial inoculation. On the day of inoculation, neutropenic mice were anesthetized with isoflurane by inhalation and then a 50 μL suspension of 1 h logarithmic-phase *P. aeruginosa* (approximately 5 ×

10^5 colony forming units (CFU) in CAMHB) or CAMHB was injected into each posterior thigh muscle. The initial inocula was quantified by viable counting using a Synbiosis protoCOL® colony counter (Don Whitley Scientific Ltd, England). At 4, 8 and 13 h after inoculation, mice were anesthetized by isoflurane and blood was aseptically collected by cardiac puncture (n = 4 at each time point) and subjected to viable counting to determine the number of bacterial cells in the blood. To induce bacteremia in non-neutropenic mice, a 50 μL suspension of 1h logarithmic-phase *P. aeruginosa* (approximately 5×10^7 CFU reconstituted in saline) or saline was injected into each posterior thigh muscle following anaesthesia. Blood samples were collected at 1, 2, 4 and 8 h after inoculation (n = 4 at each time point) to determine at what time point bacteremia had been achieved. The blood samples collected from both neutropenic and non-neutropenic mice at each time point were serially diluted in sterile saline and plated on nutrient agar plates which were incubated overnight at 37^oC. Colonies were then counted.

4.3.5 Effect of systemic infection on brain uptake of colistin

An aliquot (200 μL) of colistin sulfate solution (equivalent to 40 mg/kg in saline) filtered through a 0.22 μm membrane (Millipore, Carrigtwohill, Country Cork, Ireland) was subcutaneously administered in the interscapular region of neutropenic or non-neutropenic mice 8 h following bacterial inoculation as this was the time demonstrated to result in reproducible bacteremia (see Results). At various times (0.5, 1, 2, or 4 h) after administration of colistin sulfate, mice (n = 4 at each time point)

were anesthetized with isoflurane, blood was collected by cardiac puncture and the whole brain was removed following cervical dislocation. Plasma and brain samples were then stored at -20°C until analysed by HPLC using an assay previously developed and validated in our laboratory (18). The standards used for quantitating colistin in mouse plasma and brain homogenate were prepared in non-infected matrices from mice. However, to ensure that the presence of bacteria in plasma and brain samples did not affect the ability to quantitate colistin, quality control (QC) samples of colistin (n=4) were also prepared in plasma and brain obtained from mice treated with cyclophosphamide and *P. aeruginosa*. The peak areas from low and high quality control (QC) samples were compared to standards prepared in non-infected matrices, and precision and accuracy were calculated.

Following determination of the colistin concentrations in brain homogenate and plasma, a brain:plasma (B:P) concentration ratio of colistin was calculated at each post-dose time point. In addition to discrete B:P ratios, the area under the plasma concentration-time curve from zero time to 4 h (AUC_{plasma}), the area under the corresponding brain concentration-time curve (AUC_{brain}), and their associated variances were determined by Bailer's approach using the linear trapezoidal rule (4), which was previously validated against WinNonLin (version 4.0, Pharsight Corporation, Mountain View, CA, USA).

4.3.6 Effect of LPS on brain uptake of colistin

Given that LPS (*S. enterica*) is known to induce BBB disruption, the following component of the study was included as a positive control. In brief, mice were administered three intraperitoneal injections (200 µl) of 0.9% (w/v) saline (control) or LPS (*S. enterica*) at 0, 6, and 24 h as previously described (19). An aliquot (200 µl) of a colistin sulfate solution (40 mg/kg in saline) was then administered subcutaneously to mice 4 h after the third saline or LPS dose. At 0.5 h after administration of colistin, plasma and brain samples (n = 4) were harvested, and concentrations of colistin in brain homogenate and plasma were determined by HPLC to obtain a B:P ratio.

4.3.7 Assessment of BBB integrity

The brain uptake of ^{14}C-sucrose was assessed to detect whether the paracellular integrity of the BBB was compromised in infected (both neutropenic and non-neutropenic) and LPS-treated mice (as a positive control). At 8 h after the initial bacterial or saline inoculation or 4 h after the third LPS or saline dose, mice were intravenously administered a 50 µL solution of ^{14}C-sucrose (2 µCi in saline) (n = 6 per group). Plasma and brain samples were collected 5 min post-dose, and radioactivity in plasma and brain determined using liquid scintillation counting (Tri-carb 2800 TR, Perkin Elmer, Boston, MA) as described previously (19).

4.3.8 Measurement of plasma cytokine concentrations

Concentrations of TNF-α, IL-1β and IL-6 were determined in the plasma obtained

from non-neutropenic mice 8 h after the initial bacterial or saline inoculation or 4 h after the third LPS or saline dose, in an attempt to correlate BBB disruption to plasma pro-inflammatory cytokine levels. Plasma (50 µL) was added to the wells of mouse cytokine 96-well kits (Ready-SET-Go!®, eBioscience, San Diego, CA) and absorbance was recorded at 450 nm with a Fluostar Optima microplate reader (BMG Labtech, Mount Eliza, Victoria, Australia). The endogenous components within plasma did not interfere with the assay, as no absorbance was obtained following incubation with plasma alone. Concentrations of the three cytokines in each plasma sample were calculated by comparison to a standard curve developed with known concentrations of each cytokine. The quantification range for the cytokines ranged between 8 and 1000 pg/mL. Plasma samples containing cytokine concentrations exceeding 1000 pg/mL were diluted in blank plasma and re-assayed.

4.3.9 Data analysis

All data are presented as mean ± SD, unless otherwise stated. A Student's *t*-test was used to compare the difference between two groups, whereas bacterial colony numbers in infected animals at different post-inoculation time points using viable counts on linear scale were compared using a one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparisons test (PASW Statistics for Windows, version 17.0, Chicago, Illinois). A p value < 0.05 was considered to be a significant difference. When comparing AUC_{plasma} and AUC_{brain} between saline-treated and infected mice, a *z*-test was used to test significant differences as

proposed by Bailer (4). A *z* value > 1.96 was considered to be a significant difference between groups.

4.4 Results

4.4.1 Bacterial burden in blood after inoculation of neutropenic and non-neutropenic mice

At 4 h after inoculating neutropenic mice with *P. aeruginosa* (5×10^5 CFU), bacteremia was relatively low (less than 4.65 Log_{10} CFU/mL of blood), and it was not until 8 h post-inoculation that bacteraemia appeared to be reproducibly established with blood levels of 5.05-5.38 Log_{10} CFU/mL (Fig. 4-1a). There was no significant difference in the level of bacteraemia between 8 and 13 h ($p > 0.05$). For this reason, all subsequent brain uptake experiments in neutropenic mice were conducted 8 h following bacterial inoculation or saline administration. When the same inoculum of *P. aeruginosa* was injected into non-neutropenic mice, no bacteremia was detectable, and therefore the inoculum was increased to approximately 5×10^7 CFU. As shown in Fig. 4-1b, bacteremia was minimal at 1 and 2 h post-inoculation (less than 2.95 Log_{10} CFU/mL), whereas at 4 h post-inoculation, blood levels reached a plateau of 4.90-5.48 Log_{10} CFU/mL which is similar to the level observed in neutropenic mice (albeit at a lower inoculum). At 8 h post-inoculation, there was no significant difference in bacteremia relative to 4 h ($p>0.05$), however, in order to keep a consistent time exposure between neutropenic and non-neutropenic studies, all brain uptake assessments in non-neutropenic mice were also undertaken at 8 h

post-inoculation.

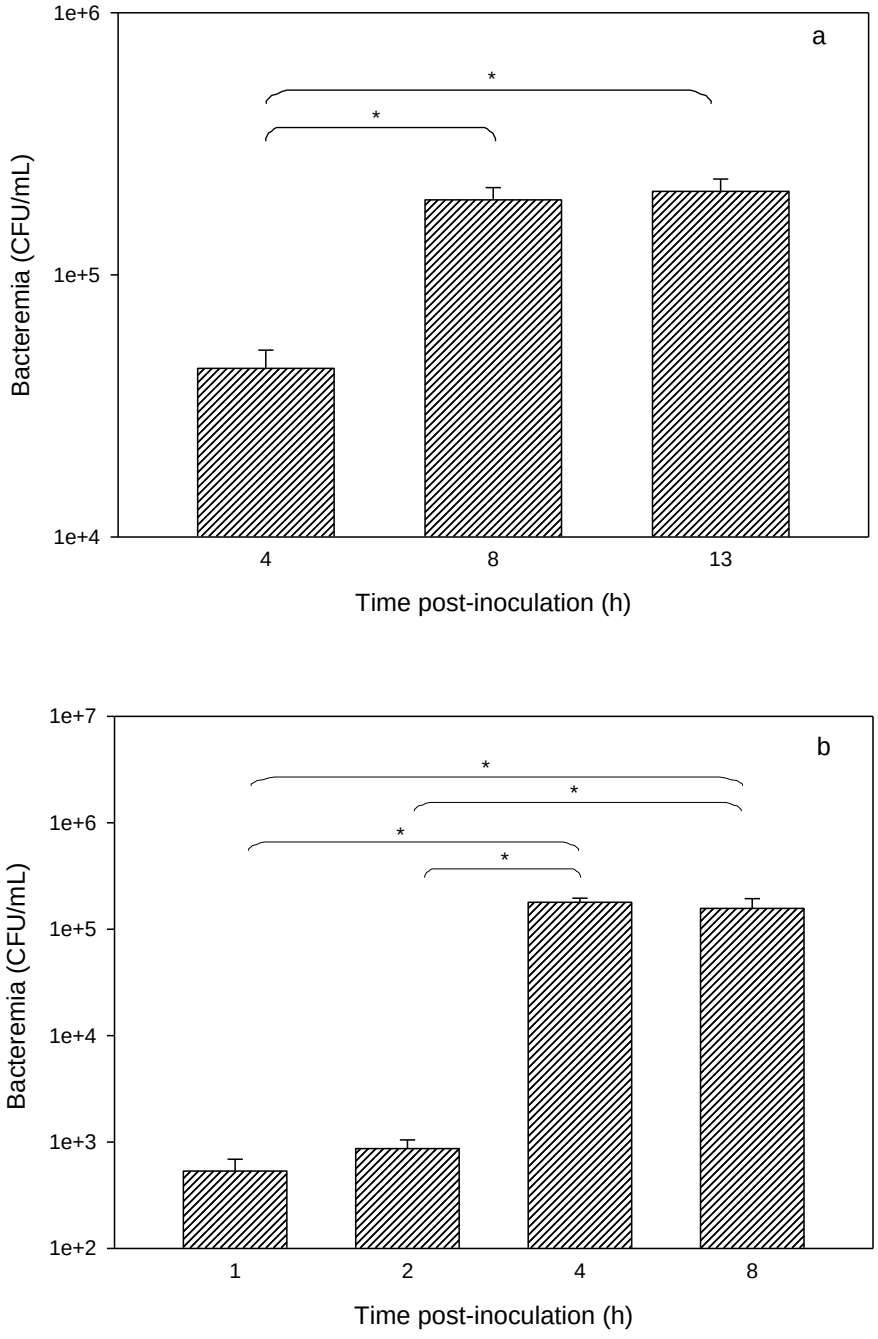

Figure 4-1. Bacteremia (CFU/mL) at various time points following intramuscular administration of *P. aeruginosa* to (a) neutropenic Swiss Outbred mice and (b) non-neutropenic Swiss Outbred mice. Data are presented as mean ± SEM (n = 4). * indicates a *p* value < 0.05, using a one-way ANOVA.

4.4.2 Brain uptake of colistin following systemic infection or LPS treatment

To ensure that *P. aeruginosa* inoculation or cyclophosphamide treatment did not interfere with HPLC analysis of colistin in plasma and brain homogenate, the HPLC methods developed previously (18) were re-validated using plasma and brain from bacteria-inoculated neutropenic mice. No changes in colistin chromatographic peak shapes or retention times were observed in colistin-spiked plasma or brain homogenate obtained from mice pretreated with cyclophosphamide and inoculated with bacteria. Using standard curve solutions of colistin prepared in plasma and brain homogenate from non-infected mice, the precision and accuracy values for QCs prepared in plasma and brain homogenate from infected mice were within acceptable ranges (as shown in Table 4-1). These results indicated that plasma and brain from non-infected mice were suitable to establish standard curves to calculate the concentrations of colistin in samples from infected animals.

Table 4-1. Precision and accuracy values of the quality control (QC) samples prepared in plasma and brain homogenate obtained from infected neutropenic mice and compared to a standard curve prepared in matrices obtained from non-infected mice.

Target colistin conc. in matrices from infected mice	Mean ± SD Measured colistin concentration (μg/mL or μg/g)	Precision (%)	Accuracy (%)
Plasma (μg/mL, n = 4)			
1.25	1.26 ± 0.044	3.2	100.8
10.00	10.9 ± 1.46	13.4	109.2
Brain (μg/g, n = 4)			
0.19	0.184 ± 0.025	13.4	96.8
3.00	3.17 ± 0.084	2.6	105.7

All mice tolerated the current single subcutaneous dose of colistin with no observed toxicity over the experimental period. The plasma and brain concentrations of colistin in infected and non-infected neutropenic mice are shown in Fig. 4-2. Plasma concentrations of colistin were not significantly affected by inoculation with *P. aeruginosa*, with AUC_{plasma} values of infected and non-infected mice being 119 ± 12 and 108 ± 12 μg·h/mL, respectively (z = 0.20). Similarly, the brain homogenate concentrations of colistin were not different between infected and non-infected animals over the 4 h post-dose period with AUC_{brain} values of 2.74 ± 0.21 and 2.58 ± 0.28 μg·h/g, respectively (z = 0.45). Similarly, when colistin was administered to non-neutropenic animals, there was no significant difference in the B:P ratio of colistin between infected and non-infected mice at 0.5 h post-administration (Fig.

4-3a), with B:P ratios of 0.043 ± 0.032 (infected mice) and 0.032 ± 0.011 (non-infected mice). Given that there was no difference in the brain uptake of colistin at this single time point, no further mice were dosed to obtain a concentration-time profile similar to that obtained for neutropenic mice (i.e. Fig 4-2). However, in mice treated with LPS from *S. enterica*, the B:P ratio of colistin at 0.5 h post-administration was 0.12 ± 0.053 (Fig. 4-3b), which was significantly higher than that of saline-treated mice (0.036 ± 0.011), confirming that when the BBB is perturbed, the brain uptake of colistin is indeed enhanced.

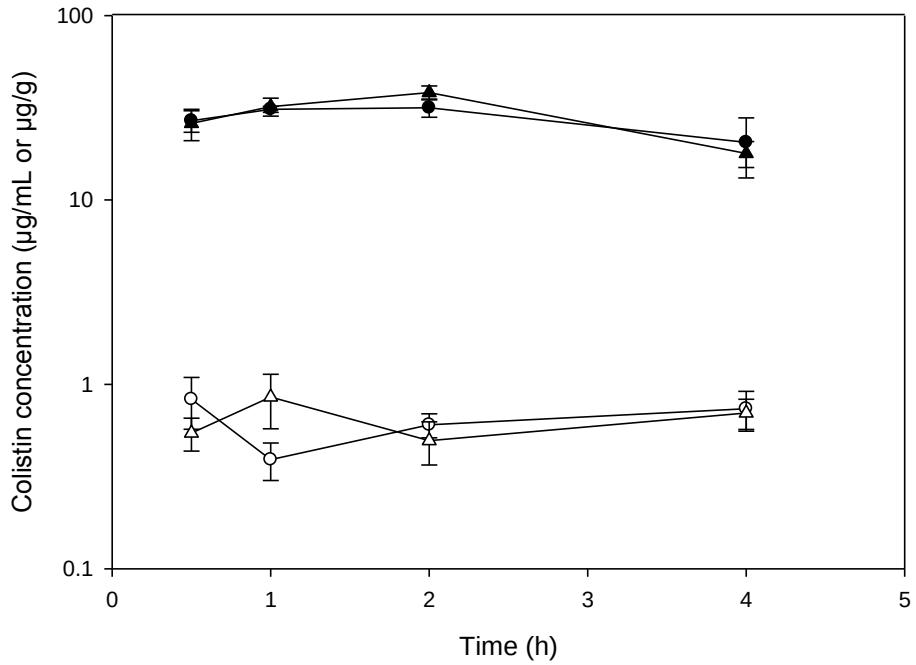

Figure 4-2. Plasma (µg/mL) and brain (µg/g) concentrations of colistin after subcutaneous administration of colistin sulfate (40 mg/kg) to neutropenic Swiss Outbred mice 8 h after inoculation with *P. aeruginosa* (~5 $\times 10^5$ CFU) or saline. The data indicate plasma (▲) and brain (Δ) from infected mice and plasma (●) and brain (○) from non-infected mice, and are presented as mean ± SEM (n = 4).

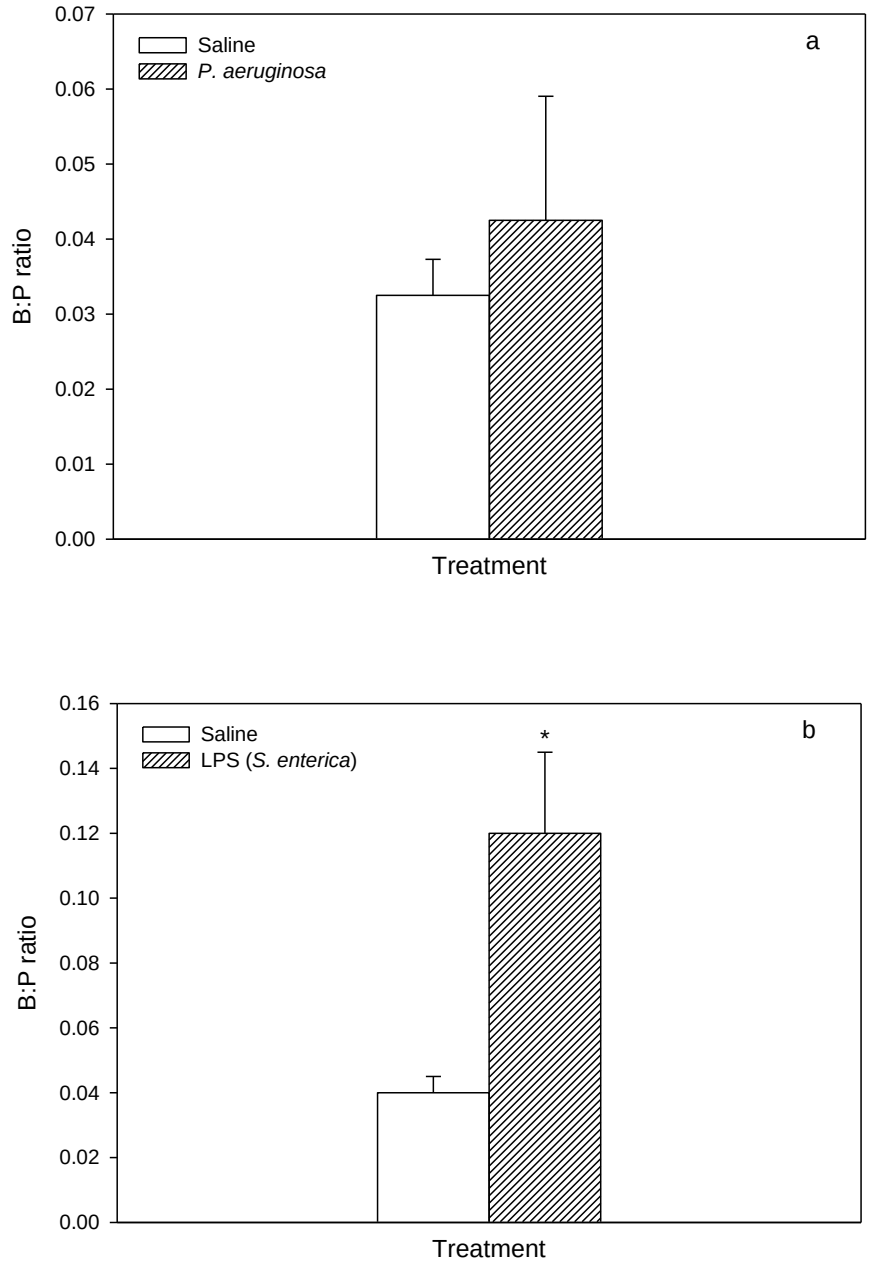

Figure 4-3. Brain:plasma **(**B:P) ratios of colistin following administration to (a) non-neutropenic Swiss Outbred mice inoculated with saline or *P. aeruginosa* (~5 × 10^7 CFU) and (b) Swiss Outbred mice administered saline or LPS from *S. enterica* (3 mg/kg at 0, 6 and 24 h). Data are presented as mean ± SEM (n = 4). * indicates $p < 0.05$ relative to saline-administered mice, using a Student's *t*-test.

4.4.3 BBB integrity following systemic infection or LPS treatment

The B:P ratio of the BBB paracellular route marker, ^{14}C-sucrose, was determined in mice during bacteremia to confirm a lack of paracellular dysfunction, as suggested by the lack of difference in colistin brain uptake between non-infected and infected mice. In both neutropenic and non-neutropenic mice, the average B:P ratios of ^{14}C-sucrose were not significantly different between infected and non-infected mice (Fig. 4-4a), suggesting that even though bacteremia was present, the tight junctions at the BBB were still functionally intact. In contrast, a small but significant difference in ^{14}C-sucrose brain uptake was observed between saline- and LPS-treated mice with B:P ratios of 0.046 ± 0.0035 (LPS-treated) and 0.039 ± 0.0024 (saline-treated) (Fig. 4-4b).

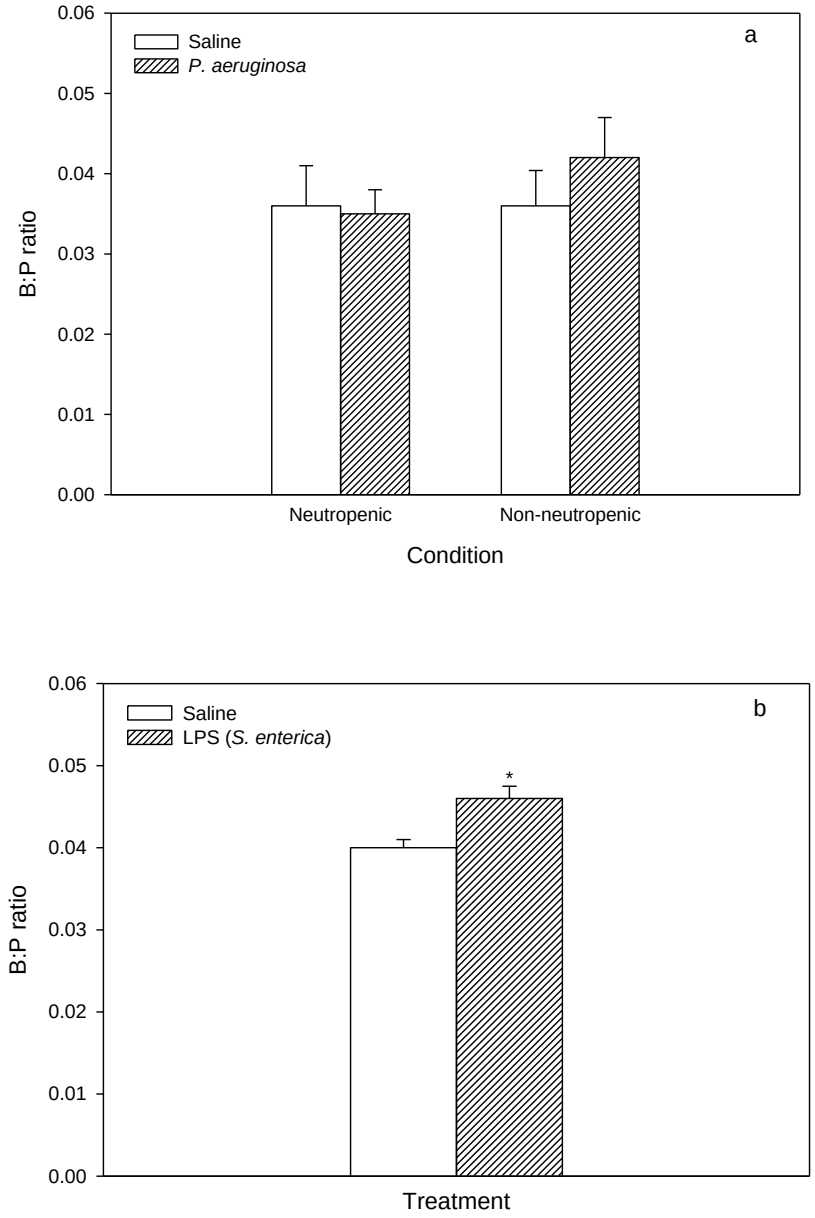

Figure 4-4. Brain:plasma (B:P) ratios of ^{14}C-sucrose following administration to (a) neutropenic or non-neutropenic Swiss Outbred mice inoculated with saline or *P. aeruginosa* (~5 x 10^5 to 5 x 10^7 CFU) and (b) Swiss Outbred mice administered saline or LPS from *S. enterica* (3 mg/kg at 0, 6 and 24 h). Data are presented as mean ± SEM (n = 6) and * indicates a significant ($p < 0.05$) difference between saline and LPS-treated mice, using a Student's *t*-test.

4.4.4 Plasma cytokine levels following systemic infection or LPS treatment

Plasma concentrations of TNF-α, IL-1β and IL-6 in infected and non-infected non-neutropenic mice are shown in Fig. 4-5a. The values of all three cytokines were very low in non-infected mice, with TNF-α and IL-1β levels being below the limit of quantitation of the assay (i.e. 8 pg/mL). After inoculation with *P. aeruginosa*, concentrations of cytokines were substantially higher with values of 774.1 ± 184.4 pg/mL for TNF-α, 110.7 ± 88.4 pg/mL for IL-1β and 1078.3 ± 235.5 pg/mL for IL-6, consistent with the pro-inflammatory mediators having been activated during systemic bacterial infection. Studies to measure the plasma cytokine levels in neutropenic mice were not conducted as results from non-neutropenic mice were more likely to resemble the clinical setting.

The levels of TNF-α, IL-1β and IL-6 were also determined in plasma of saline- and LPS-treated mice (Fig. 4-5b), given that *S. enterica* LPS induced BBB disruption therefore allowing for a better correlation to be made between BBB disruption and plasma cytokine levels. The plasma levels of these cytokines from LPS-treated mice were 84.8 ± 14.9 pg/mL for TNF-α, 9.3 pg/mL for IL-1β (SD value could not be calculated as concentrations of only two samples were above 8 pg/mL) and 916.5 ± 310.4 pg/mL for IL-6. The concentrations of cytokines in saline-treated mice were in all cases below the limit of quantitation of the assays. While statistical analysis could not be conducted, given that saline-treated mice exhibited non-quantifiable plasma cytokine levels, it was obvious that plasma concentrations of these three cytokines

were higher after LPS treatment. In addition, the TNF-α plasma levels in infected mice were substantially higher than the corresponding levels in LPS-treated mice ($p < 0.05$), even though BBB disruption and enhanced colistin brain uptake was only observed in LPS-treated mice, suggesting a lack of correlation between BBB disruption and plasma levels of this cytokine.

4.5 Discussion

As a consequence of the development of antibiotic resistance, there has been increasing clinical use of the cationic polypeptide antibiotic colistin for the treatment of Gram-negative bacterial infections (31). Associated with this clinical use are reports of neurotoxicity, suggesting that colistin has the potential to permeate the BBB. We have demonstrated that colistin exhibits minimal penetration across the healthy BBB, however, has the ability to traverse the BBB during systemic inflammation due to LPS-mediated perturbation of the paracellular route (17). To reflect the clinical scenario more closely, the impact of systemic infection with *P. aeruginosa* on the brain uptake of colistin was assessed in a mouse model. The strain of *P. aeruginosa* selected to induce bacteremia (ATCC27853) was initially isolated from a patient and has been widely used to determine the antimicrobial activities of various antibiotics (15, 35), therefore, it is clinically relevant.

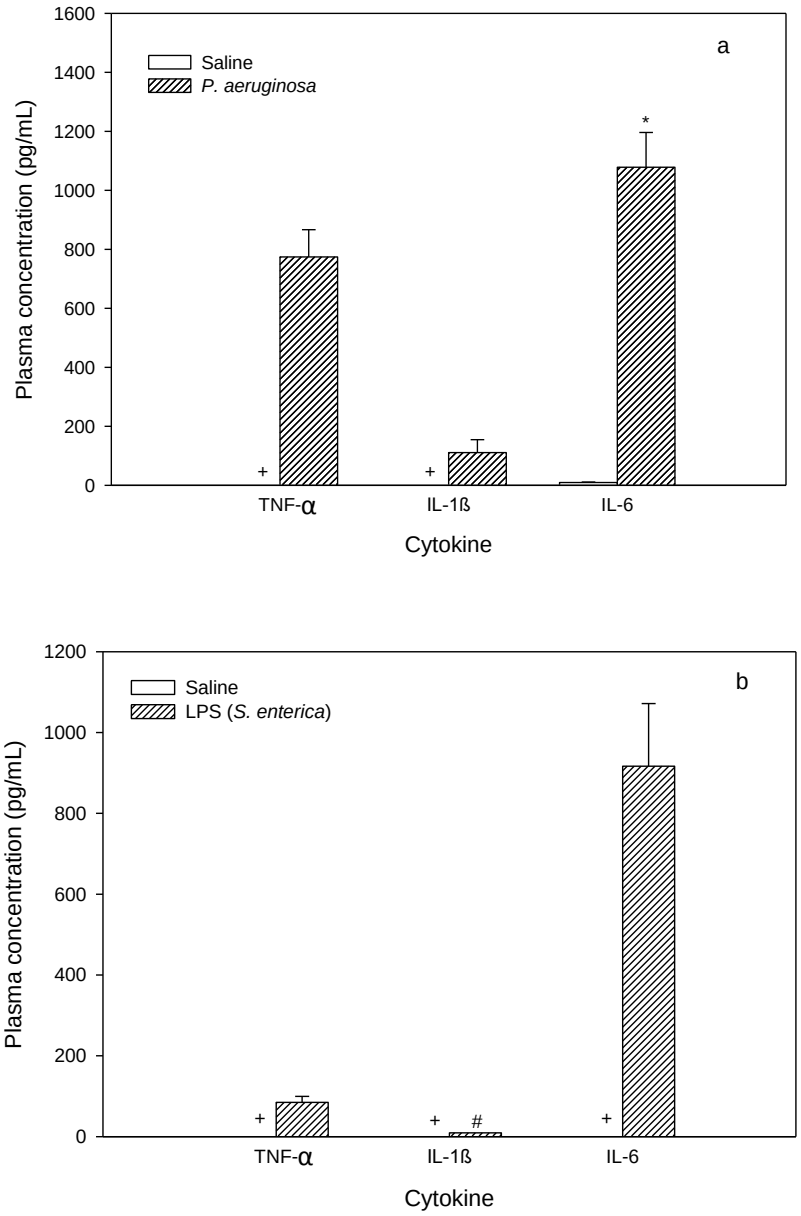

Figure 4-5. Plasma concentration (pg/mL) of TNF-α, IL-1β and IL-6 from non-neutropenic mice (a) 8 h after inoculation with saline or *P. aeruginosa* (~5×10^7 CFU) and (b) 4 h after last dose of LPS (*S. enterica*) or saline. Data are presented as mean ± SEM (n=4). * indicates $p < 0.05$ between bacteria-inoculated and saline-treated animals using a Student's *t*-test, $^{+}$ indicates that plasma levels were below 8 pg/mL and $^{\#}$ indicates n = 2 (as the remaining replicates were below 8 pg/mL).

It has been shown that inoculation of mice with *Escherichia coli* and *Streptococcus pneumoniae* at sepsis-inducing doses leads to BBB disruption, which was demonstrated by brain access of the impermeable horseradish peroxidise (38). Although a different bacterial strain, an increased BBB permeability and enhancement in colistin brain uptake was therefore expected following inoculation with *P. aeruginosa*. Interestingly, despite a high level of bacteremia similar to that achieved following inoculation with *E. coli* and *S. pneumoniae* (38), the plasma and brain concentrations of colistin did not differ between *P. aeruginosa*-infected and non-infected mice. Moreover, the B:P ratios of the BBB paracellular route marker, ^{14}C-sucrose, were not different between the two groups suggesting that the tight junctions were not disturbed following *P. aeruginosa* inoculation. However, administration of LPS from *S. enterica* led to an increase in the brain uptake of both colistin and ^{14}C-sucrose demonstrating that our model was able to discriminate between an intact and disturbed BBB, suggesting the observations with *P. aeruginosa* were indicative of a negative impact on BBB dynamics.

The lack of effect of *P. aeruginosa* on BBB integrity and colistin brain uptake was first assessed in neutropenic animals, whose immune system and ability to produce pro-inflammatory cytokines may be affected (9). Cytokines are pro-inflammatory mediators of the immune system that are released in order to respond to acute infection. These mediators are known to modify BBB permeability during neuroinflammatory disease states such as multiple sclerosis, meningitis and

encephalitis and this is associated with their ability to affect the expression and function of inter-endothelial tight junction proteins including occludin and zonula occludens-1 (21, 30, 41). Various *in vitro* studies have demonstrated that pro-inflammatory mediators such as TNF-α, IL-1β and IL-6 are able to cause BBB paracellular dysfunction by altering the cytoskeleton structure of endothelial cells (10). It has also been demonstrated that direct administration of such cytokines *in vivo* could lead to BBB disruption (2, 36). Given the role of pro-inflammatory cytokines in mediating BBB disruption, and the altered inflammatory response of neutropenic mice, it was considered that the neutropenic state may have prevented the ability for bacteremia to induce BBB dyfsunction. For this reason, we then developed a bacterial infection model in non-neutropenic (i.e. non-cyclophosphamide-treated) mice to determine whether the presence of neutrophils (and the subsequent altered release of pro-inflammatory cytokines) would lead to BBB dysfunction and enhanced colistin brain uptake. Such a scenario is also more likely to be representative of that observed in otherwise healthy patients who have contracted a bacterial infection. Not surprisingly, a higher inoculum of *P. aeruginosa* was required to induce bacteremia in non-neutropenic mice. However, similar to that observed in neutropenic mice, no significant difference in the brain uptake of colistin or ^{14}C-sucrose was found between infected and non-infected mice suggesting the paracellular route was not disrupted in the infected mice. These studies suggested, that even in the presence of an intact immune system, bacteremia induced by *P. aeruginosa* was not able to induce BBB dysfunction.

To ensure that there was indeed an inflammatory response induced by bacteremia, and to examine possible relationships between inflammatory mediators and BBB disruption, the plasma levels of TNF-α, IL-1β and IL-6 were measured in infected and non-infected mice. For all three cytokines, the concentration in plasma was higher in the infected mice relative to non-infected mice. Despite the higher plasma cytokine levels, the BBB integrity was not affected during bacterial infection, suggesting that there was no clear correlation between plasma cytokine levels and BBB dysfunction, at least for the three cytokines examined. To further confirm this correlation, we measured the plasma cytokine levels in mice which we demonstrated did have a disturbed BBB (i.e. mice treated with LPS from *S. enterica*). It showed that even though we observed a significant increase in colistin and ^{14}C-sucrose brain uptake, plasma cytokine levels were actually lower than those observed following bacterial infection (where there was no BBB disruption). These data confirmed that for these three plasma cytokines, there did not appear to be a direct link between plasma levels and extent of BBB disruption.

There are various reasons as to why there appeared to be differences between the BBB-disrupting effects of our two treatment paradigms. Firstly, it is important to note that bacterial infection with *P. aeruginos*a led to a larger pro-inflammatory response (relative to *S. enterica* LPS administration), yet the BBB disrupting effects of LPS appeared greater. This suggests that there may be a direct effect of LPS species on the BBB, independent of release of plasma cytokines, as has been suggested elsewhere

(16). Alternatively, given the levels of cytokines released in these studies were well below those previously shown to induce BBB disruption both *in vitro* and *in vivo* (10-12, 36, 38), it is possible that a more representative correlation may be observed if higher bacterial doses were administered. Indeed, the levels of TNF-α and IL-6 released in patients with non-bacterial induced acute pancreatitis (where enhanced BBB permeability was detected) were substantially higher than the levels observed in our studies following *P. aeruginosa* infection (14). Whether such levels and their resultant BBB disrupting effects can be obtained with higher inocula of *P. aeruginosa* ATCC 27853 or with administration of other clinically-relevant bacterial strains need therefore be investigated. It should also be noted that we only measured the levels of IL-1β, IL-6 and TNF-α and it is possible that other cytokines such as IFN-γ or IL-10 may also play an important role in BBB disruption (32). Furthermore, colistin is administered parenterally as its inactive pro-drug colistin methanesulfonate (CMS) (5). Due to the stability issues (22, 23), it is difficult to accurately determine whether CMS has the potential to traverse the BBB or whether the brain uptake of CMS is altered during bacterial infection.

Nevertheless, we have demonstrated that the brain uptake of colistin does not appear to be substantially affected during bacteremia induced with a clinically-relevant strain. We have also confirmed that such an enhancement in the brain uptake of colistin is possible following administration of LPS from *S. enterica*, which suggests that the CNS exposure of this antibiotic may be increased during infection with other bacterial

strains.

4.6 Conclusion

These studies have demonstrated that, despite increasing the plasma concentrations of three pro-inflammatory cytokines, induction of bacteremia by *P. aeruginosa* did not alter the BBB paracellular route or the ability of colistin to enter the brain parenchyma following systemic administration. Furthermore, a direct relationship between plasma levels of TNF-α, IL-1β and IL-6 and BBB disruption was not evident *in vivo*, in contrast to that which has been suggested from *in vitro* studies.

4.7 Acknowledgements

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Declaration for Thesis Chapter 5

Declaration by candidate

In the case of Chapter 5, the nature and extent of my contribution to the work was the following:

- Design of the study
- All laboratory experiments
- Data analysis and interpretation
- Preparation of the initial draft of the manuscript; and
- Formulation of conclusions and hypotheses arising from the results of the study

The extent of my contribution was 70%. The following co-authors also contributed to the work.

Name	Nature of contribution
Joseph A. Nicolazzo	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts; and • Formulation of conclusions and hypotheses arising from the results of the study
Roger L. Nation	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts; and • Formulation of conclusions and hypotheses arising from the results of the study
Jian Li	• Supervision and advice regarding the concept and design of the studies, data analysis and interpretation • Review of manuscript drafts; and • Formulation of conclusions and hypotheses arising from the results of the study

Candidate's Signature	[redacted]	**Date** 06/08/12

Declaration by co-authors

The undersigned hereby certify that:

(1) the above declaration correctly reflects the nature and extent of the candidate's contribution to this work, and the nature of the contribution of each of the co-authors.
(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;

(3) they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
(4) there are no other authors of the publication according to these criteria;
(5) potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit; and
(6) the original data are stored at the following location(s) and will be held for at least five years from the date indicated below:

Location(s) Drug Delivery, Disposition and Dynamics, Monash Institute of Pharmaceutical Sciences

	Signature	Date
Signature 1	[redacted]	06/08/2012
Signature 2		6/8/2012
Signature 3		06/8/2012

Chapter Five

Species-dependent lipopolysaccharide-induced blood-brain barrier disruption: amelioration by colistin *in vitro* and *in vivo*

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Manuscript in preparation

5.1 Abstract

The aim of this study was to use *in vivo* and *in vitro* models to assess the impact of lipopolysaccharide (LPS) from two different bacterial species on blood-brain barrier (BBB) integrity and colistin brain uptake. Following repeated administration of LPS from *Salmonella enterica* and *Pseudomonas aeruginosa*, the brain-to-plasma (B:P) ratio of ^{14}C-sucrose was significantly increased (1.3 fold) only in *S. enterica* LPS-treated mice relative to saline-treated mice ($p < 0.05$), indicative of a disrupted BBB in *S. enterica* LPS-treated mice. Furthermore, the brain uptake of colistin in mice following intraperitoneal administration was increased 3-fold with administration of LPS from *S. enterica*, but not *P. aeruginosa*. This species-dependent effect did not appear to result from differences in plasma cytokine levels as the concentrations of tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) following administration of each LPS was not different between strains ($p > 0.05$). To clarify whether this species-specific effect of LPS was due to direct effects on the BBB, human brain capillary endothelial cells (hCMEC/D3) were treated with LPS from *P. aeruginosa* or *S. enterica* at concentrations from 3.75 to 30 µg/mL and claudin-5 expression was measured by Western blotting. While *S. enterica* LPS significantly ($p < 0.05$) reduced claudin-5 expression (by 33.3%) at low concentrations (i.e. 7.5 µg/mL), *P. aeruginosa* LPS only decreased claudin-5 expression at the highest experimental concentration (i.e. 30 µg/mL). Co-administration of the clinically-used antibiotic colistin was able to ameliorate the *S. enterica* LPS-induced reduction in claudin-5 expression in hCMEC/D3 cells and the *S. enterica* LPS-induced BBB

perturbation in mice. This study demonstrates that the BBB disruption induced by LPS is species- and dose-dependent, likely due to a direct effect on brain endothelial cells, which can be ameliorated by colistin.

5.2 Introduction

The blood-brain barrier (BBB) is formed by specialized endothelial cells of cerebral microvessels. Unlike those in other organs, the layer of endothelial cells in the central nervous system (CNS) constitutes a physical barrier between blood and brain tissue by a complex network of tight junctions (40). The tight junctions (TJs) consist of transmembrane proteins spanning the intercellular cleft; the proteins include occludin, claudins and several cytoplasmic proteins including zonula occludens (18). Claudin-5 is one of the primary transmembrane proteins from the claudin family. Studies have demonstrated that claudin-5 is crucial for maintaining the structural and functional integrity of the endothelium. The loss of claudin-5 causes increased BBB permeability and such dysfunction cannot be compensated by other TJ proteins, suggesting it is likely the most critical TJ protein at the BBB (33). Under normal conditions, these TJ proteins seal the paracellular route of the BBB, thus preventing the movement of hydrophilic molecules from the blood into the brain, and therefore, ensuring homeostatic regulation of the CNS environment (51).

However, alterations to TJ proteins and increases in BBB permeability are observed during various disease states including peripheral hyperalgesia, bacterial meningitis,

systemic inflammation and bacterial infection (13, 28, 36, 47). These observations have been made in animal models of such conditions, where for example, systemic challenge with bacterial lipopolysaccharide (LPS) or lambda-carrageenan leads to enhanced brain uptake of normally-impermeable BBB markers such as ^{125}I-albumin and ^{14}C-sucrose (28, 34). Indeed, we have also demonstrated that systemic administration of LPS from *Salmonella enterica* (as a model of bacteria-induced inflammation) leads to BBB dysfunction, resulting in increased brain uptake of ^{14}C-sucrose (22). Interestingly, in a subsequent study, using a systemic bacterial infection mouse model (induced by inoculation with *Pseudomonas aeruginosa*), the integrity of the BBB was not affected, and this suggested the possibility of a species-dependent effect of LPS on BBB dynamics.

One proposed mechanism of BBB dysfunction in response to LPS involves elevated plasma concentrations of cytokines, especially tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6). This mechanism has been supported by studies demonstrating that following treatment with cytokines, the BBB permeability in animals and endothelial monolayers was significantly enhanced (1, 8, 9, 15, 41). However, in our previous study involving inoculation with *P. aeruginosa* in mice, the BBB paracellular integrity was not compromised despite significant increases in the plasma concentrations of the above-mentioned three cytokines (as shown in Section 4.4.2), suggesting a limited correlation between plasma cytokine levels and BBB disruption. Although our results appear to contrast those of previous

studies, it should be noted that despite bacterial infection causing increases in plasma cytokine levels, these increased levels (i.e. < 2 ng/mL) are substantially lower than those shown to cause BBB disruption (i.e. 10 to 800 ng/mL) (4, 10, 47). Therefore, such correlations between plasma cytokine levels and BBB disruption may only be observed when the plasma levels of these pro-inflammatory molecules reach a certain threshold.

Although, as discussed above, bacterial infection with *P. aeruginos*a led to a large pro-inflammatory response, LPS from *S. enterica* with a lower pro-inflammotory response (as shown in section 4.4.4) caused greater BBB disruption. It is possible, therefore, that there may be a direct effect of LPS on the BBB via LPS receptors at the endothelium, and that this direct effect may be dependent upon the bacterial species from which the LPS is derived. Indeed, studies have shown that LPS can directly cause increased BBB permeability in animals and decreased integrity in endothelial monolayers (4, 12, 13, 19, 48). By binding to its receptors such as Toll-like receptor (TLR) 4 or CD14, LPS is reported to activate intracellular signalling pathways in brain endothelial cells (i.e. RhoA, mitogen-activated protein kinase (MAPK), myosin light chain kinase, Nuclear Factor-KappaB (NF-kB)). These pathways lead to altered actin cytoskeleton architecture and induce local release of cytokines, which is thought to lead to altered expression of TJ proteins at the brain endothelial barrier (13, 19, 35, 45) (see Section 1.3.2.7.1). It is possible that the response of the BBB to different species of LPS may vary and details of this effect still require further clarification.

In the present study, the BBB-disrupting effects of LPS from both *S. enterica* and *P. aeruginosa* were assessed in mice using ^{14}C-sucrose as a BBB integrity marker. To determine whether the distinguishing effects of LPS on the BBB integrity were related to a differential induction in systemic cytokine levels, plasma concentrations of TNF-α, IL-1β and IL-6 were measured as they are the main cytokines involved in immune responses to infection (23) and are reported to decrease the integrity of the BBB (8). To further determine whether any BBB-disruption differences were due to specific effects of LPS on the BBB, the effects of each LPS on the expression of the TJ protein claudin-5 were measured in an immortalized human brain capillary endothelial cell line (hCMEC/D3). The hCMEC/D3 cell line has been used in recent studies to assess BBB integrity following various treatments (2, 42).

Given that BBB disruption can be induced by systemically-circulating LPS in sepsis (32), it is of great importance to develop strategies to attenuate its cytotoxic effects, potentially preventing neurotoxicity associated with LPS. For example, epigallocatechin gallate has been shown to attenuate LPS-induced inflammatory cytokine production in hCMEC/D3 cells through the inhibition of NF-kappaB activation (26), which may ameliorate LPS-induced BBB damage. Another possible way to achieve attenuation is to co-administer compounds which are able to bind to LPS directly, effectively reducing the free concentration of LPS able to lead to BBB disruption. Polymyxin B has been shown to inactivate the BBB-disrupting effect of LPS from *Haemophilus influenzae* through binding to the lipid A component (35).

Moreover, human serum amyloid P component, which is a LPS-binding protein, alleviated the symptoms of *S. enterica* LPS-induced septic shock, and significantly inhibited the enhanced permeability of two BBB integrity markers, sodium fluorescein and albumin both tracers (49). A negatively charged polysaccharide, pentosan polysulfate, has also been demonstrated to reduce the deleterious effects of LPS on the permeability in brain endothelial cells (48). Colistin, which is another polypeptide antibiotic belonging to the polymyxin family, is now increasingly used to treat Gram-negative bacterial infections as last line salvage therapy (29). In addition to its bactericidal effect, colistin has also been shown to bind to LPS and prevent the pathophysiologic effects of the endotoxin in the circulation (27) (see Section 1.2.3). Therefore, the final aim of this study was to assess claudin-5 expression *in vitro* and BBB dysfunction *in vivo* following simultaneous administration of LPS and colistin at therapeutically relevant concentrations, to determine whether colistin could indeed ameliorate LPS-induced BBB disruption.

5.3 Materials and methods

5.3.1 Chemicals and reagents

Colistin sulfate was supplied by Zhejiang Shenghua Biok Biology Co., Ltd (EP5 grade, Zhejiang, China). ^{14}C-Sucrose was provided by American Radiolabeled Chemicals (St Louis, MO). Lipopolysaccharide (LPS) from both *Salmonella enterica serotype Typhimurium* and *Pseudomonas aeruginosa*, and Trizma·HCl were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia).

Solid-phase extraction cartridges (C_{18} Sep-Pak®, 100 mg) were purchased from Waters (Milford, MA). Tween 20 was purchased from Aldrich Chemical Company (Milwaukee, WI). Endothelial Basal Medium-2 (EBM-2) and Endothelial Growth Media Single Quots kit were purchased from Lonza (Walkersville, MD). Fetal bovine serum (FBS) and Dulbecco's Phosphate Buffered Saline were purchased from Invitrogen (Auckland, New Zealand) and rat tail collagen Type I was purchased from BD Biosciences (Bedford, MA). The primary rabbit anti-claudin-5 and mouse anti-β-actin antibodies were purchased from Abcam (Cambridge, MA) and the secondary goat anti-mouse and donkey anti-rabbit antibodies were purchased from LI-COR® Biosciences (Lincoln, NE). All other reagents were of analytical and/or HPLC grade and water was obtained from a Millipore purification system (Millipore Corporation, Billerica, MA).

5.3.2 Effect of different LPS strains on BBB integrity and brain uptake of colistin

Animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were performed in accordance with the Australian National Health and Medical Research Council (NHMRC) guidelines for the care and use of animals for scientific purposes. Male Swiss Outbred mice (6-8 weeks of age, 25-30 g) were used in all studies. Mice had free access to food and water during all experimental periods.

Mice were administered intraperitoneal (i.p.) injections (200 μL) of 0.9% (w/v) saline

(control) or LPS (*S. enterica* or *P. aeruginosa*, 3 mg/kg of body weight in saline) at 0, 6, and 24 h. At 4 h after the last administration, mice (n = 4) were intravenously administered a 50 μL solution of ^{14}C-sucrose (2 μCi in saline), plasma and brain samples were collected at 5 min post-dose, and radioactivity in plasma and brain was determined using liquid scintillation counting (Tri-carb 2800 TR; Perkin Elmer, Boston, MA) as detailed previously (22). A 5 min post-dose brain-to-plasma (B:P) ratio was calculated using the following formula: B:P = (number of disintegrations per minute (dpm)/g of brain homogenate)/(dpm/mL of plasma). To determine whether the brain uptake of colistin differed in mice treated with different LPS strains, an aliquot (200 μL) of a colistin sulfate solution (40 mg/kg) was administered subcutaneously to mice at 4 h after the third LPS or saline dose. Plasma and brain samples (n = 4) were harvested 0.5 h later, and concentrations of colistin in brain homogenate and plasma were determined by HPLC to obtain B:P ratios (21).

5.3.3 Effect of different LPS strains on plasma cytokine levels

Plasma concentrations of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) at 4 h after the last injection of saline or LPS (*S. enterica* or *P. aeruginosa*) were measured by mouse cytokine kits (Ready-SET-Go!®, eBioscience, San Diego, CA). According to the company instructions, a 50 μL aliquot of plasma was added to a 96-well plate and the absorbance was recorded at 450 nm with a Fluostar Optima microplate reader (BMG Labtech, Mount Eliza, Victoria, Australia). Standard curves were established using cytokines of known concentrations available in the kits with a quantification

range between 8 to 1000 pg/mL. No absorbance was detected following plasma treatment alone, suggesting the assay was not affected by the endogenous components within plasma.

5.3.4 Impact of LPS strains on claudin-5 expression *in vitro*

The hCMEC/D3 cells (passage 36-37) were seeded on rat tail collagen-coated 6-well plates (at a density of 50,000 cells/cm^2) and cultured in EBM-2 media supplemented with vascular endothelial growth factor, insulin-like growth factor 1, epidermal growth factor, basic fibroblast growth factor, hydrocortisone, ascorbate, and penicillin-streptomycin from Endothelial Growth Media Single Quots kit and 2.5% v/v FBS as recommended by the manufacturer. The cells were maintained at 37℃ in an atmosphere of 5% CO_2/95% air. Culture medium was changed every 1-2 days until cells reached a confluent monolayer (which on average, took 4-5 days).

Once confluent, the hCMEC/D3 cells were treated with serum-free medium or LPS (*S. enterica* or *P. aeruginosa* in serum-free medium) at concentrations of 3.75, 7.5, 15 and 30 μg/mL. These concentrations of LPS were predicted based on the likely bioavailability from the i.p. cavity, representing approximately 10% of the injected i.p. dose (23). To match the time period of exposure in the previous *in vivo* studies, the cells were exposed to serum-free medium or LPS for 28 h. The cells were then lysed using ice cold radio-immunoprecipitation assay buffer (150 mM sodium chloride, 1.0% v/v Triton X-100, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulfate,

50 mM Tris Base, pH 8.0) supplemented with 4% of the Complete Protease Inhibitor Cocktail (Roche Pharmaceuticals, Basel, Switzerland) and 1 mM of phenylmethanesulfonyfluoride and the lysate was stored at -20℃. A Western blot was used to measure the expression of the TJ protein claudin-5. Total protein levels from each sample lysate were quantified with a BCA Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin as a standard, and these samples were then loaded at 7.5 μg/well and run on a 12% tris-glycine polyacrylamide gel. Proteins were separated by electrophoresis at 120 V for 1.5 h in a mini-gel apparatus (Bio-Rad Laboratories, Hercules, CA) and the formulae for polyacrylamide gel is shown in Appendix 2. After separation, proteins were transferred onto a nitrocellulose membrane (0.22 μm pore size, Bio-Rad Laboratories GmbH, Munich, Germany), the membranes were washed in TBS-T (Tris-buffered saline, 1 mM Tris·HCl, 15 mM sodium chloride, pH = 7.6 supplemented with 0.05% (v/v) Tween 20), and blocked with 5% (w/v) bovine serum albumin (BSA) dissolved in TBS-T. Two hours later, the 5% BSA solution was removed and the membrane was incubated with the primary antibodies diluted in TBS-T, overnight at 4°C. The membranes were rinsed again with TBS-T and then incubated with the secondary antibodies diluted in TBS-T for 2 h at room temperature. Bands were visualized by exposure to Licor Odyssey scanner (Lincoln, NE) and optical density was quantified using Image J analysis software (National Institute of Health, USA). The expression of claudin-5 was measured relative to the levels of the housekeeping protein β-actin.

5.3.5 Ameliorative effect of colistin on LPS-mediated BBB disruption

Studies investigating whether colistin can ameliorate the BBB-disrupting effect of LPS from *S. enterica* were performed in hCMEC/D3 cells and confirmed *in vivo*. hCMEC/D3 cells were grown to confluence (as detailed above) and treated with either (i) serum-free medium, (ii) LPS from *S. enterica* (15 μg/mL in serum-free medium); (iii) colistin sulfate (30 μg/mL in serum-free medium), or (iv) LPS from *S. enterica* (15 μg/mL in serum-free medium) and colistin sulfate (30 μg/mL in serum-free medium) (n=3 per treatment). The concentration of colistin chosen was based on the average plasma concentration obtained after subcutaneous administration of a 40 mg/kg dose to mice (22). At 28 h after treatment, cells were lysed and the expression of claudin-5 was quantified, as described above.

To confirm whether any ameliorative effect of colistin observed *in vitro* was reflective of the *in vivo* setting, male Swiss Outbred mice (n=4 per treatment) were randomly divided into 4 groups and each mouse in each group was administered an i.p. dose of LPS (3 mg/kg in saline) or saline either with a subcutaneous dose of colistin (40 mg/kg in saline) or saline at 0, 6 and 24 h. At 4 h after the last dose, mice were intravenously administered a 50 μL solution of ^{14}C-sucrose (2 μCi in saline) as a BBB integrity marker, and plasma and brain samples were collected at 5 min post-dose. The radioactivity in plasma and brain homogenate was determined using liquid scintillation counting as described above, and B:P ratios of ^{14}C-sucrose were determined for each treatment group. In addition, plasma cytokine levels (TNF-α,

IL-1β and IL-6) were also measured as described above.

5.3.6 Data analysis

All data are presented as mean ± SD, unless otherwise stated. For comparisons of the B:P ratios (^{14}C-sucrose or colistin) or cytokine concentrations between saline-treated and LPS (*S. enterica* or *P. aeruginosa*)-treated animals, a Student's t test was used. When comparing the hCMEC/D3 actin-normalized expression of claudin-5 between different LPS concentrations, a one-way analysis of variance followed by a Newman-Keuls multiple comparisons test (PASW Statistics for Windows, version 17.0; Chicago, Illinois) was used. The one-way analysis of variance was also used when comparing the claudin-5 expression or B:P ratios of ^{14}C-sucrose between saline-, LPS (*S. enterica*)- and colistin-treated hCMEC/D3 cells or mice, respectively. A p value < 0.05 was considered to be a significant difference.

5.4 Results

5.4.1 Plasma cytokine levels following LPS treatment

Plasma concentrations of TNF-α, IL-1β and IL-6 following administration of different LPS strains are shown in Fig. 5-1. The levels of all three cytokines were below the lower limit of quantification (LLQ) (i.e. < 8 pg/mL) in saline-treated mice. The IL-1β levels in plasma following administration of LPS from *P. aeruginosa* were also below the LLQ, and plasma samples from only 2 of 4 animals demonstrated quantifiable levels of IL-1β following *S. enterica* LPS administration. However, the concentrations

of the other two cytokines (i.e. TNF-α and IL-6) were considerably higher in both *P. aeruginosa* and *S. enterica* LPS-treated mice, although a statistical enhancement (relative to control) could not be demonstrated given that the corresponding cytokine levels in saline-treated mice were all below the LLQ. Importantly, the plasma cytokine levels of TNF-α and IL-6, while increased relative to those in saline-treated mice, were not significantly different between mice treated with LPS from *P. aeruginosa* and *S. enterica* ($p > 0.05$). Although the result was generated from a small number of replicates (i.e. n=4), it appeared consistent with observations from our *in vitro* results where we measured the released concentrations of TNF-α and IL-6 in hCMEC/D3 cells following incubation with LPS from *P. aeruginosa* or *S. enteric*. (see Appendix 5); As observed in the previous chapter, these results suggest that the LPS-disrupting effect on the BBB *in vivo* has limited relationship with plasma cytokine levels of TNF-α and IL-6, and may be due to direct effects of these LPS species on the BBB.

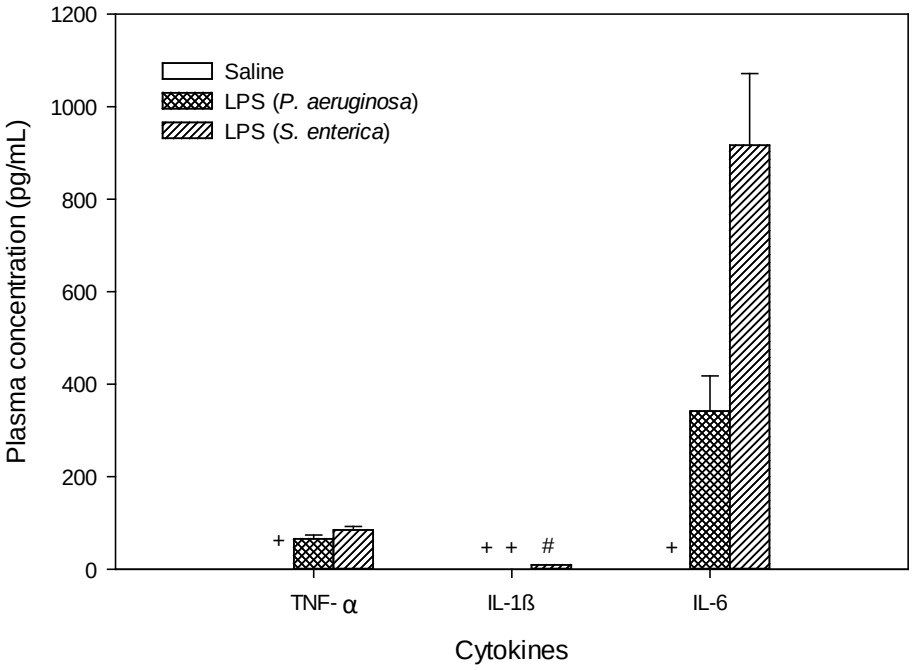

Figure 5-1. Plasma concentration (pg/mL) of TNF-α, IL-1β and IL-6 in Swiss Outbred mice at 4 h after the last dose of saline or LPS alone (*S. enterica* or *P. aeruginosa*, 3 mg/kg at 0, 6 and 24 h). Data are presented as mean ± SEM (n=4). $^{+}$ indicates that plasma levels were below the lower limit of quantification (8 pg/mL). $^{\#}$ indicates n=2 (as the remaining replicates were below 8 pg/mL).

5.4.2 Effect of different LPS on BBB integrity and brain uptake of colistin

The B:P ratios of ^{14}C-sucrose in saline- and LPS (*S. enterica* or *P. aeruginosa*)-treated mice are shown in Fig. 5-2a and Fig. 5-2b. While there was a slight, but significant, enhancement in the B:P ratio of ^{14}C-sucrose with LPS from *S. enterica*, no significant difference in the B:P ratio of ^{14}C-sucrose was detected between saline- and LPS (*P. aeruginosa*)-treated mice with B:P ratios of 0.032 $\pm$ 0.004 and 0.043 $\pm$ 0.015, respectively. Consistent with this species-dependent effect on BBB integrity, the B:P

ratios of colistin in saline- or LPS (*P. aeruginosa*)-treated mice were very low with the values of 0.033 ± 0.005 and 0.024 ± 0.006, respectively (Fig. 5-3a), whereas a substantially higher colistin B:P ratio (0.12 ± 0.053) was detected in LPS (*S. enterica*)-treated mice (Fig. 5-3b). These results demonstrated that at the current dose, the BBB integrity is disturbed by *S. enterica* LPS but not by *P. aeruginosa* LPS.

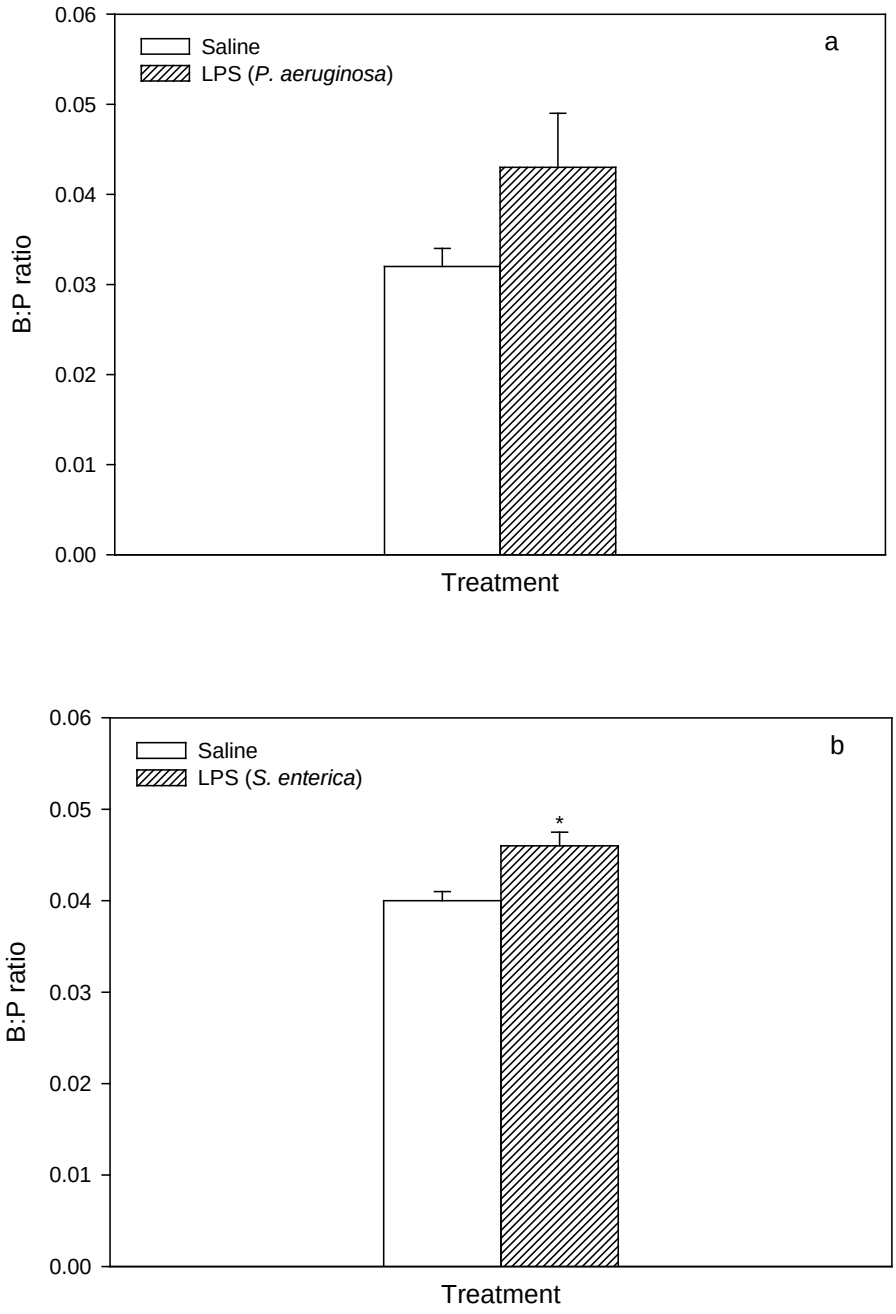

Figure 5-2. Brain-to-plasma (B:P) ratios of ^{14}C-sucrose in Swiss Outbred mice at 4 h after the last dose of saline or LPS (3 mg/kg at 0, 6 and 24 h) from (a) *P. aeruginosa* and (b) *S. enterica*. Data are presented as mean ± SEM (n = 6). * indicates $p < 0.05$ relative to saline-administered mice, using a Student's *t*-test.

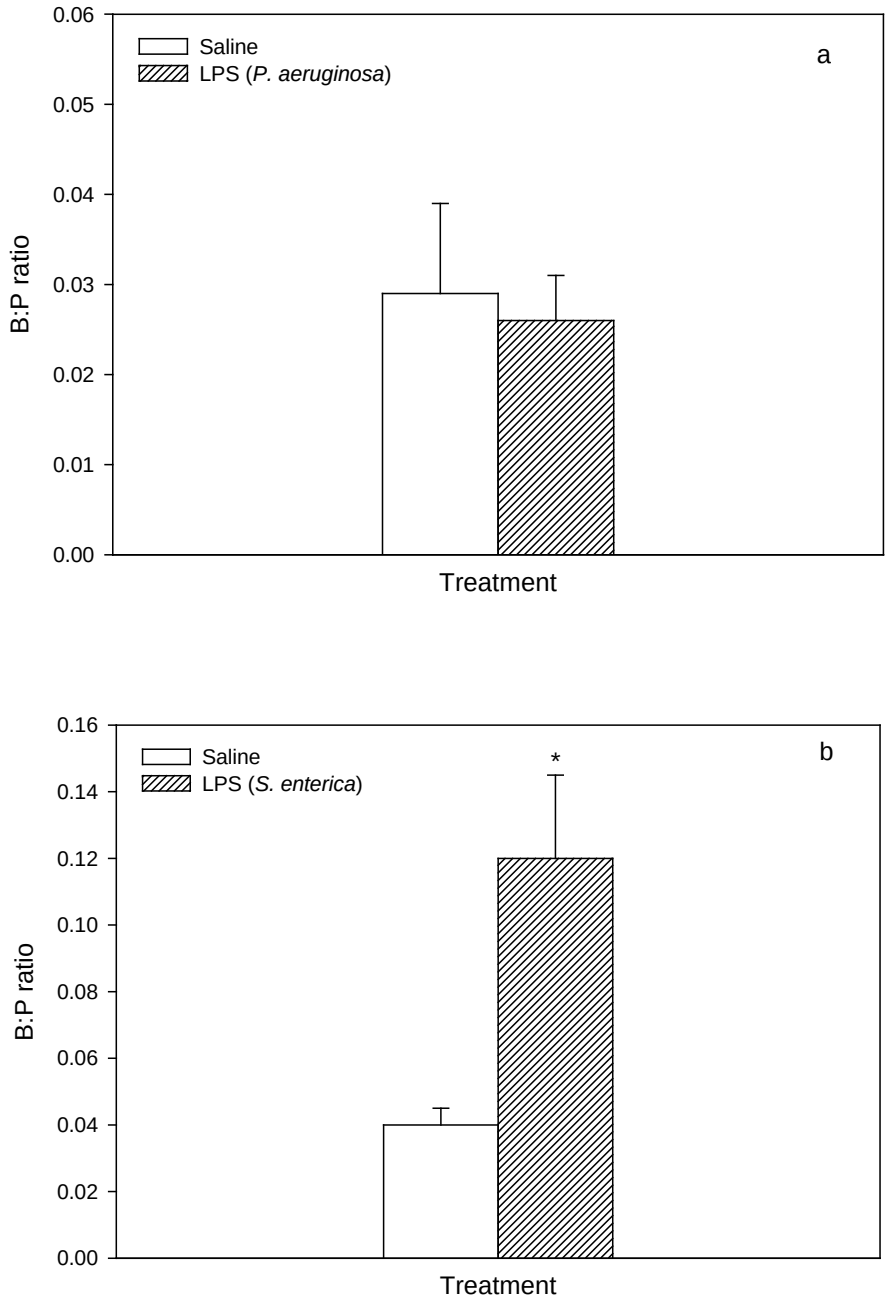

Figure 5-3. B:P ratios of colistin in Swiss Outbred mice at 4 h after the last dose of saline or LPS (3 mg/kg at 0, 6 and 24 h) from (a) *P. aeruginosa* and (b) *S. enterica*. Data are presented as mean ± SEM (n = 4). * indicates $p < 0.05$ relative to saline-administered mice, using a Student's *t*-test.

5.4.3 Effect of LPS on claudin-5 expression

At 28 h after the hCMEC/D3 cells were treated with medium or medium containing LPS from different strains, the expression levels of the TJ protein claudin-5 were determined by western blot. As shown in Fig. 5-4a, a one-way ANOVA demonstrated that the claudin-5:β-actin ratios were no different between brain endothelial cells treated with medium or *S. enterica* LPS at 3.75 μg/mL (1.17 ± 0.17 vs 1.07 ± 0.06). However, as the concentration of *S. enterica* LPS increased to 7.5 μg/mL and above, the claudin-5:β-actin ratios decreased substantially relative to control-treated cells ($p < 0.05$). In contrast, no difference in the claudin-5:β-actin ratio was observed between cells treated with medium and *P. aeruginosa* LPS except at the highest concentration of LPS assessed (i.e. at 30 μg/mL) (Fig. 5-4b). Representative immunoreactive bands were shown in Appendix 3. These results were suggestive of a concentration-dependent effect of both LPS strains on the ability to reduce claudin-5 expression, and in line with that observed *in vivo* (section 5.4.2), a species-dependent effect of LPS on the BBB paracellular route.

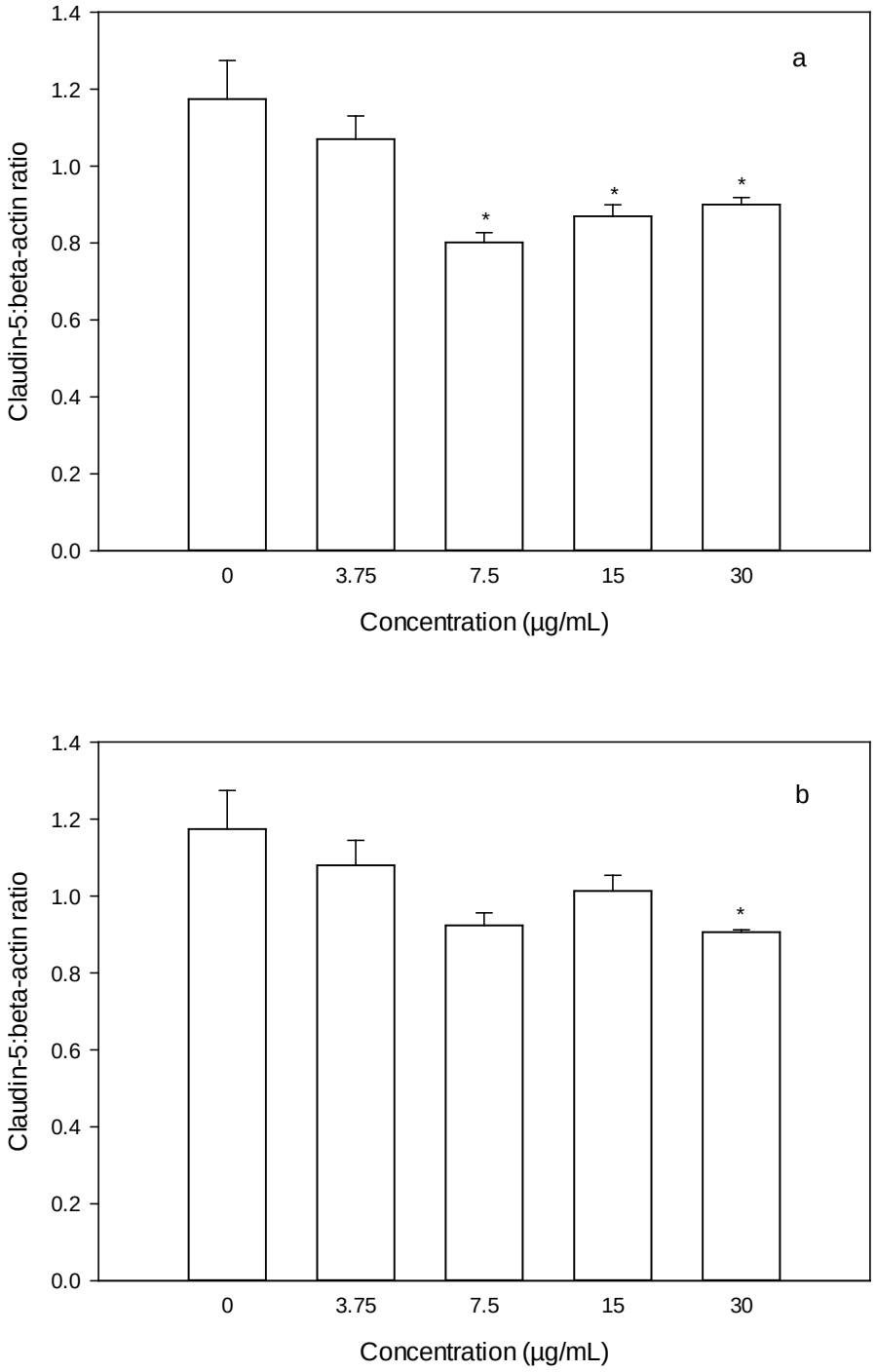

Figure 5-4. Claudin-5:β-actin ratios in hCMEC/D3 cells 28 h after treatment with (a) LPS (*S. enterica*) and (b) LPS (*P. aeruginosa*) at concentrations of 0, 3.75, 7.5, 15 and 30 µg/mL. Data are presented as mean ± SEM (n=3). * indicates a p value < 0.05 compared to control using a one-way ANOVA.

5.4.4 Amelioration of LPS effect on BBB integrity by colistin

As shown in Fig. 5-5, the claudin-5:β-actin level in hCMEC/D3 cells was not affected by treatment with colistin alone, however, treatment with *S. enterica* LPS at a concentration of 15 μg/mL, resulted in a significant (p<0.05) reduction in the expression of this TJ protein. Importantly, when LPS-treated hCMEC/D3 cells were also treated with colistin at 30 μg/mL, the expression of claudin-5 returned to baseline level, being no different to control-treated hCMEC/D3 cells, suggesting that the disruptive effect of LPS was abolished by colistin treatment. Representative immunoreactive bands were shown in Appendix 3.

Similarly, the integrity of the BBB (as measured by the B:P ratio of ^{14}C-sucrose) was no different between saline-treated and colistin-treated mice (Fig. 5-6) with B:P ratios of 0.034 ± 0.003 for saline-treated mice and 0.032 ± 0.003 for colistin-treated mice. The B:P ratio of ^{14}C-sucrose was significantly increased following *S. enterica* LPS pre-treatment with ^{14}C-sucrose B:P ratios of 0.044 ± 0.002. However, when LPS was co-administered with colistin at 40 mg/kg, the brain uptake of ^{14}C-sucrose returned to baseline levels, as was observed with hCMEC/D3 cells *in vitro*, with ^{14}C-sucrose B:P ratios of 0.026 ± 0.001. In fact, the B:P ratios of ^{14}C-sucrose were significantly lower than those in the saline-treated mice.

To determine whether the cytokine-inducing ability of *S. enterica* LPS would be restored by co-administration of colistin, cytokine levels were measured in mice

following treatment with *S. enterica* LPS alone or with colisitin and *S. enterica* LPS, as shown in Fig. 5-7. While IL-1β levels were below the LLQ (i.e. 8 pg/mL) in mice treated with colistin and *S. enterica* LPS simultaneously, TNF-α and IL-6 concentrations in mice treated with *S. enterica* LPS alone or colistin and *S. enterica* LPS were not different from each other. These results indicate that the BBB-restoring effects of colistin were not related to an effect on plasma cytokines levels, at least for TNF-α and IL-6.

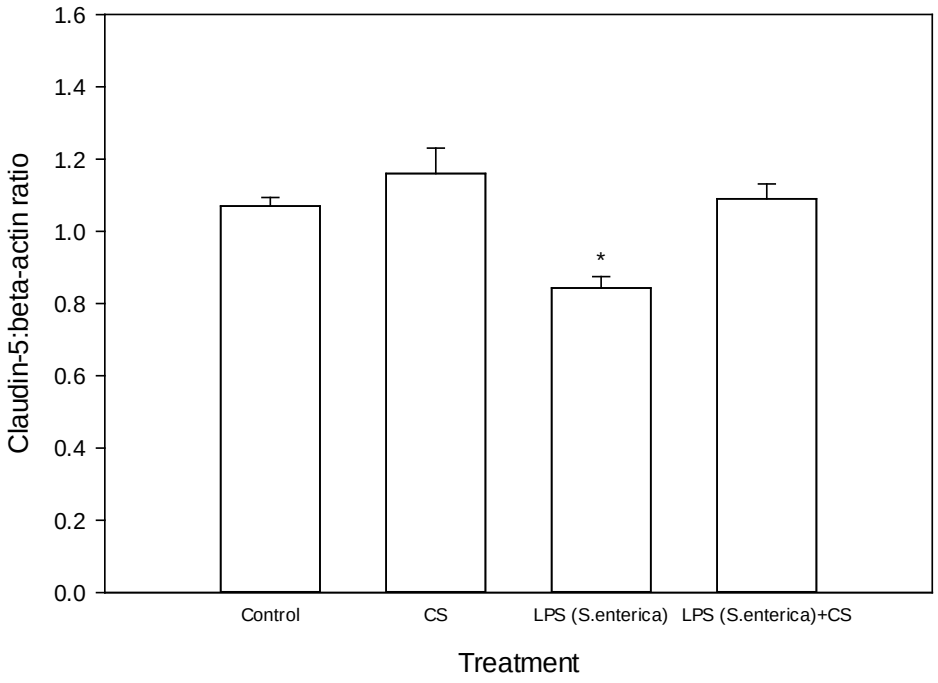

Figure 5-5. Claudin-5:β-actin ratios in hCMEC/D3 cells 28 h after treatment with serum-free medium (control), colistin sulfate (CS, 30 µg/mL in serum-free medium), LPS (*S. enterica*, 15 µg/mL in serum-free medium), and LPS (*S. enterica*, 15 µg/mL in serum-free medium) with CS (30 µg/mL in serum-free medium). Data are presented as mean ± SD (n=3).

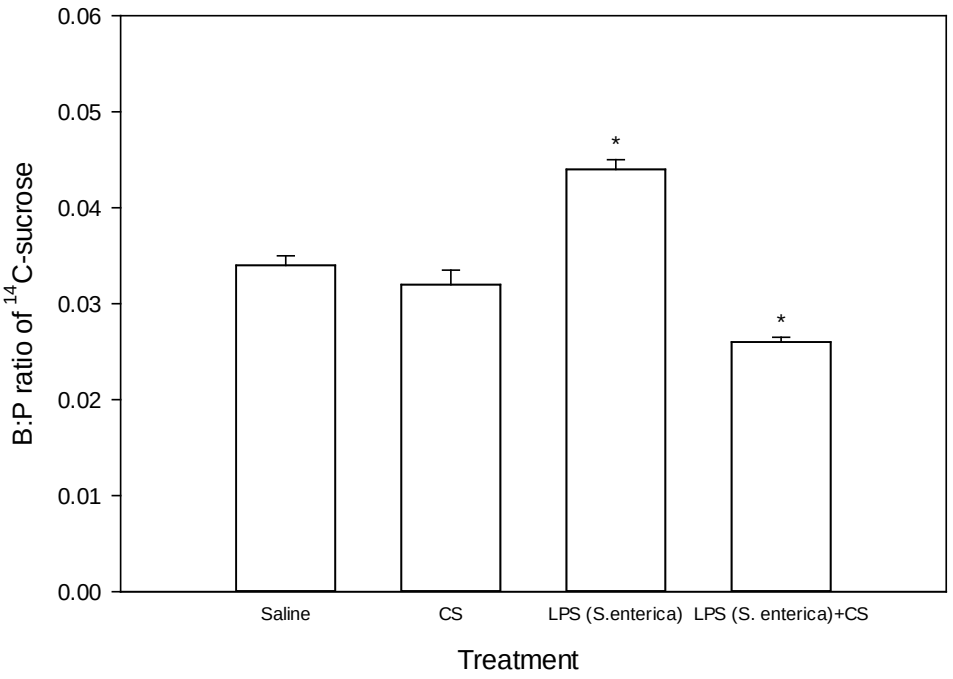

Figure 5-6. B:P ratios of ^{14}C-sucrose in Swiss Outbred mice at 4 h after the last dose of the i.p. saline-subcutaneous (s.c.) saline regimen; i.p. saline and s.c. CS (40 mg/kg) at 0, 6 and 24 h; i.p. *S. enterica* LPS (3 mg/kg) and s.c. saline at 0, 6 and 24 h; and intraperitoneal *S. enterica* LPS (3 mg/kg) with CS (40 mg/kg) at 0, 6 and 24 h. Data are presented as mean ± SEM (n=4).

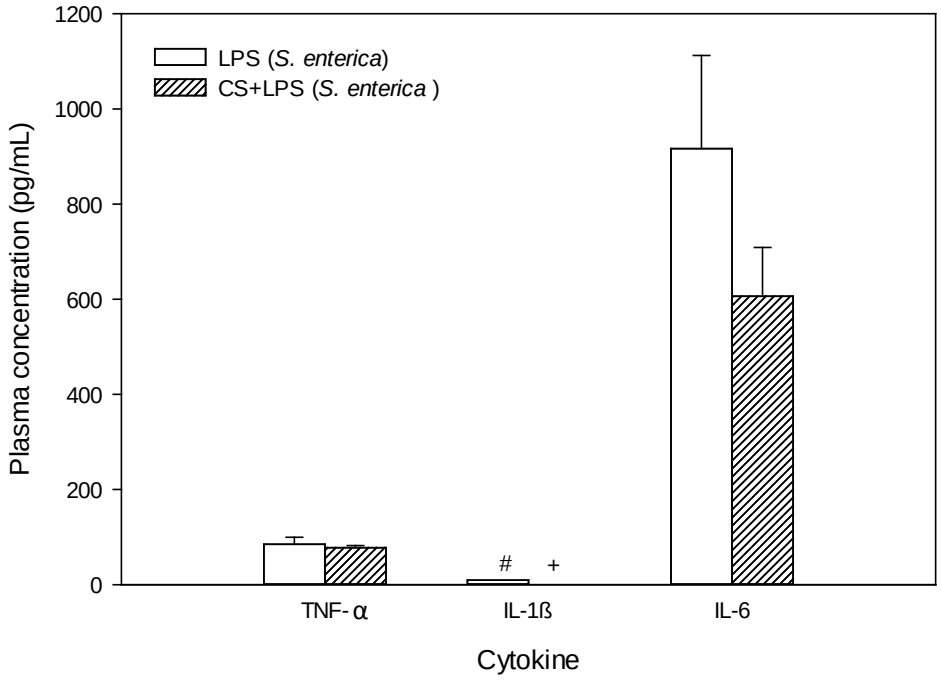

Figure 5-7. Plasma concentration (pg/mL) of TNF-α, IL-1β and IL-6 in Swiss Outbred mice at 4 h after the last dose of LPS alone (*S. enterica*, 3 mg/kg at 0, 6 and 24 h) or LPS (*S. enterica*, 3 mg/kg at 0, 6 and 24 h) with colistin (CS) (40 mg/kg at 0, 6 and 24 h). Data are presented as mean ± SEM (n=4). $^{+}$ indicates that plasma levels were below the lower limit of quantification (8 pg/mL). $^{\#}$ indicates n=2 (as the remaining replicates were below 8 pg/mL).

5.5 Discussion

Due to the unique TJ structure exhibited by endothelial cells forming the BBB, the biochemical homeostasis within the CNS is well maintained. However, as a result of certain diseases such as systemic inflammation and bacterial infection (see Section 1.3.2.7), an opening of the BBB TJs and the subsequent hyperpermeability may potentially lead to neurotoxicity, given that the brain exposure of

normally-impenetrable compounds may be increased (52). To this end, we have assessed the impact of systemic inflammation on the CNS exposure of the poorly-permeable antibiotic colistin, and have shown increased brain uptake following repeated i.p. injections of LPS from *S. enterica* (Section 3.4.3) (22). This suggested that the brain uptake of this antibiotic may also be enhanced during bacterial infection, however, following bacteremia induced by *P. aeruginosa*, BBB integrity and brain uptake of colistin remained unaltered (Section 4.4.2 and 4.4.3). As bacterial infection with *P. aeruginosa* led to elevated plasma cytokine levels (TNF-α, IL-1β and IL-6), with no increase in BBB permeability, it was hypothesized that different LPS strains (i.e. *P. aeruginosa* vs *S. enterica*) may have a different ability to directly affect the BBB, independent of effects on plasma cytokine levels.

In order to address whether the above-mentioned disparity resulted from the differences between the bacterial strains, the effect of LPS from both *P. aeruginosa* and *S. enterica* was assessed. After being administered the same dosing regimen as used for LPS from *S. enterica* (i.e. 3 mg/kg at 0, 6, 24 h), the brain uptake of ^{14}C-sucrose and colistin in mice treated with *P. aeruginosa* LPS was no different to that in saline-treated mice. In addition, the elevated concentrations of TNF-α and IL-6 in the plasma in *P. aeruginosa* LPS treated-mice were not different from those in *S. enterica* LPS-treated mice, suggesting a limited relationship between plasma levels of TNF-α and IL-6 and BBB disruption (albeit this was undertaken in a small number of animals). Moreover, the fact that co-administration with colistin was not able to

decrease the plasma levels of TNF-α and IL-6 induced by *S. enterica* LPS, but still ameliorate the BBB-disrupting effect of *S. enterica* LPS, further indicated that LPS may be causing a direct BBB disruption independent of TNF-α and IL-6 plasma levels. The results may appear to contrast other studies showing that altered BBB integrity is observed in brain endothelial cells and animals following treatment with cytokines such as TNF-α, IL-1β and IL-6 (8, 15), however, the BBB-disrupting effect of cytokines in those studies were only observed at concentrations far exceeding the levels we have observed *in vivo* with both LPS administration and bacterial infection (1, 10). Our results suggested that, at the current dosing regimen, LPS from *P. aeruginosa* is not as BBB-disrupting as LPS from *S. enterica*. However, it should be noted that the disruptive effect on the BBB induced by LPS is dose-dependent and the effective dose of each LPS may vary from strain to strain (7, 17, 48). Significantly increased sucrose permeability across brain capillary endothelial cells was observed following treatment with *E. coli* LPS at a higher experimental concentration of LPS (i.e. 5 ng/mL) (11). Another study has demonstrated that transendothelial electrical resistance values in rat brain endothelial cell monolayers treated with *S. enterica* LPS were not altered until the concentration of LPS reached 0.1 μg/mL (48). Therefore, it is possible that *P. aeruginosa* LPS may perturb the BBB when present at high concentrations/doses.

It has been well reported that LPS can affect the integrity of the BBB and alter the paracellular permeability through binding to its BBB cellular receptors such as TLR4

or CD14 (39, 45). Several cytoplasmic signalling molecules such as RhoA, NF-kB, PI3 kinase, myosin light chain kinase, protein kinase C and MAPK have been suggested to be involved in this process, either directly or indirectly via the rearrangement of actin cytoskeleton (13, 19, 42). Studies have also shown that different LPS strains may impact on inflammatory mediator production (38), and therefore, we assessed whether *P. aeruginosa* and *S. enterica* LPS exhibited differences in their ability to alter TJ expression in a relevant *in vitro* BBB model. Recently generated by immortalizing primary human brain endothelial cells (50), the hCMEC/D3 cells show a morphology that closely resembles primary cells in culture and they form confluent monolayers that express important *in vivo* BBB characteristics such as TJ proteins (37). Therefore, the hCMEC/D3 cells were treated with LPS from both bacterial species at a series of concentrations and the levels of claudin-5, which is one of the primary TJ proteins limiting the paracellular movement of molecules (see Section 1.3.2.2), were then quantified. As studies have shown that the level of LPS in the general circulation represents approximately 10% of the injected i.p. dose (25), the estimated plasma concentration of LPS in our animal study following i.p. administration of 3 mg/kg was expected to be approximately 15 µg/mL. To reflect these likely concentrations, LPS concentrations ranging from 3.75-30 µg/mL were chosen to measure the impact of different LPS strains on claudin-5 expression in the cell culture model. The results demonstrated that, while *S. enterica* LPS can affect the expression of claudin-5 at a relatively low concentration, the required concentration of *P. aeruginosa* LPS to decrease claudin-5 expression was

four times higher. Our findings from the *in vitro* studies corroborate the results from our *in vivo* studies, and suggest that *S. enterica* LPS may also exhibit a greater ability to decrease TJ function at the BBB *in vivo*. However, further studies would be required to confirm this, given the *in vitro* model used in our studies was of human origin and our *in vivo* studies were performed in mice and the small number of replicates in the present study (i.e. n=3).

The first step for LPS to exert its BBB-disrupting effect is through the binding of LPS to the receptors at the BBB and lipid A is the binding moiety of LPS (14). Therefore, the specific BBB-disrupting effect of *S. enterica* LPS, relative to *P. aeruginosa* LPS, may be due to their different structures, particularly the lipid A component of LPS. Indeed, LPS from different bacterial species exhibit different biological activities. Zughaier et al. have shown that LPS from *Escherichia coli* has a TNF-α-inducing activity eight-fold greater than LPS from *P. aeruginosa* (53). It is interesting to note that lipid A which adopts a conical conformation is more active in inducing cytokine production through binding to receptors than lipid A that adopts a cylindrical shape (31). There is evidence suggesting that lipid A of LPS from *P. aeruginosa* maintains a cylindrical shape (16), while lipid A of LPS from *E. coli* or *S. enterica* exhibits a conical shape (43). Moreover, lipid A with side chains of 12-14 carbons in length has been shown to stimulate immunological responses more efficiently, whereas altering the number or length of the attached fatty acids in lipid A can reduce the magnitude of the signal (30, 46). As most side chains of lipid A from *P. aeruginosa*

LPS contain 12 or even less carbons, whereas there are 14 carbon atoms in most side chains of lipid A from *S. enterica* LPS, it is likely that LPS from *S. enterica* may be able to bind to its receptors at the BBB more effectively to trigger the downstream signalling pathway to cause BBB disruption. However, while the data suggested that *S. enterica* LPS was more disruptive, the mechanisms behind this and which signalling molecules were involved remained unknown.

Given that colistin, an antibiotic from the polymyxin family, is able to bind to the LPS within the outer membrane of Gram-negative bacteria, and indeed this is the initial step of its antibacterial effect (6) (see Section 1.2.3), we investigated whether the BBB-disruptive effects of LPS from *S. enterica* could be ameliorated by colistin. Treatment of hCMEC/D3 cells with *S. enterica* LPS led to a significant reduction in claudin-5 expression. However, when co-treated with colistin sulfate at 30 μg/mL, a concentration similar to that we have observed in mice after repeated administration of colistin sulfate (22), the claudin-5-reducing effects of LPS were prevented. It should be noted that alterations of BBB functions are not always accompanied by changes in the endothelial expression of TJ proteins (24, 44) and the decreased expression of claudin-5 may not necessarily affect BBB function. Therefore, it was necessary to determine whether a similar effect would be observed *in vivo*. Thus, the BBB penetration of ^{14}C-sucrose in mice was assessed under a range of different pre-treatments. The brain uptake of ^{14}C-sucrose in mice treated with colistin alone did not differ from that in saline-treated mice suggesting repeated subcutaneous

administrations of colistin did not affect BBB integrity. An opening of the BBB paracellular route was observed in LPS (*S. enterica*)-treated mice as the B:P ratio of ^{14}C-sucrose increased significantly; however, co-administration of colistin with LPS (*S. enterica*) restored the BBB integrity, indicating colistin could negate the LPS effect. The deprival of BBB-disrupting effect of LPS (*S. enterica*) may result from the binding of lipid A with colistin. As mentioned above, the lipid A component is the moiety of LPS responsible for binding to receptors and triggering the downstream signalling pathways responsible for the damage of TJ function (20, 45). Therefore, the avid binding of colistin to the lipid A portion of LPS (5) may decrease the free concentration of circulating LPS available to the receptors (i.e. TLR4 or CD14), thus preventing the ability of the LPS to decrease expression of claudin-5 *in vivo*. It should noted that co-administration of LPS with colistin did not significantly reduce the release of cytokines, at least for TNF-α and IL-6. The results appearing to contrast to what was observed in other studies may result from the different bacteria species inducing the release of cytokines (see Section 1.2.3), as *S. enterica* LPS was used in our study whereas *P. aeruginosa* was employed in the other study (3). Overall, this observation may suggest a new potential therapeutic role for colistin; namely, prevention of LPS-induced BBB disruption that is observed in various disorders such as sepsis and acquired immune deficiency syndrome (11).

5.6 Conclusion

The ability of LPS to induce BBB disruption *in vitro* and *in vivo* has been

demonstrated to be species-dependent, with LPS from *S. enterica* inducing greater BBB disruption than LPS from *P. aeruginosa*. Moreover, it has been shown that colistin, a polymyxin antibiotic, has the ability to reduce the LPS-mediated BBB paracellular disruption, suggesting another potential therapeutic application of colistin in Gram-negative bacteria-caused sepsis.

5.7 Acknowledgements

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Chapter Six

Summary and future directions

6.1 Summary

Applying a novel HPLC method, this PhD project first investigated the factors that limit the brain uptake of colistin in healthy mice. The impact of systemic inflammation and infection on the BBB transport of colistin was then assessed using models developed in mice. Finally, the factors which may lead to the BBB alterations during systemic inflammation, and the potential for colistin to reverse this, were evaluated in hCMEC/D3 cells and in mice.

For quantifying colistin concentration in mouse brain homogenate, a HPLC method involving protein precipitation with TCA and derivatization with FMOC-Cl was developed. This reliable and reproducible HPLC method has been applied to evaluate the brain uptake of colistin in healthy mice following a single intravenous bolus of colistin sulfate (5 mg/kg), which was shown to be minimal with B:P ratios of colistin ranging from 0.02 to 0.06.

To investigate whether the minimal brain uptake of colistin was due to the rapidly decreased plasma concentration following intravenous administration, single and multiple subcutaneous injections of colistin were administered, as is used clinically. While the plasma concentrations of colistin were sustained at higher levels for a longer period of time with these approaches, the brain uptake of colistin still remained very low with average B:P ratios of 0.03 and 0.02 for single and multiple subcutaneous injections, respectively. Another reason that may contribute to the low

brain uptake of colistin is the P-gp transporter at the BBB, given colistin has some physicochemical properties such as a positive charge at physiological pH and a molecular mass of > 400 Da, characteristics which are commonly shared by P-gp substrates. However, the B:P ratios of colistin remained unaffected in the presence of P-gp inhibitors (i.e. PSC833 or GF120918) ($p > 0.05$). The results suggested that the low brain uptake of colistin in healthy mice was not attributed to the plasma concentration present or the efflux transporter-P-gp. It is therefore likely that the physicochemical properties of colistin (i.e. large Mw and ionized nature etc) and the inability to penetrate paracellular route was reason for low brain uptake of colistin (or something to this effect).

In a surrogate mouse model of systemic inflammation established by intraperitoneal injections of LPS from *S. enterica*, a significant increase in the brain uptake of colistin was observed following both *in vivo* (intravenous) and *in situ* (trans-cardiac perfusion) administrations of colistin sulfate. For the first time, we demonstrated that the enhanced brain uptake of colistin is due to the opening of tight junctions at the BBB, which may resemble the scenario encountered in infected patients.

To mimic the clinical setting more closely, the brain uptake of colistin was assessed in the presence of a systemic bacterial infection. The systemic infection model was developed in mice following intramuscular administration of *P. aeruginosa*, which is a clinically-relevant MDR Gram-negative bacteria, for which colistin is used to treat.

Though bacteremia was successfully achieved, the BBB integrity and the brain uptake of colistin was not affected regardless of the elevated plasma concentrations of three cytokines, which have been showed to cause decreased expression and re-organization of tight junction proteins (TNF-α, IL-1β and IL-6). The results suggested that either not enough *P. aeruginosa* LPS were released during bacterimia to exert its BBB-disrupting effect or *P. aeruginosa* LPS was not as virulent as *S. enterica* LPS in disrupting the BBB.

The interesting observation that increased BBB permeability could be induced by LPS (a major outer membrane component of Gram-negative bacteria) from *S. enterica* but not by *P. aeruginosa* bacteria provided the first suggestion that there may be species-dependent bacterial effects on the BBB. Indeed, LPS derived from *P. aeruginosa* was unable to affect the integrity of the BBB or the brain uptake of colistin, an effect observed with LPS derived from *S. enterica*. Such findings were corroborated using an *in vitro* model, where we demonstrated decreased expression of claudin-5 with LPS from *S. enterica* at low concentrations, whereas much higher concentrations of LPS from *P. aeruginosa* were required to induce the same effect. Therefore, these studies showed that there is indeed a species- and dose-dependent BBB-disrupting effect of LPS and the effective concentration of LPS may vary from strain to strain.

Importantly, co-administration of colistin sulfate with LPS from *S. enterica* was able

to restore BBB integrity in mice and ameliorate the claudin 5 reduction in hCMEC/D3 cells induced by *S. enterica* LPS. This is likely due to the ability of colistin to bind to the lipid A portion and neutralize the BBB disrupting effect of LPS, suggesting that colistin has the potential to ameliorate endotoxemia-related BBB disruption in various disorders such as sepsis and acquired immune deficiency syndrome.

This study not only provides a better understanding of the BBB transport of a clinical-relevant antibiotic and its potential to induce centrally-mediated neurotoxicity, but also provides insight into the impact of systemic inflammation and bacterial infection on the BBB transport of drugs, which normally do not cross the BBB. The species-dependent effects of LPS suggest that there may be increased CNS exposure of antibiotics or other drugs during certain infections and not others, potentially placing patients at increased risk of neurotoxicity while infected with such bacteria.

6.2 Future directions

This PhD project mainly focused on the BBB transport of colistin sulphate, the active form of colistin, under healthy and disease condition. Given that CMS, the inactive pro-drug of colistin, is another clinical form that is parenterally dosed, it will be of interest to assess the impact of infection on the brain uptake of CMS. The possible altered BBB transport of CMS and/or converted colistin may contribute to the potentially centrally-mediated neurotoxicity of colistin

Our study has suggested direct effects of LPS on the BBB, which may involve several signalling molecules including RhoA, NF-kB, PI3, and MAPK. Given we have demonstrated a species-specific effect of LPS, it may be possible to use these two strains of LPS to further assess which of these signalling molecules are involved in inducing BBB disruption (i.e. certain signalling molecules may be enhanced with one LPS and not another), which may provide insight into the factors responsible for BB disruption.

It was observed in our studies that there was no direct correlation between BBB disruption and plasma concentrations of three cytokines (TNF-α, IL-1β and IL-6) in mice, which could be simply because the levels of the cytokines were not high enough (as has been used in other *in vitro* studies). Quantifying the expression of tight junction proteins in cells treated with a series of concentrations of each cytokine can help to address this discrepancy. In addition, a more effective correlation may be achieved between brain cytokine levels and BBB disruption following bacterial inoculation and LPS administration, and therefore, it would be of great interest to measure the cytokines levels in the brain homogenate following administration of each LPS. Furthermore, the possibilities that other cytokines such as IFN-γ or IL-10, chemokines, MMPs and ROS may play an important role in BBB disruption can not be ruled out, and it is worth detecting their concentrations in the plasma and brain homogenate following both LPS administration and bacterial infection.

While increased BBB permeability was observed following intraperitoneal injections of LPS (from *S. enterica*) but not with inoculation of *P. aeruginosa*, it remains unclear whether the BBB transporters are affected by these treatments. Previous studies have shown that treatment with the cytokines (TNF-α, IL-1β and IL-6), which were substantially increased after both treatments, will alter P-gp and BCRP function at the BBB if their levels are high enough. Therefore, whether the function of efflux transporters is altered in LPS-treated or *P. aeruginosa*-inoculated mice will require further investigation.

Although increased BBB permeability was not detected in the presence of *P. aeruginosa*-induced *bacteremia*, it remains unknown whether similar results will be observed when the bacteraemia is induced by *A. baumannii* or *K. pneumonia* or indeed other strains. As *A. baumannii* and *K. pneumonia* are also two clinically-relevant MDR Gram-negative bacteria which are treated by the polymyxins, it will be of interest to assess the BBB condition (i.e. integrity and function of transporters) and BBB transport of antibiotics (and other drugs) during systemic infection induced by these two bacterial strains.

Appendix 1: HPLC method validation in Swiss Outbred mouse blood and brain homogenate described in Chapter 3

Table A2-1. Precision and accuracy values of the quality control (QC) samples prepared in plasma and brain homogenate obtained from LPS-treated mice and compared to a standard curve prepared in matrices obtained from healthy mice.

Target colistin conc. in matrices from infected mice	Mean ± SD Measured colistin concentration (μg/mL or μg/g)	Precision (%)	Accuracy (%)
Plasma (μg/mL, n = 4)			
1.25	1.34 ± 0.10	7.4	107.2
5.00	4.97 ± 0.13	2.6	99.2
20.00	19.93 ± 2.42	12.1	99.7
Brain (μg/g, n = 4)			
0.19	0.18 ± 0.012	6.7	94.7
0.75	0.67 ± 0.035	5.2	89.3
3.00	2.87 ± 0.161	5.6	95.7

Appendix 2: Formulae for polyacrylamide gel described in Chapter 5

A2-1. Stacking gel mixture (4% acrylamide)

	Volume
40% (w/v) Acrylamide/bis-acrylamide	0.5 mL
Milli-Q Water	3.1 mL
0.5 M Tris-HCl (pH 6.8)	1.25 mL
10% SDS	50 µL
10% ammonium persulfate	50 µL
TEMED	5 µL

A2-2. Stacking gel mixture (12% acrylamide)

	Volume
40% (w/v) Acrylamide/bis-acrylamide	2.4 mL
Milli-Q Water	3.4 mL
1.5 M Tris-HCl (pH 8.8)	2 mL
10% SDS	80 µL
10% ammonium persulfate	80 µL
TEMED	8 µL

TEMED (polymerization catalyst) was added immediately before pouring gel into the plate fixture.

Appendix 3: Immunoreactive bands for claudin-5 in hCMEC/D3 cells described in Chapter 5

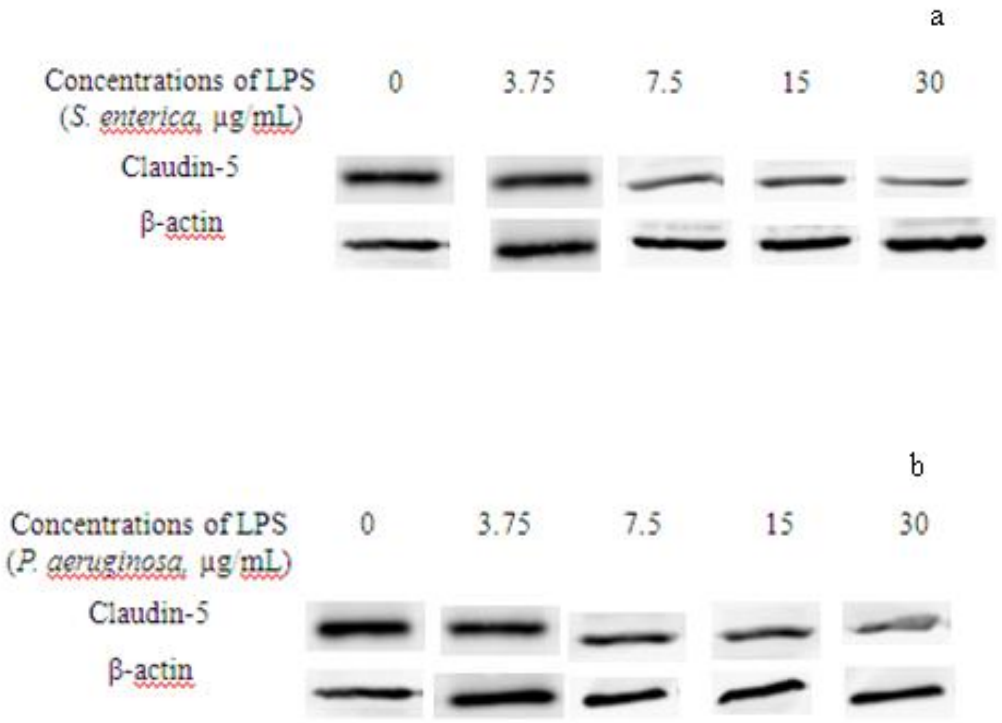

Figure A3-1. Representative immunoreactive bands for claudin-5 in hCMEC/D3 cells treated with (a) LPS (*S. enterica*) and (b) LPS (*P. aeruginosa*) at the concentrations of 3.75, 7.5, 15 and 30 µg/mL.

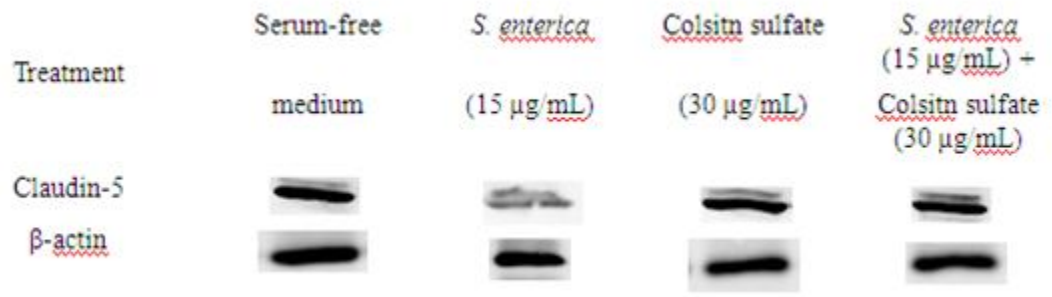

Figure A3-2. Representative immunoreactive bands for claudin-5 in hCMEC/D3 cells treated with serum-free medium, LPS (*S. enterica*, 15 µg/mL), colistin sulfate (CS, 30 µg/mL) and LPS (*S. enterica*, 15 µg/mL) with CS (30 µg/mL).

Appendix 4: Development of bacteremia following higher inocula of *P. aeruginosa*

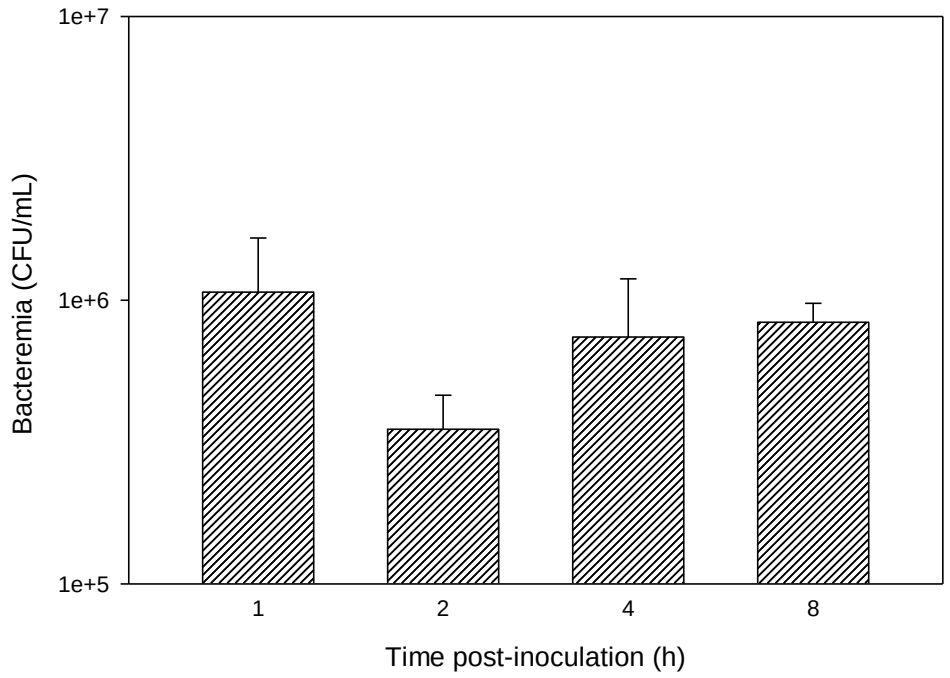

Figure A7-1. Bacteremia (CFU/mL) at various time points following intramuscular administration of *P. aeruginosa* (~6×10^8 CFU) to non-neutropenic Swiss Outbred mice. Data are presented as mean ± SEM (n = 4). As the bacteremia at post time points was much higher than that of neutropenic mice following intramuscular administration of *P. aeruginosa* (~5×10^5 CFU), it was decided to use a lower inoculum (~5×10^7 CFU) in non-neutropenic mice to render the disease condition similar between non-neutropenic and neutropenic mice (see Section 4.3.4).

Appendix 5: Cytokines released from hCMEC/D3 cells following various treatments

Concentrations of pro-inflammatory cytokines (TNF-α and IL-6) were measured by human cytokine kits (Ready-SET-Go!®, eBioscience, San Diego, CA). According to the company instructions, a 50 µL aliquot of medium at 28 h following a series of treatment was added to a 96-well plate and the absorbance was recorded at 450 nm with a Fluostar Optima microplate reader (BMG Labtech, Mount Eliza, Victoria, Australia). Standard curves were established using cytokines of known concentrations available in the kits with quantification ranges between 5 to 5000 pg/mL for TNF-α and between 5 to 250 pg/mL for IL-6. No absorbance was detected in LPS (*S. enterica* or *P. aeruginosa*) and colistin sulfate-contained medium, suggesting the assay was not affected by the endogenous components within plasma.

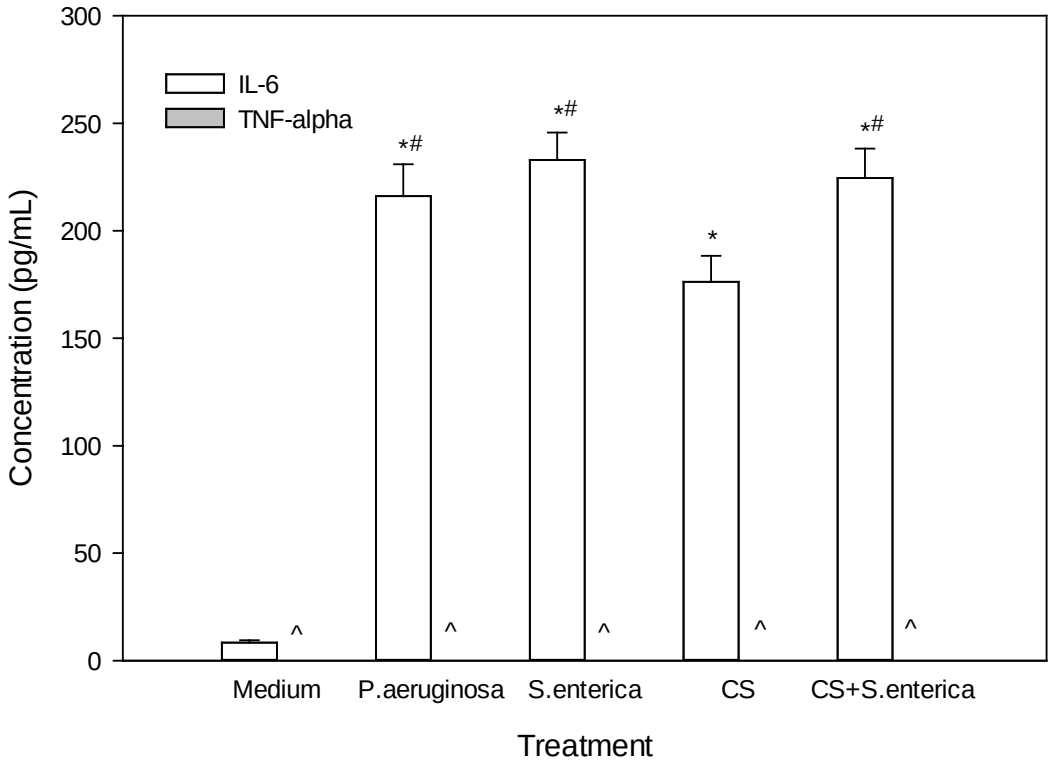

Figure A5-1. Release of IL-6 and TNF-α from hCMEC/D3 cells following a series of treatments (cell culture and treatment are same as Section 5.3.4 and 5.3.5) The concentrations of both LPS are 15 μg/mL and the concentration of colistin is 30 μg/mL. Data are presented as mean ± SD (n=3). * indicates $p < 0.05$ relative to cells treated with medium and # indicates $p < 0.05$ relative to cells treated with CS alone, using a one-way ANOVA. ^ indicates TNF-alpha released from hCMEC/D3 cells following a series of treatment were all below detection limit (i.e. 5 pg/mL).

Appendix 6: Publications in support of this thesis

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Brain Penetration of Colistin in Mice Assessed by a Novel High-Performance Liquid Chromatographic Technique▽

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A sensitive and reliable liquid chromatographic method was developed and validated for the determination of colistin concentrations in mouse brain homogenate. With a mobile phase consisting of acetonitrile-tetrahydrofuran-water (50:25:25 [vol/vol]) at a flow rate of 1 ml/min, a linear correlation between peak area and colistin concentration was observed over the concentration range of 93.8 to 3,000 ng/g in brain tissue (R^2 > 0.994). Intra- and interday coefficients of variation were 5.1 to 8.3% and 5.8 to 8.5%, respectively, and the recovery ranged from 85% to 94%. This assay was then utilized to determine the amount of colistin that permeated the blood-brain barrier over a 2-h period following bolus intravenous administration of colistin sulfate to mice. After a single dose of 5 mg/kg of body weight to mice, brain homogenate concentrations of colistin were very low, relative to plasma colistin concentrations, suggesting that colistin permeability across the healthy blood-brain barrier is negligible during this experimental period.

Colistin (also known as polymyxin E) is a cationic polypeptide antibiotic and is bactericidal against multidrug-resistant gram-negative pathogens (8). Like other members of the polymyxin family, colistin consists of two major components (colistins A and B), with colistins A and B differing only in the fatty acid side chain. In studies conducted several decades ago, colistin was shown to result in adverse effects after intramuscular or intravenous administration to patients (6, 14); nephrotoxicity and neurotoxicity were the most commonly observed adverse effects. It is possible that the prevalence of such toxicity may have been exaggerated due to a lack of understanding of colistin pharmacology and the use of inappropriate doses (22). Over the last few years, however, resistance to many other antibiotics and limited development of new antibiotics have resulted in an increasing use of colistin, administered in the form of its inactive prodrug, colistin methanesulfonate (CMS) (1), for the treatment of infections caused by gram-negative pathogens, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (5). With its increased use, interest in the pharmacology of this old antibiotic has been rekindled and optimization of dosage regimens is therefore urgently required in order to maximize its efficacy while minimizing potential toxicity.

Gram-negative bacterial species are increasingly reported to cause severe central nervous system (CNS) infections, such as meningitis (11, 24, 26) and ventriculitis (7, 30), which have a high mortality rate if untreated or treated inappropriately. There have been several clinical case reports of successful treatment of meningitis and ventriculitis by parenteral administration of CMS, either alone or in combination with other antibacterials (11, 12, 18), resulting in eradication of the gram-negative pathogens from the cerebrospinal fluid (CSF). These studies suggest that CMS, or colistin that is generated from CMS in vivo, has the potential to permeate the blood-CSF barrier (BCSFB). Indeed, using a microbiological assay, Jiménez-Mejías et al. were able to detect "colistin" in human CSF following intravenous administration of CMS (12). However, less is known about the ability of colistin to penetrate the blood-brain barrier (BBB), the endothelial lining of the cerebral blood vessels—a process which would be required in order to treat infections of the brain parenchyma, such as meningoencephalitis (27). A more thorough understanding of colistin penetration into the brain parenchyma, therefore, would be useful for optimizing treatment of such infections. In addition, the neurotoxicity associated with colistin (2) warrants a better understanding of colistin penetration across the BBB so that more rational dosing regimens can be designed with minimal potential for CNS-mediated toxicity. However, in order to accurately determine brain penetration of colistin, a robust and reliable analytical technique is required.

Numerous methods based on microbiological (15, 16, 19), thin-layer chromatographic (TLC) (32), immunological (13), and liquid chromatography and mass spectrometry (10, 25) techniques have been developed to quantitate the polypeptide antibiotics in plasma and tissues. While liquid chromatography and mass spectrometry methods are quite sensitive, they are often not very accessible, and the other techniques listed above often lack sensitivity or selectivity required for accurate quantitation. With respect to colistin, several methods for assaying this polypeptide in rat or human plasma have been developed utilizing derivatizing reagents such as orthophthalaldehyde (4, 17) and 9-fluorenylmethyl chloroformate (FMOC-Cl) (20, 21). While these assays have been successfully used to measure concentrations of colistin in human and rodent plasma and some tissue samples (34), quantitative assays for measuring the concentration of this antibiotic in animal and human brain tissues are not available.

The purpose of this study, therefore, was to develop a reli-

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able and sensitive method to quantify colistin in mouse brain homogenate. Our method consists of protein precipitation with trichloroacetic acid (TCA), solid-phase extraction (SPE) of colistin from brain tissue, and derivatization with FMOC-Cl, followed by liquid chromatography with fluorescence detection. This method was then employed in our laboratory to determine the concentration of colistin in mouse brain homogenate following intravenous administration of colistin sulfate, to determine the potential of colistin to penetrate the BBB in healthy mice.

MATERIALS AND METHODS

Chemicals and reagents. Colistin sulfate was purchased from Zhejiang Shenghua Biok Biology Co., Ltd. (EP5 grade; Zhejiang, China). FMOC-Cl, TCA, boric acid, and sodium hydroxide were all of analytical grade and obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia), and sodium hydrogen carbonate (analytical grade) was from Merck Chemicals (Kilsyth, Victoria, Australia). Acetonitrile (ACN), methanol, and acetone of high-performance liquid chromatography (HPLC) grade were all obtained from Merck KGaA (Darmstadt, Germany), and tetrahydrofuran was purchased from Honeywell Burdick & Jackson (Muskegon, MI). Water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Carbonate buffer (1% [wt/wt], pH 10) was prepared as described previously (20). SPE cartridges (C_{18} Sep-Pak; 100 mg) were purchased from Waters Corporation, Milford, MA.

Preparation of stock solutions, working standards, and QC solutions. A stock solution (1.00 mg/ml) of colistin sulfate was prepared in water and stored at 4°C. Working standard solutions with concentrations of 0.47, 0.94, 1.88, 3.75, 7.50, and 15.0 μg/ml were prepared by serial dilution of the stock solution with water. Quality control (QC) solutions were prepared separately at 0.47, 1.88, and 15.0 μg/ml, using an independently prepared stock solution (1.00 mg/ml).

Choice of protein precipitant: ACN versus TCA. While ACN is often used to precipitate brain homogenate proteins, extraction recovery of the compound of interest may be low, and so we compared the efficiency of both ACN and TCA as brain homogenate protein precipitants for this assay. Blank mouse brains were homogenized in a volume of Milli-Q water (in ml) equal to twice the weight (in g) of the tissue using a Kinematica Polytron PT-DA 3007/2EC homogenizer (Luzernerstrasse, Switzerland). A 3,000-ng/g colistin-spiked homogenate was prepared by adding 20 μl of a 15.0-μg/ml working standard into 280 μl of blank brain homogenate. Aliquots of this homogenate sample were subjected to either an ACN or TCA-ACN pretreatment to precipitate proteins, as follows. To 300 μl of 3,000 ng/g colistin-spiked brain homogenate was added ACN at various volumes (300, 600, 900, or 1,200 μl) or various TCA solutions (750 μl of 2% [wt/vol] TCA, 300 μl of 5% TCA, 150 μl of 10% TCA, or 75 μl of 20% TCA). To TCA-treated homogenates, ACN was added at the same volume of TCA. Both ACN and TCA-ACN-treated brain homogenates underwent tissue processing as detailed below.

Preparation of calibration and QC samples. Calibration and QC samples were prepared by adding a 20-μl aliquot of each working standard or QC solution into 280 μl of blank brain homogenate to achieve concentrations of 93.8, 188, 375, 750, 1,500, and 3,000 ng/g. To each mixture was added 300 μl of 5% TCA, given that this was found to be the most appropriate tissue precipitant (see Results). This mixture was vortex mixed for 5 min, and another 300 μl of ACN was added prior to further vortex mixing and centrifugation at 16,100 × *g* for 15 min. The entire supernatant was transferred to an SPE cartridge which had been conditioned by washing with 1 ml of acetone and 1 ml of methanol, followed by 1 ml of carbonate buffer. An aliquot (30 μl) of 100 mM FMOC-Cl and 80 μl of methanol were then added to the SPE cartridge, and after 10 min, colistin-FMOC derivatives were eluted with 900 μl of acetone. The eluent was vortex mixed for 1 min, and 40 μl of this solution was injected onto the HPLC column. The peak areas of the FMOC derivatives of colistins A and B were summed and employed for calibration (20). The low-, medium-, and high-QC samples of 93.8, 375, and 3,000 ng/g were prepared in the same manner, and intraday accuracy and precision were assessed. Intraday assay performance was determined by replicate analysis of six QC samples at each concentration, and interday assay parameters were determined by analysis of the QC samples on 3 separate days.

Chromatographic conditions. All chromatographic analyses were performed on a Shimadzu HPLC system consisting of an LC-10AT*vp* pump, a SIL-10ADvp autoinjector, a DGU-14A degasser, and an RF-10AXL fluorescence detector (Shimadzu, Kyoto, Japan) connected to a data acquisition and processing system (Shimadzu CLASS VP 6.14 SP1). The analyses were performed at 25°C. Samples were injected onto a Phenomenex Onyx monolithic C_{18} column (5 by 4.6 mm) using a mobile phase of ACN-tetrahydrofuran-water (50:25:25 [vol/vol]). The flow rate was set at 1 ml/min, and colistin-FMOC was detected at an excitation wavelength of 265 nm and an emission wavelength of 315 nm. For the linear least-squares regression analysis of the calibration curve, data were weighted according to 1/response.

TABLE 1. Average HPLC peak areas (mvolts · min) of colistin obtained following addition of TCA and ACN to colistin-spiked brain homogenate[a]

TCA concn (% [wt/vol])	Vol (μl) of:		Mean ± SD peak area of colistin (10^7)[b]
	TCA	ACN	
2	750	750	3.2 ± 0.3
5	300	300	3.1 ± 0.1
10	150	150	3.0 ± 0.4
20	75	75	1.9 ± 0.1

[a] Shown are results from various combinations of TCA and ACN added to 300 μl of 3,000 ng/g colistin-spiked brain homogenate.
[b] $n = 3$.

Determination of recovery. Duplicate sets of colistin-spiked samples were prepared in either 300 μl of blank brain homogenate or the same volume of water. The concentrations used for determining recovery were 93.8, 375, and 3,000 ng/g. The recovery was calculated by comparing the peak areas of colistin derivatives extracted from brain homogenate-spiked samples to those obtained from water-spiked samples.

To mimic the extraction scenario that would be encountered with samples obtained from in vivo mouse brain uptake studies, another set of colistin-spiked brain homogenate samples were prepared and the recovery of colistin was measured after a 4-h incubation at 37°C. The reason for incubating colistin for 4 h in brain homogenate is that this would provide an opportunity for colistin to diffuse into the brain tissue and more closely resemble what would be expected in an in vivo brain uptake study. Brain homogenate (300 μl) at 93.8, 375, and 3,000 ng/g colistin was incubated in a 37°C shaking water bath for 4 h (120 rpm). After this period, water-spiked and brain homogenate-spiked samples underwent the extraction procedure described above and recovery was calculated in the same manner.

Mouse brain uptake study. Animal experiments were approved by the Faculty of Pharmacy and Pharmaceutical Sciences Animal Ethics Committee and performed in accordance with the National Health and Medical Research Council guidelines for the care and use of animals for scientific purposes. On the day of the study, a colistin sulfate dosing solution was prepared by dissolving colistin sulfate in saline, which was filtered with a 0.2-μm-pore syringe filter (Minisart; Sartorius). The brain uptake study was carried out with 16 Swiss Outbred male mice (6 to 8 weeks of age and weighing 22 to 28 g). A 50-μl bolus dose of colistin was administered to each mouse by tail vein injection at a dose of 5 mg/kg. Approximately 4 min prior to tissue harvesting, mice were anesthetized with an intraperitoneal dose of 133 mg/kg ketamine and 10 mg/kg xylazine. At 5, 30, 60, or 120 min ($n = 4$ mice at each time point), blood was collected by cardiac puncture and centrifuged immediately to obtain plasma (~200 μl per sample). Animals were humanely killed by cervical dislocation, and then the whole brain was removed. Plasma and whole brain were stored at −20°C until analysis.

On the day of analysis, the brain was thawed and homogenized as described above. A 280-μl sample was taken, and 20 μl of water was added, to match the homogenate standards. These brain homogenate samples were then processed as detailed above. Concentrations of colistin in mouse plasma were determined using the reported HPLC method (21), which was validated for mouse plasma in the present study. Precision and accuracy results from this assay validation (data not shown) demonstrate that the method for assaying colistin in rat plasma is transferable to mouse plasma.

RESULTS AND DISCUSSION

Choice of precipitant (ACN versus TCA) and chromatography. Previously, HPLC methods for the measurement of colistin in rat and human plasma have been developed and validated using ACN to precipitate the proteins in plasma (20, 21). However, we were unable to use ACN for brain homogenate

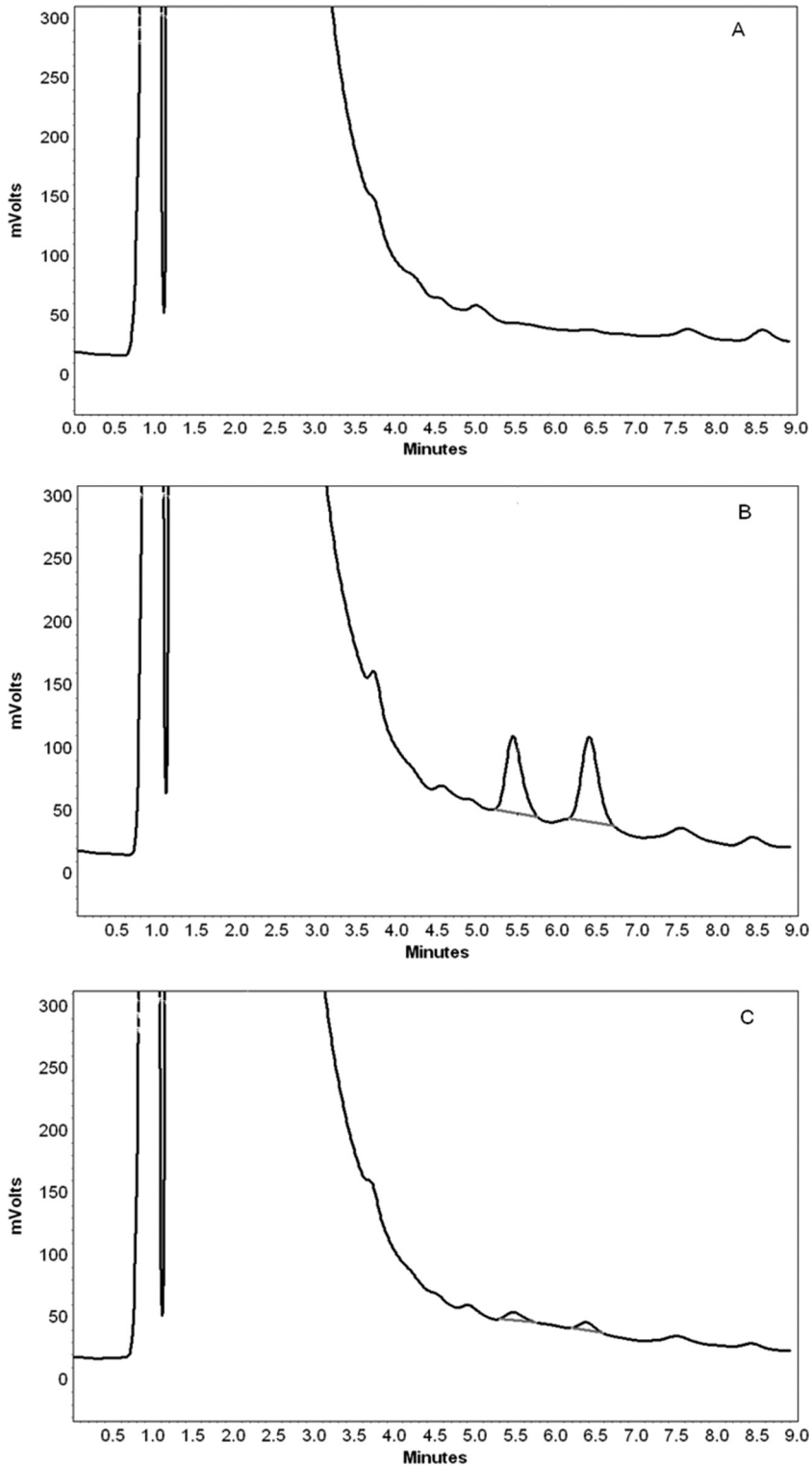

FIG. 1. Chromatograms of blank brain homogenate (A), brain homogenate spiked with 3,000 ng/g colistin (B), and brain homogenate at 5 min after intravenous administration of colistin sulfate to a mouse at a dose of 5 mg/kg (C). The colistin concentration in brain homogenate in this mouse was 220 ng/g.

protein precipitation due to poor recovery of colistin, even at the highest concentration of colistin (3,000 ng/g) and the largest volume of ACN (1,200 μl). TCA, a widely used precipitating reagent in brain homogenate assays (3, 35), was consequently assessed for its ability to extract colistin at different concentrations and volumes, and the extents of recovery of colistin from each of the volumes and concentrations of TCA are shown in Table 1. An aliquot of 75 μl of 20% (wt/vol) TCA

TABLE 2. Precision and accuracy values of the assay quantitating colistin in mouse brain homogenate

Parameter and target colistin concn (ng/g)	Mean ± SD measured colistin concn (ng/g)	Precision (%)	Accuracy (%)
Intraday (n = 6)			
93.8	91.6 ± 7.2	7.9	97.7
375	384 ± 31.8	8.3	102
3,000	3,264 ± 167	5.1	108
Interday (n = 3)			
93.8	97.5 ± 8.3	8.5	103.9
375	345 ± 27.6	8.0	92
3,000	3,210 ± 186	5.8	107

provided the lowest extraction of colistin, whereas 750 μl of 2% (wt/vol) TCA provided the highest extraction. However, to minimize the liquid handling on the SPE cartridge, it was deemed most appropriate to use 300 μl of 5% (wt/vol) TCA combined with the same volume of ACN, and this protocol was applied to carry out the assay validation. This extraction method provided satisfactory recovery at low, medium, and high concentrations of colistin (see below).

Under the current chromatographic conditions, well-resolved and symmetric peaks of FMOC derivatives of colistin A and colistin B were observed and there was no interference from components of the brain homogenate. The average retention times of FMOC derivatives of colistin B and colistin A were 5.6 and 6.5 min, respectively, while the run time was 9 min. Figure 1 shows representative chromatograms of blank brain homogenate, a colistin-spiked brain sample, and a brain homogenate sample obtained following intravenous administration of colistin sulfate (5 mg/kg) to a mouse.

Linearity, precision, and accuracy. The calibration curve relating colistin response to brain homogenate concentration exhibited good linearity ($R^2 > 0.994$) over the range of 93.8 to 3,000 ng/g. The lower limit of quantitation was 93.8 ng/g, as demonstrated by the satisfactory precision and accuracy values presented in Table 2. Intraday and interday levels of precision for QCs at three different concentrations were less than 8.3% and 8.5%, respectively, and the accuracy ranged from 92 to 108%. These performance characteristics demonstrate that this novel method was robust and reliable for the determination of colistin concentration in brain homogenate following in vivo administration.

Recovery. The values of recovery for colistin at concentrations of 93.8, 375, and 3,000 ng/g ranged from 85% to 94%. After a 4-h incubation of colistin sulfate in brain homogenate at 37°C, the values of recovery for the three corresponding concentrations were 87%, 86%, and 96%, respectively, indicating that colistin could be adequately recovered from brain tissue obtained from in vivo studies. While several HPLC methods have been reported for measuring colistin concentrations in human and rodent plasma (20, 21) and some tissue samples (34), the present method allows for excellent recovery and quantitative analysis of colistin in brain tissue. With some minor modification, this method may also be utilized to quantify the concentration of colistin (and other polymyxins) in other tissues from rodents.

Brain uptake of colistin following intravenous administration to mice. The brain and plasma concentrations of colistin at various times after intravenous administration of colistin sulfate to mice at a nominal dose of 5 mg/kg are shown in Fig. 2. Mice appeared to tolerate this dose with no observed toxicity during the whole experimental period, which is consistent with earlier findings (31). While the concentration of colistin in plasma declined rapidly over the 2-h experiment, the concentration of colistin in the brain homogenate remained relatively constant over a 90-min period, albeit the concentrations were relatively low. This resulted in an increasing brain/plasma ratio with time (ranging from 0.02 to 0.06), suggestive of potential accumulation of colistin within the brain. However, further studies with multiple dosing or intravenous infusions would be required to more clearly identify the potential of colistin to accumulate within the brain, as there are limited data in the present study to conclusively confirm this hypothesis.

To accurately determine if a compound has permeated the BBB using brain homogenate studies, it is common to account for the concentration of compound remaining within the brain microvasculature (33). Our group has determined the brain microvascular volume in Swiss Outbred mice to be 0.026 ml/g, using [^{14}C]inulin as a vascular marker (unpublished data), and when this volume is taken into account, the concentrations of colistin within the brain parenchyma are close to zero. This indicates that any colistin detected in the mouse brain homogenate following a single intravenous dose of colistin sulfate is likely to be restricted within the brain microvessels, suggesting poor penetration across the healthy BBB. However, as mentioned above, multiple doses of colistin (as prescribed clinically) may result in appreciable accumulation of this compound within the brain parenchyma, and future studies may be directed at addressing this issue.

The limited penetration of colistin into brain parenchyma following a single intravenous dose could be due to several factors. First, the average molecular mass of colistin (1,163 Da)

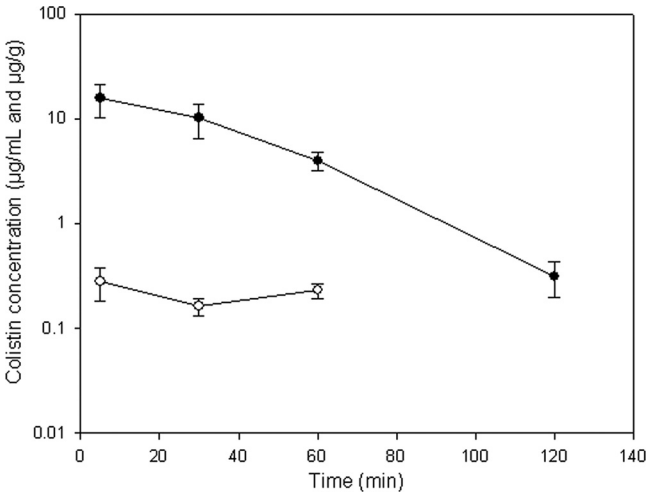

FIG. 2. Plasma (●; μg/ml) and brain homogenate (○; μg/g) concentrations of colistin after intravenous administration of colistin sulfate to Swiss Outbred mice at a dose of 5 mg/kg. At 120 min, the concentration of colistin in brain homogenate was below the lower limit of quantitation. Data are presented as means ± standard deviations (n = 4).

far exceeds the "threshold" for optimum BBB permeability (450 Da) (9). Second, the logP values (where P is the octanol-to-water partition coefficient) of the two main components of colistin (i.e., colistin A and colistin B) are −3.15 and −3.68 (28), respectively, which are below the range of the logP values recommended for passive permeation of the BBB (9). Third, like many large cationic compounds, colistin may be actively excluded from the brain parenchyma by active efflux systems, such as P-glycoprotein (23). The mechanisms limiting the BBB penetration of colistin warrant further investigation so that appropriate measures can be taken not only to enhance colistin exposure for the treatment of life-threatening CNS infections, but also to minimize the potential for CNS-induced neurotoxicity when colistin is being used for the treatment of systemic infections.

While colistin has been reported to penetrate the human BCSFB (12), the current study demonstrates that colistin has minimal penetration into the brain parenchyma of healthy mice. The reason for this observation could be due to differences between the BBB and BCSFB transport of colistin, species differences associated with CNS exposure, or the fact that the BBB and BCSFB are intact in the healthy mice, which during infection may become compromised to allow greater CNS exposure of colistin (29). Therefore, further studies may be performed to determine whether the penetration of colistin into the brain parenchyma is enhanced during meningitis and/or meningoencephalitis, which may be considered beneficial when treating meningoencephalitis and other infections of the brain parenchyma.

The use of protein precipitation with TCA and derivatization with FMOC-Cl has allowed for the accurate and reproducible quantitation of colistin in mouse brain homogenate. This HPLC method is reliable and has been successfully applied to evaluate colistin penetration across the BBB in healthy mice. This method can be conveniently applied to determine whether the brain uptake of colistin is altered during meningitis and meningoencephalitis, which more closely mirrors the scenario encountered in patients, and may assist in the understanding of colistin efficacy and/or neurotoxicity in these infections.

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Impact of P-Glycoprotein Inhibition and Lipopolysaccharide Administration on Blood-Brain Barrier Transport of Colistin in Mice[▽]

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The aim of this study was to investigate the factors limiting the blood-brain barrier (BBB) transport of colistin in healthy mice and to assess the impact of systemic inflammation on the transport of this antibiotic across the BBB. Colistin sulfate (40 mg/kg) was administered subcutaneously to Swiss outbred mice as single and multiple doses to determine any relationship between brain uptake and plasma concentrations of colistin. To assess the effect of P-glycoprotein (P-gp) on BBB transport, colistin sulfate (5 mg/kg) was concomitantly administered intravenously with PSC833 or GF120918 (10 mg/kg). Systemic inflammation was induced by three intraperitoneal injections of lipopolysaccharide (LPS; 3 mg/kg), and BBB transport of colistin was subsequently measured following subcutaneous administration and by an *in situ* brain perfusion. The brain uptake of colistin was low following single and multiple subcutaneous doses, with brain-to-plasma concentration ratios ranging between 0.021 and 0.037, and this was not significantly enhanced by coadministration of GF120918 or PSC833 ($P > 0.05$). LPS significantly increased the brain uptake of subcutaneously administered colistin with area under the brain concentration time curve (AUC_{brain}) values of 11.7 ± 2.7 μg · h/g and 4.0 ± 0.3 μg · h/g for LPS- and saline-treated mice, respectively (mean ± standard deviation). Similarly, *in situ* perfusion of colistin led to higher antibiotic brain concentrations in LPS-treated animals than in saline-treated animals, with colistin brain-to-perfusate concentration ratios of 0.019 ± 0.001 and 0.014 ± 0.001, respectively. This study demonstrates that the BBB transport of colistin is negligible in healthy mice; however, brain concentrations of colistin can be significantly enhanced during systemic inflammation, as might be observed in infected patients.

Colistin (polymyxin E) is one of two polymyxins clinically used to treat infections caused by Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, and *Klebsiella pneumoniae* (12). Although the clinical use of colistin waned in the 1970s due to concerns related to its adverse effects (10), colistin has reemerged as a last-line therapy for Gram-negative bacterial infections and is increasingly employed against multidrug-resistant Gram-negative bacteria (9). One of the adverse effects of colistin that has attracted the attention of clinicians and scientists is neurotoxicity (6, 8); however, it remains unknown as to whether this toxicity is centrally or peripherally mediated. If colistin were to exert any centrally mediated toxicity, it would need to permeate the blood-brain barrier (BBB), the endothelial lining separating the brain parenchyma from the blood. It has been clinically demonstrated that intravenous administration of colistin methanesulfonate, an inactive prodrug of colistin (5), leads to detectable levels of colistin in cerebrospinal fluid (CSF) (1, 15, 21); however, this is an indicator of blood-CSF barrier penetration and not BBB penetration. We have previously demonstrated that the brain uptake of colistin following a single intravenous dose to mice was negligible (16), suggesting minimal penetration across an intact BBB. However, the mechanisms governing the low BBB penetration of colistin remain unknown, and, furthermore, it is unclear as to whether brain accumulation of colistin occurs with repeated doses (as is used clinically), given that our previous study assessed colistin brain penetration after only a single intravenous dose (16).

It is generally accepted that only small molecules with low molecular mass (<450 Da) and high lipid solubility permeate the healthy BBB by a passive transcellular process (13). The tight junctions of the interendothelial domains restrict the passage of large hydrophilic molecules through the paracellular route (14), and this is expected to be the main reason for the low BBB penetration of colistin observed (given a molecular mass of 1,163 Da) (19). However, even for compounds that possess the appropriate physicochemical properties for transcellular permeation, efficient BBB transport is not always guaranteed due to the presence of active efflux proteins expressed at the luminal surface of brain endothelial cells (29). P-glycoprotein (P-gp) is one such efflux transporter and is responsible for restricting the brain penetration of a wide range of substrates, including anticancer drugs, analgesics, and antibiotics (27). Many of these substrates share some common physicochemical properties such as a positive charge at physiological pH and a molecular mass of >400 Da (7). Given that the primary amines of colistin possess a pK_a value of approximately 10 and that colistin has a high molecular weight (19), it is plausible that the low brain uptake of colistin observed in our previous study (16) may also be due, in part, to P-gp-mediated efflux.

While it is important to understand the mechanisms limiting colistin brain uptake in healthy subjects, it is crucial to identify whether colistin has a higher propensity to access the central nervous system (CNS) during bacterial infections, where the

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integrity of the BBB may be compromised (26). Indeed, other investigators have detected colistin in the CSF of infected patients following systemic administration (1, 15, 21), whereas we have previously observed limited brain uptake in healthy mice although this could also be attributed to species differences and/or differences between BBB and blood-CSF barrier penetration profiles. While the impact of infection on BBB transport may be investigated by inducing bacteremia in mice, bacterial lipopolysaccharide (LPS) is often used to mimic the infective state as it results in the release of proinflammatory cytokines (25, 31). Several studies have shown that BBB disruption can be induced by bacterial LPS, leading to increased brain uptake of compounds that normally exhibit limited access into the CNS (3, 22). Whether a similar enhancement in BBB penetration would occur with colistin remains unknown; however, it is of importance to assess this phenomenon as this would better reflect the scenario encountered in infected patients. Therefore, the aims of the current study were as follows: (i) to identify whether the brain uptake of colistin increases with repeated administration, under conditions where sustained colistin plasma concentrations may be achieved; (ii) to determine the impact of coadministration of P-gp inhibitors on the brain uptake of colistin; and (iii) to examine if the BBB penetration of colistin is enhanced following administration of LPS.

MATERIALS AND METHODS

Chemicals and reagents. Colistin sulfate was purchased from Zhejiang Shenghua Biok Biology Co., Ltd. (EP5 grade; Zhejiang, China). PSC833 (Valspodar) was a gift from Novartis (Basel, Switzerland) and GF120918 (Elacridar) was a gift from GlaxoSmithKline (Middlesex, United Kingdom). [^{3}H]digoxin, Ultima Gold scintillation fluid, and Solvable were purchased from Perkin Elmer (Boston, MA), and [^{14}C]inulin and [^{14}C]sucrose were obtained from American Radiolabeled Chemicals (St. Louis, MO). Lipopolysaccharide from *Salmonella enterica* serotype Typhimurium was obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Solid-phase extraction (SPE) cartridges (C_{18} Sep-Pak; 100 mg) were purchased from Waters (Milford, MA). All other reagents were of analytical and/or high-performance liquid chromatography (HPLC) grade, and water was obtained from a Millipore purification system (Billerica, MA).

Animal studies. Animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were performed in accordance with the Australian National Health and Medical Research Council (NHMRC) guidelines for the care and use of animals for scientific purposes. Male Swiss outbred mice (6 to 8 weeks of age; 25 to 30 g) were used in all studies. Mice had free access to food and water during all experimental periods.

Brain uptake of colistin following subcutaneous administration. An aliquot (200 μl) of a sterile-filtered solution of colistin sulfate (equivalent to 40 mg/kg of body weight in saline) was administered to mice subcutaneously over the interscapular region. At 0.5, 1, 2, and 4 h, mice (n = 4 at each time point) were anesthetized with an intraperitoneal dose of 133 mg/kg of body weight of ketamine and 10 mg/kg xylazine, blood was collected by cardiac puncture, and the whole brain was removed following cervical dislocation. Plasma and brain samples were stored at −20°C until the day of analysis. To determine whether the brain uptake of colistin increased with repeated administration, subcutaneous injections of colistin sulfate were administered (at 0, 6, and 12 h) at the same dose and using the same method described above. Two hours after the last dose, plasma and brain samples were collected and stored at −20°C until analysis. Concentrations of colistin in brain homogenate and in plasma samples were measured using HPLC (16), and a brain-to-plasma concentration (B/P) ratio of colistin was calculated at each postdose time point.

Impact of P-gp inhibitors on brain uptake of colistin. An aliquot (50 μl) of a colistin sulfate solution was administered to mice by tail vein injection (5 mg/kg of body weight) immediately after a 50-μl intravenous injection of PSC833 (10 mg/kg), GF120918 (10 mg/kg), or blank vehicle (n = 6 for each pretreatment). The blank vehicle consisted of 20% (vol/vol) ethanol, 60% (vol/vol) polyethylene glycol 400 (PEG 400), and 20% (wt/vol) glucose. Mice were anesthetized (as detailed above), and plasma and brain samples were harvested 5 min after administration of colistin sulfate. Samples were analyzed for colistin (16), and B/P ratios were calculated.

To ensure that these doses of PSC833 and GF120918 were effective in inhibiting P-gp function at the BBB, the brain uptake of the P-gp substrate digoxin was assessed in the presence and absence of the inhibitors. An aliquot (50 μl) of a [^{3}H]digoxin solution (40 μCi/ml) was administered to mice by tail vein injection in a vehicle consisting of 20% (vol/vol) ethanol, 60% (vol/vol) PEG 400, and 20% (wt/vol) glucose with or without PSC833 or GF120918 (10 mg/kg) (n = 6 mice for each group). Plasma and brain samples were collected at 5 min postdose, and radioactivity in plasma and brain was determined using liquid scintillation counting. Briefly, 50 μl of plasma was placed into a 5-ml scintillation vial, and 2 ml of Ultima Gold scintillation fluid was added, followed by brief vortex mixing. Brain samples were placed into 20-ml scintillation vials containing 2 ml of Solvable and were maintained at 50°C overnight. The next day, 200 μl of hydrogen peroxide (30%, vol/vol) was added to the digested brains to bleach the samples, and they were allowed to sit for 30 min at 50°C. An aliquot (10 ml) of Ultima Gold scintillation fluid was then added to each vial containing brain digest, followed by brief vortex mixing. Radioactivity in both brain digest and plasma samples was then measured by liquid scintillation counting (Tri-carb 2800 TR; Perkin Elmer, Boston, MA) and a B/P ratio was calculated by the following formula: B/P = (number of disintegrations per minute [dpm]/g of brain)/(dpm/ml of plasma).

Effect of LPS on brain uptake of colistin. Mice were administered intraperitoneal injections (200 μl) of 0.9% (wt/vol) saline (control) or LPS (3 mg/kg of body weight in saline) at 0, 6, and 24 h. An aliquot (200 μl) of a colistin sulfate solution (40 mg/kg in saline) was then administered subcutaneously to mice over the interscapular region 4 h after the third LPS or saline dose. At various time points after administration of colistin (0.5, 1, 2, and 4 h), plasma and brain samples (n = 4 mice at each time point) were harvested, and concentrations of colistin in brain homogenate and plasma were determined by HPLC to obtain B/P ratios at each time point. The area under the plasma concentration-time curve from 0 to 4 h ($AUC_{plasma,\ 0–4}$), the area under the brain concentration-time curve from 0 to 4 h ($AUC_{brain,\ 0–4}$), and their associated variances were also determined using Bailer's approach (2).

It is possible that any enhancement in colistin brain uptake following LPS administration could have been due to a direct effect of LPS on BBB integrity or an indirect effect (e.g., an alteration to plasma protein binding and unbound fraction of colistin). To delineate these effects, the BBB transport of colistin was measured in saline- and LPS-treated animals using a modified *in situ* perfusion technique (4). The perfusion fluid used was carbonated Krebs buffer consisting of the following components (per liter): 128.0 mmol of NaCl, 24.0 mmol of $NaHCO_3$, 4.2 mmol of KCl, 2.4 mmol of NaH_2PO_4, 1.5 mmol of $CaCl_2$, 0.9 mmol of $MgSO_4$, and 9.0 mmol of D-glucose. The buffer was carbonated with 5% CO_2–95% O_2, the pH was adjusted to 7.4, and the buffer was warmed to 37°C before the experiment. Mice were pretreated with LPS or saline, as described above, and 4 h after the last administration, mice were anesthetized. After the thorax was opened, the heart was exposed, and the descending thoracic aorta was clamped, followed by severing of both the left and right jugular veins. A 21-gauge butterfly needle (attached to polyethylene tubing and perfusion solution) was inserted into the left ventricle of the heart. To mimic the plasma concentrations obtained after multiple subcutaneous administrations of colistin, 40 μg/ml of colistin was infused transcardially at a rate of 2 ml/min for 4 min. At the completion of the perfusion, brains were removed and stored at −20°C until the day of analysis. Three aliquots of the perfusion fluid eluting from the polyethylene tubing were also collected and stored at −20°C until the day of analysis. The concentrations of colistin in brain homogenate were then determined by an HPLC method described previously (16). The concentration of colistin in the perfusate solution was also determined using the above assay with minor modifications. Briefly, 50 μl of acetonitrile was added to 50 μl of perfusion fluid, and the mixture was vortex mixed for 1 min and centrifuged at 16,100 × g for 10 min before being loaded onto an SPE cartridge. All other derivatization and analytical parameters were identical to those reported previously (16). The calibration curve relating chromatographic peak area to perfusate colistin concentration exhibited good linearity (r^2 of >0.993) over the range of 0.31 to 10.0 μg/ml. The coefficient of variation for quality control samples (n = 6) prepared at 0.63 μg/ml and 10.0 μg/ml was less than 4.4%, and the accuracy values of these replicates ranged from 108.8% to 110.5% of the nominal concentrations.

Effect of LPS on BBB integrity. To ensure that the LPS regimen used in the studies above was affecting the integrity of the BBB, the brain uptake and BBB transport of impermeable markers were determined following intravenous administration and *in situ* perfusion, respectively. At 4 h after the last saline or LPS administration, mice were intravenously administered a 50-μl solution of [^{14}C]inulin (2 μCi in saline) or [^{14}C]sucrose (2 μCi in saline) (n = 6 per group).

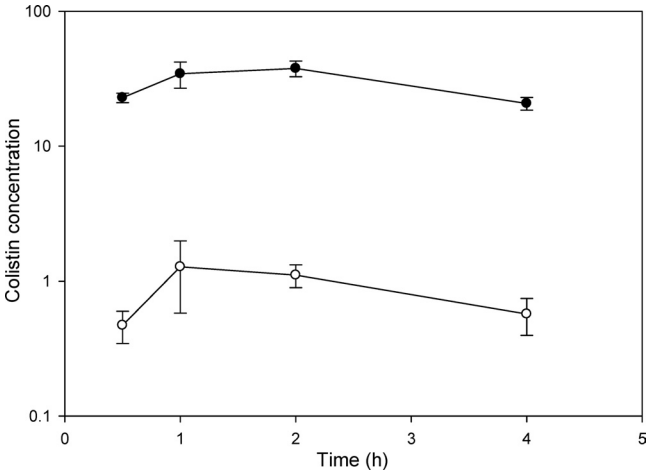

FIG. 1. Plasma (●; μg/ml) and brain (○; μg/g) concentrations of colistin following a subcutaneous dose of colistin sulfate (40 mg/kg) to Swiss outbred mice. Data are presented as means ± standard errors of the means ($n = 4$).

Plasma and brain samples were obtained 5 min following intravenous administration, and samples were measured for radioactivity, as described previously. Similarly, 4 h after the last pretreatment with saline or LPS, the BBB penetration of [^{14}C]sucrose or [^{14}C]inulin was determined following perfusion of each compound (0.25 μCi/ml) at a rate of 2 ml/min for 4 min ($n = 3$ per group). Brain and perfusate were collected, and samples were prepared for liquid scintillation counting, as described previously. The brain-to-plasma concentration or brain-to-perfusate concentration ratio of [^{14}C]sucrose and [^{14}C]inulin (as a measure of BBB integrity) was calculated by comparing the concentration of compound in brain (dpm/g) to that in plasma or perfusate (dpm/ml).

Data analysis. All data are presented as means ± standard deviations (SD) unless otherwise stated. For comparisons of the brain-to-plasma concentration (B/P) or brain-to-perfusate concentration ratios between saline-treated (control) and LPS-treated animals, Student's t test was used, whereas B/P ratios between vehicle-, PSC833-, and GF120918-treated animals were compared using a one-way analysis of variance followed by a Newman-Keuls multiple comparisons test (PASW Statistics for Windows, version 17.0; Chicago, Illinois). A P value of <0.05 was considered to be a significant difference. For comparison of the AUC_{plasma} or AUC_{brain} between saline- and LPS-treated groups using Bailer's approach, a z test was used to determine significant differences. A z value of >1.96 was considered to be a significant difference between two groups.

RESULTS

Brain uptake of colistin after single and repeated subcutaneous administrations. Mice tolerated a subcutaneous dose of 40 mg/kg of body weight of colistin sulfate with no observed toxicity over the experimental period. Subcutaneous administration of colistin sulfate at this dose resulted in sustained plasma concentrations of colistin over the 4-h sampling period, with maximum plasma concentrations at 1 to 2 h postdose, as shown in Fig. 1. Despite these high and sustained plasma concentrations, the resulting concentration of colistin in brain was very low at all four postdose time points, with B/P ratios of 0.021 ± 0.009 (0.5 h), 0.037 ± 0.014 (1 h), 0.029 ± 0.005 (2 h), and 0.028 ± 0.007 (4 h). Similar results were also obtained following multiple subcutaneous dosing to mice. Two hours after the last of three subcutaneous doses of colistin sulfate, the plasma concentration of colistin was 32.0 ± 3.5 μg/ml, yet the brain colistin concentration at this time was 0.8 ± 0.1 μg/g, leading to a B/P ratio of 0.024 ± 0.002 ($n = 4$). It should be noted that in all studies, the amount of colistin remaining in the cerebral vasculature was not subtracted for calculation of B/P ratios as this resulted in ratios close to zero (or negative).

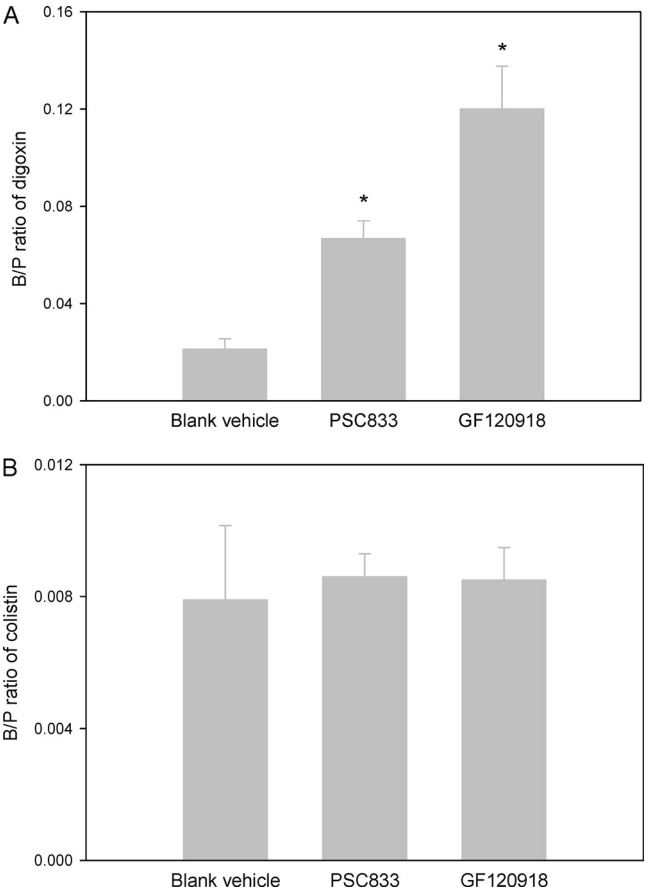

FIG. 2. (A) B/P ratios of [^{3}H]digoxin 5 min after intravenous administration (2 μCi) to Swiss outbred mice with and without coadministration of PSC833 or GF120918 (10 mg/kg). Data are presented as means ± standard errors of the means ($n = 6$; *, $P < 0.05$ between PSC833 or GF120918 and vehicle group using a one-way analysis of variance and Newman-Keuls multiple comparison test). (B) B/P ratios of colistin 5 min after intravenous administration of colistin sulfate (5 mg/kg) to Swiss outbred mice with and without coadministration of PSC833 or GF120918 (10 mg/kg). Data are presented as means ± standard errors of the means ($n = 6$).

Impact of P-gp inhibitors on brain uptake of colistin. The B/P ratios of [^{3}H]digoxin in the presence and absence of P-gp inhibitors are shown in Fig. 2A. Both of these inhibitors significantly ($P < 0.05$) enhanced the brain uptake of [^{3}H]digoxin, demonstrating that the dose of inhibitors (10 mg/kg) was valid for assessing the involvement of P-gp in colistin brain uptake. However, the average B/P ratio of colistin following administration of the same dose of P-gp inhibitors was still negligible (Fig. 2B); there was no significant difference in the B/P ratios of colistin between mice treated with vehicle, PSC833, or GF120918 ($P > 0.05$).

Brain uptake of colistin after pretreatment with LPS. The plasma and brain concentrations of colistin after subcutaneous administration of colistin sulfate to saline- and LPS-treated mice are shown in Fig. 3A. The plasma concentrations of colistin were not affected by administration of LPS at any postdose time points, with AUC_{plasma} values not significantly different between LPS- and saline-treated groups (values of

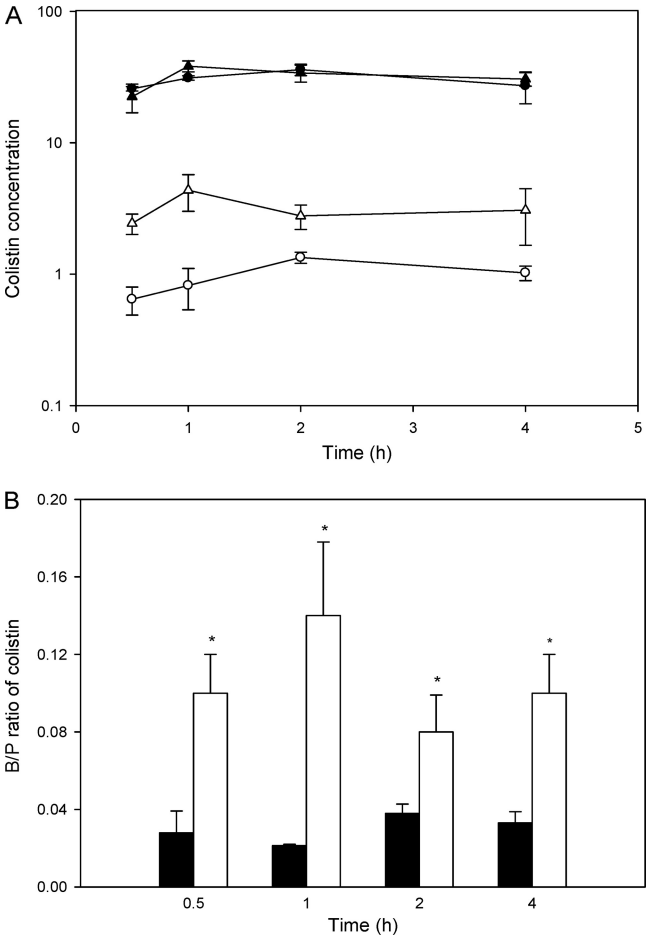

FIG. 3. (A) Plasma (μg/ml) and brain (μg/g) concentrations of colistin after subcutaneous administration of colistin sulfate (40 mg/kg) to Swiss outbred mice 4 h after the final dose of a pretreatment regimen (comprising administration at 0, 6, and 24 h) with intraperitoneal LPS (3 mg/kg) (▲, plasma; △, brain) or saline (●, plasma; ○, brain). Data are presented as means ± standard errors of the means ($n = 4$). (B) Corresponding B/P ratios of colistin from data depicted in panel A after intraperitoneal LPS (3 mg/kg) (□) or saline (■) administration. Data are presented as means ± standard errors of the means ($n = 4$; *, $P < 0.05$ between saline- and LPS-treated animals using Student's t test).

117.5 ± 9.2 μg · h/ml and 121.4 ± 8.2 μg · h/ml, respectively; $z = 0.33$). Even though plasma concentrations were unaffected by LPS treatment, the brain concentrations of colistin in LPS-treated mice were significantly higher than in saline-treated mice at all postdose time points, as shown in Fig. 3A. The average B/P ratios of colistin at the designated time points in saline- and LPS-treated animals are shown in Fig. 3B. In addition, the overall brain exposure to colistin was significantly enhanced following LPS pretreatment, with AUC_{brain} values of 11.7 ± 2.7 μg · h/g and 4.0 ± 0.3 μg · h/g for LPS- and saline-treated mice, respectively ($z = 2.81$). To ensure that LPS did not interfere with the HPLC analysis of colistin and that the increased peak areas observed in brain homogenate were indeed due to increased brain concentration of colistin, the HPLC methods were also validated using brain and plasma from LPS-treated mice not exposed to colistin. No changes in

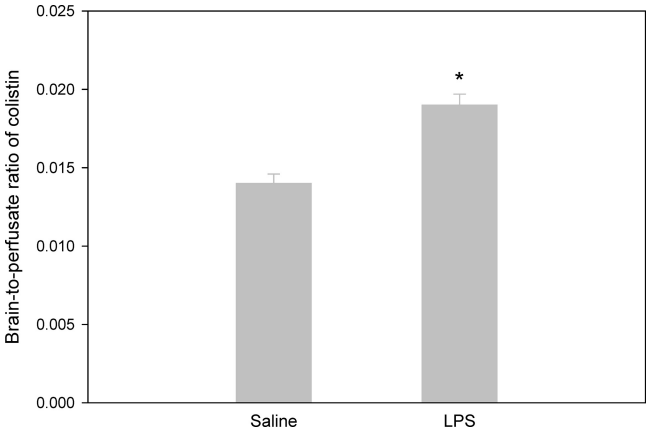

FIG. 4. Brain-to-perfusate concentration ratios following *in situ* perfusion of colistin (40 μg/ml) at a rate of 2 ml/min for 4 min in Swiss outbred mice pretreated with LPS (3 mg/kg) or saline (0, 6 and 24 h). Data are presented as means ± standard errors of the means ($n = 4$; *, $P < 0.01$ using Student's t test).

colistin chromatographic peak areas or retention times were observed in the presence of LPS (data not shown), indicating that the increased peak areas detected in brain homogenates following LPS pretreatment were indeed reflective of increased brain concentrations of colistin.

Using the *in situ* perfusion technique, the BBB permeability of colistin was shown to be significantly ($P < 0.01$) enhanced in LPS-treated mice (Fig. 4), with brain-to-perfusate colistin concentration ratios of 0.019 ± 0.001 and 0.014 ± 0.001 for LPS- and saline-treated mice, respectively. However, the increase in the brain uptake of colistin mediated by LPS as measured by the *in situ* perfusion (36% increase) was not as large as the effect measured following subcutaneous administration of colistin (average 286% increase). These findings suggested that either (i) the effect of LPS may have been due to an event imparted in the systemic circulation (e.g., altered plasma protein binding of colistin) or (ii) that the *in situ* perfusion method is a less sensitive technique than the *in vivo* brain uptake technique for detecting changes to BBB permeability for poorly penetrating compounds. Therefore, the effect of LPS on the integrity of the BBB was compared following *in vivo* administration and *in situ* perfusion of the BBB integrity markers [^{14}C]sucrose and [^{14}C]inulin. The B/P ratios of [^{14}C]sucrose and [^{14}C]inulin following intravenous administration in saline- and LPS-treated mice are shown in Table 1. The average B/P ratios of [^{14}C]sucrose and [^{14}C]inulin were 0.035 to 0.038 in

TABLE 1. B/P ratios of [^{14}C]inulin and [^{14}C]sucrose following intravenous administration to saline- and LPS-pretreated Swiss outbred mice

Pretreatment	B/P ratio[a]	
	[^{14}C]inulin	[^{14}C]sucrose
Saline	0.038 ± 0.004	0.035 ± 0.007
LPS	0.065 ± 0.016*	0.069 ± 0.007*

[a] [^{14}C]inulin and [^{14}C]sucrose were administered in a dose of 2 μCi. Data are presented as means ± SD ($n = 6$). *, $P < 0.05$ compared to saline pretreatment using Student's t test.

TABLE 2. Brain-to-perfusate concentration ratios of [^{14}C]inulin and [^{14}C]sucrose following *in situ* perfusion to saline- and LPS-pretreated Swiss outbred mice

Pretreatment	Brain-to-perfusate concentration ratio[a]	
	[^{14}C]inulin	[^{14}C]sucrose
Saline	0.019 ± 0.001	0.024 ± 0.002
LPS	0.025 ± 0.002*	0.029 ± 0.003*

[a] Perfusion was conduced at a rate of 0.25 μCi/ml/min for 4 min. Data are presented as means ± SD ($n = 3$). *, $P < 0.05$ compared to saline pretreatment using Student's t test.

saline-treated animals, whereas pretreatment with LPS resulted in average apparent B/P ratios of 0.065 to 0.069. This approximate doubling in the B/P ratios of [^{14}C]sucrose and [^{14}C]inulin following administration of LPS is suggestive of a significant disturbance in the integrity of the BBB. However, when assessed using the *in situ* perfusion technique, the extent of BBB damage (as measured by the brain-to-perfusate concentration ratio of [^{14}C]sucrose and [^{14}C]inulin) did not appear to be as marked as that observed following intravenous administration of these BBB markers (Table 2). Compared with the LPS-induced doubling in the apparent B/P ratio of the intravenously administered BBB markers, the *in situ* perfusion technique detected a 20 to 32% increase in the brain-to-perfusate concentration ratio of the BBB markers following LPS pretreatment, albeit this increase was still significant ($P < 0.05$).

DISCUSSION

The relentless increase in resistance to almost all currently available antibiotics in Gram-negative bacteria, in particular, *P. aeruginosa*, *A. baumannii*, and *K. pneumoniae*, together with the shortage of new antibiotics with activity against these pathogens, has caused substantial worldwide concern (17). Consequently, the relatively old polymyxin antibiotic colistin is being increasingly used as a salvage therapy (20, 24). While there are a number of clinical cases and reports on the use of colistin (1, 15, 18), there is little information available on the BBB transport of this antibiotic, which may assist in understanding the general CNS disposition of this increasingly important antibiotic.

Consistent with the physicochemical properties of colistin, our previous study demonstrated that the brain penetration of colistin was negligible in healthy mice after a single intravenous dose (16). It is possible that the low brain uptake of colistin observed following this dosage regimen is not representative of that which may occur under circumstances where the colistin plasma concentrations are sustained at a relatively high level. Therefore, in the current studies, colistin sulfate was administered subcutaneously, and plasma colistin concentrations were maintained at high levels over the 4-h experimental period and were approximately double the concentration detected following a single intravenous dose at 5 mg/kg (15.8 ± 2.8 μg/ml) (16). Despite these higher and prolonged plasma concentrations, the mean B/P ratio of colistin was no higher than that observed in the previous single-dose intravenous study (16). Similar results were also obtained after colistin was administered in a multiple-dose regimen, more reflective of the scenario encountered clinically. Therefore, it is likely that in patients with an intact BBB, penetration of colistin into the brain would be minimal, even if this antibiotic was dosed multiple times to allow for sustained plasma concentrations. This negligible entry of colistin across a healthy BBB is most likely to be a result of the tight junctions sealing the paracellular route, hindering the penetration of this, and other, high-molecular-weight compounds.

While the interendothelial tight junctions minimize paracellular penetration of compounds, the transcellular absorption of many compounds can be limited by efflux transporters, including P-gp (27). Given that colistin exhibits some of the characteristics possessed by known P-gp substrates (cationic charge at physiological pH and high molecular weight) (19), we investigated whether efflux by P-gp was also contributing to the low brain uptake of colistin. In this study, the brain uptake of [^{3}H]digoxin, a commonly used P-gp substrate (28), significantly increased when it was coadministered with PSC833 or GF120918, which indicated that the function of P-gp could be effectively blocked with the dose of inhibitors used. However, when these inhibitors were administered at the same dose, they had no effect on the brain uptake of colistin. These studies suggested that P-gp does not contribute to the low brain uptake of colistin and that it is likely that the existence of the tight junctions sealing the paracellular route is the main contributor to the low BBB penetration of colistin.

To further confirm that tight junctions were the major hindrance to colistin BBB penetration, we intentionally disturbed the BBB paracellular integrity by systemic administration of LPS. The administration of LPS not only causes dysfunction of the tight junctions and decreased resistance of the paracellular route (32) but has also been shown to cause a downregulation of efflux transporters, such as P-gp (11). However, given that we have demonstrated that the BBB transport of colistin does not appear to be limited by the action of P-gp, any increase in the brain uptake of colistin following LPS administration is likely to be a result of increased paracellular diffusion. Administration of LPS also served another important purpose in this study in that its effects can mirror the infected state in which colistin is normally clinically used (25). The reduced BBB integrity during the infected state may be beneficial as it may lead to increased antibiotic concentrations in the brain. This may be an advantage in treating brain infections or a disadvantage if the site of infection is outside the CNS and if the antibiotic causes centrally mediated adverse effects such as neurotoxicity. Indeed, the brain uptake of colistin was significantly enhanced after administration of LPS, and this increase was similar in extent to that observed for [^{14}C]sucrose and [^{14}C]inulin, suggesting that the enhanced colistin brain uptake was likely a result of increased BBB paracellular permeability, as might occur during the infected state (32). Moreover, this conclusion was supported by the results from the *in situ* brain perfusion technique showing a significant increase in the brain uptake of colistin and the BBB markers following LPS pretreatment. Interestingly, the effect of LPS on the brain uptake of colistin, [^{14}C]sucrose, and [^{14}C]inulin, as measured by *in situ* perfusion, was not as marked as that observed following *in vivo* administration of these compounds. Such observations have also been made by others for BBB-impermeable compounds (23, 30), suggesting that the *in situ* perfusion technique may be less sensitive for detecting LPS-induced changes to the BBB

transport of poorly penetrating, high-molecular-weight compounds. Nevertheless, it has been demonstrated that the BBB transport of colistin is significantly enhanced during systemic inflammation, which may ultimately lead to higher CNS concentrations of colistin in infected patients. Further studies clarifying whether a similar increase in BBB transport of colistin occurs during an actual bacterial infection remain to be undertaken. Such studies may provide some insight into the disposition of this antibiotic in the clinical setting.

In summary, this study demonstrated that colistin exhibits minimal BBB penetration in healthy mice, regardless of the plasma concentrations present, and that this low brain uptake is not attributed to efflux by P-gp. Additionally, this study has provided evidence for the first time that the brain uptake of colistin is significantly increased in mice when the BBB is perturbed by employing LPS, which may resemble the scenario encountered in infected patients.

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The Effect of Systemic Infection Induced by *Pseudomonas aeruginosa* on the Brain Uptake of Colistin in Mice

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Abstract

In view of reports of colistin-induced neurotoxicity in infected patients, the aim of this study was to assess whether the integrity of the blood-brain barrier (BBB) and the brain uptake of colistin are altered in the presence of systemic *Pseudomonas aeruginosa* infection. Bacteremia was confirmed 8 h after intramuscular administration of *P. aeruginosa* ATCC27853 to Swiss Outbred mice, at which time a single subcutaneous dose of colistin sulfate (40 mg/kg) or intravenous dose of ^{14}C-sucrose (2 μCi) was administered. Despite a substantial elevation in plasma levels of the pro-inflammatory cytokines tumor necrosis factor-α, interleukin-1β and interleukin-6 during bacterial infection, the brain uptake of colistin was similar between infected and non-infected mice with AUC_{brain}/AUC_{plasma} ratios of 0.023 and 0.024, respectively. Similarly, the brain-to-plasma ratio of ^{14}C-sucrose was no different between infected and non-infected mice, consistent with a lack of effect of bacteremia on BBB integrity. To further correlate any relationship between BBB disruption and plasma levels of pro-inflammatory cytokines, BBB integrity, colistin brain uptake and plasma pro-inflammatory cytokines were measured following administration of *Salmonella enterica* lipopolysaccharide (LPS), an agent known to induce BBB disruption. Despite LPS inducing a 4-fold increase in colistin brain uptake and a significant ($p < 0.05$) 1.2-fold increase in ^{14}C-sucrose BBB penetration, plasma cytokine levels were lower with LPS treatment relative to those obtained with bacterial infection with *P. aeruginosa*. This study demonstrates that the brain uptake of colistin is not increased in mice during *P. aeruginosa*-induced systemic bacteremia despite a significant increase in plasma levels of three pro-inflammatory cytokines.

Introduction

The spread of multidrug resistance in Gram-negative bacteria presents a critical problem to physicians attempting to treat systemic infections, in particular those caused by *Pseudomonas aeruginosa, Acinetobacter baumannii* and *Klebsiella pneumoniae* (28). A decreased drive for the discovery of novel antibiotics has dramatically narrowed the available therapeutic options for such infections and has led to the reappraisal of colistin (polymyxin E). However, the use of colistin waned during the 1970s due to concerns related to its adverse effects (37) including neurotoxicity, manifesting as dizziness, numbness, vertigo and lower limb weakness (28, 34). It is still unclear whether these side effects are centrally or peripherally mediated (6), however, if the neurotoxicity induced by colistin is indeed centrally mediated, colistin or its inactive pro-drug colistin methanesulfonate (CMS) would be required to cross the blood-brain barrier (BBB).

The BBB, formed by the endothelial cells lining the cerebral microvessels, is the interface between the blood and the cerebral tissue, and acts as a major hindrance to the movement of molecules from the bloodstream into the central nervous system (CNS) (1). The endothelial cells of these cerebral microvessels have minimal pinocytotic activity and a lack of membrane fenestrations (8). Under normal conditions, the restrictive nature of the BBB is mediated by intercellular tight junctions preventing paracellular diffusion and various efflux transport systems limiting transcellular movement (20). In an attempt to understand the potential for colistin to traverse the BBB following systemic administration, we have previously assessed the brain uptake of this antibiotic following single and multiple injections to healthy mice, and these studies demonstrated minimal CNS penetration of this antibiotic (18, 19). This is not surprising given that, in addition to its relatively large molecular weight (1163), the free γ-amino groups of the five α,γ-diaminobutyric acid residues in the structure give colistin multiple positive charges at physiological pH, rendering it quite hydrophilic.

These physicochemical properties would therefore limit the ability of colistin to traverse the BBB via the transcellular or paracellular routes (33). However, the integrity of the BBB paracellular route is known to be perturbed in a variety of diseases including acute bacterial infection (39), which may be a result of elevated plasma concentrations of pro-inflammatory cytokines (7). Indeed, previous studies have demonstrated that cytokines such as tumour necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) can lead to decreased expression and re-organization of tight junction proteins, resulting in BBB disruption (2, 10).

Therefore, in the presence of a bacterial infection, it is likely that colistin may penetrate the BBB due to perturbation of the paracellular route. Indeed, colistin has been reported to penetrate the blood-cerebrospinal fluid (CSF) barrier in infected patients (3, 17, 25), however, whether enhanced BBB penetration of colistin would occur during a bacterial infection has not been demonstrated. As a surrogate model of bacterial infection, we have shown that systemic administration of *Salmonella enterica* lipopolysaccharide (LPS) to mice leads to a significant enhancement in colistin BBB transport, and this is associated with increased brain uptake of the normally impenetrable ^{14}C-sucrose, a finding consistent with perturbation of the BBB paracellular route (19). This suggests that brain exposure to colistin could be substantially higher in infected patients during treatment and could potentially lead to centrally mediated neurotoxicity. However, whether a similar increase in brain uptake of colistin will be observed in the presence of a systemic bacterial infection (rather than following administration of LPS) remains unknown.

In order to assess the brain uptake of colistin during systemic infection, a mouse thigh infection model involving intramuscular administration of *Pseudomonas aeruginosa* was established. During the last decade, the opportunistic *P. aeruginosa* (40) has developed the multidrug resistance phenotype and sometimes is only susceptible to colistin (27), which has

been shown to have a cure rate of approximately 70% to infections caused by this bacterium (26, 29). Therefore, *P. aeruginosa* was considered a clinically relevant bacterial species to develop the bacterial thigh infection model. The impact of systemic bacterial infection on the integrity of the BBB and colistin brain uptake was then determined, and related to the plasma levels of the pro-inflammatory cytokines, TNF-α, IL-1β and IL-6. Furthermore, we measured the plasma cytokine levels in mice pre-treated with LPS from *Salmonella enterica,* a treatment we have shown leads to increased BBB paracellular permeability and increased brain uptake of colistin (19), to determine any relationship between plasma pro-inflammatory cytokine levels and BBB disruption.

Materials and methods

Bacterial strain and media

P. aeruginosa ATCC 27853 (American Type Culture Collection, Rockford, MD) was stored in tryptone soy broth (Oxoid, Thebarton, South Australia, Australia) with 20% v/v glycerol at -80^{o}C. The bacterial strain was subcultured onto nutrient agar plates (Media Preparation Unit, The University of Melbourne, Parkville, Australia) prior to each experiment and incubated overnight at 37^{o}C. A colony was then selected and grown overnight in 10 mL of cation-adjusted Mueller-Hinton broth (CAMHB, Oxoid, Hampshire, England) from which the logarithmic-phase growth was obtained.

Chemicals and reagents

Colistin sulfate was purchased from Zhejiang Shenghua Biok Biology Co., Ltd (EP5 grade, Zhejiang, China). Lipopolysaccharide from Salmonella enterica serotype Typhimurium was obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). ^{14}C-Sucrose was obtained from American Radiolabeled Chemicals (St Louis, MO). Solid-

phase extraction (SPE) cartridges (C_{18} Sep-Pak®, 100 mg) were purchased from Waters (Milford, MA). Cyclophosphamide was obtained from Baxter Healthcare Pty Ltd (Old Toongabbie, New South Wales, Australia). Glycerol was purchased from Ajax Finechem (Seven Hills, New South Wales, Australia). All other reagents were of analytical and/or HPLC grade and water was obtained from a Millipore purification system (Millipore Corporation, Billerica, MA).

Animal studies

Animal experiments were approved by the Monash Institute of Pharmaceutical Sciences Animal Ethics Committee and were performed in accordance with the Australian National Health and Medical Research Council guidelines for the care and use of animals for scientific purposes. Male Swiss Outbred mice (6-8 weeks of age, 25-30 g) were used in all studies. Mice had free access to food and water during all experimental periods.

Induction of systemic infection in neutropenic and non-neutropenic mice

Systemic bacterial infection was induced through the development of a thigh infection as described previously, with minor modification (13). The thigh infection was induced in both neutropenic and non-neutropenic mice to elucidate whether the presence of neutrophils (and the associated inflammatory cascade) was essential for any bacterial-induced BBB disturbance. To induce neutropenia in mice, cyclophosphamide was injected intraperitoneally 4 days (at a dose of 150 mg/kg) and 1 day (at a dose of 100 mg/kg) prior to the bacterial inoculation. On the day of inoculation, neutropenic mice were anesthetized with isoflurane by inhalation and then a 50 µL suspension of 1 h logarithmic-phase *P. aeruginosa* (approximately 5×10^5 colony forming units (CFU) in CAMHB) or CAMHB was injected

into each posterior thigh muscle. The initial inocula were quantified by viable counting using a Synbiosis protoCOL® colony counter (Don Whitley Scientific Ltd, England). At 4, 8 and 13 h after inoculation, mice were anesthetized by isoflurane and blood was aseptically collected by cardiac puncture (n = 4 at each time point) and subjected to viable counting to determine the number of bacterial cells in the blood. To induce bacteremia in non-neutropenic mice, a 50 µL suspension of 1h logarithmic-phase *P. aeruginosa* (approximately 5×10^7 CFU reconstituted in saline) or saline was injected into each posterior thigh muscle following anaesthesia. Blood samples were collected at 1, 2, 4 and 8 h after inoculation (n = 4 at each time point) to determine at what time point bacteremia had been achieved. The blood samples collected from both neutropenic and non-neutropenic mice at each time point were serially diluted in sterile saline, plated on nutrient agar plates (which were incubated overnight at 37°C), followed by counting of bacterial colonies.

Effect of systemic infection on brain uptake of colistin

An aliquot (200 µL) of colistin sulfate solution (equivalent to 40 mg/kg in saline) filtered through a 0.22 µm membrane (Millipore, Carrigtwohill, Country Cork, Ireland) was subcutaneously administered in the interscapular region of neutropenic or non-neutropenic mice 8 h following bacterial inoculation as this was the time demonstrated to result in reproducible bacteremia (see Results). At various times (0.5, 1, 2, or 4 h) after administration of colistin sulfate, mice (n = 4 at each time point) were anesthetized with isoflurane, blood was collected by cardiac puncture and the whole brain was removed following cervical dislocation. Plasma and brain samples were then stored at -20°C until analysed by HPLC using an assay previously developed and validated in our laboratory (18). The standards used for quantitating colistin in mouse plasma and brain homogenate were prepared in non-

infected matrices from mice. However, to ensure that the presence of bacteria in plasma and brain samples did not affect the ability to quantitate colistin, quality control (QC) samples of colistin (n=4) were also prepared in plasma and brain obtained from mice treated with cyclophosphamide and *P. aeruginosa*. The peak areas from low and high quality control (QC) samples were compared to standards prepared in non-infected matrices, and precision and accuracy were calculated.

Following determination of the colistin concentrations in brain homogenate and plasma, a brain:plasma (B:P) concentration ratio of colistin was calculated at each post-dose time point. In addition to discrete B:P ratios, the area under the plasma concentration-time curve from zero time to 4 h (AUC_{plasma}), the area under the corresponding brain concentration-time curve (AUC_{brain}), and their associated variances were determined by Bailer's approach using the linear trapezoidal rule (4), which was previously validated using WinNonLin (version 4.0, Pharsight Corporation, Mountain View, CA, USA).

Effect of LPS on brain uptake of colistin

Given that LPS (*S. enterica*) is known to induce BBB disruption, the following component of the study was included as a positive control. In brief, mice were administered three intraperitoneal injections (200 µl) of 0.9% (w/v) saline (control) or LPS (*S. enterica*) at 0, 6, and 24 h as previously described (19). An aliquot (200 µl) of a colistin sulfate solution (40 mg/kg in saline) was then administered subcutaneously to mice 4 h after the third saline or LPS dose. At 0.5 h after administration of colistin, plasma and brain samples (n = 4) were harvested, and concentrations of colistin in brain homogenate and plasma were determined by HPLC to obtain a B:P ratio.

Assessment of BBB integrity

The brain uptake of ^{14}C-sucrose was assessed to detect whether the paracellular integrity of the BBB was compromised in infected (both neutropenic and non-neutropenic) and LPS-treated mice (as a positive control). At 8 h after the initial bacterial or saline inoculation or 4 h after the third LPS or saline dose, mice were intravenously administered a 50 µL solution of ^{14}C-sucrose (2 µCi in saline) (n = 6 per group). Plasma and brain samples were collected 5 min post-dose, and radioactivity in plasma and brain determined using liquid scintillation counting (Tri-carb 2800 TR, Perkin Elmer, Boston, MA) as described previously (19).

Measurement of plasma cytokine concentrations

Concentrations of TNF-α, IL-1β and IL-6 were determined in the plasma obtained from non-neutropenic mice 8 h after the initial bacterial or saline inoculation or 4 h after the third LPS or saline dose, in an attempt to correlate BBB disruption to plasma pro-inflammatory cytokine levels. Plasma (50 µL) was added to the wells of mouse cytokine 96-well kits (Ready-SET-Go!®, eBioscience, San Diego, CA) and absorbance was recorded at 450 nm with a Fluostar Optima microplate reader (BMG Labtech, Mount Eliza, Victoria, Australia). The endogenous components within plasma did not interfere with the assay, as no absorbance was obtained following incubation with plasma alone. Concentrations of the three cytokines in each plasma sample were calculated by comparison to a standard curve developed with known concentrations of each cytokine. The quantification range for the cytokines ranged between 8 and 1000 pg/mL. Plasma samples containing cytokine concentrations exceeding 1000 pg/mL were diluted in blank plasma and re-assayed.

Data analysis

All data are presented as mean ± SD, unless otherwise stated. A Student's *t*-test was used to compare the difference between two groups, whereas bacterial colony numbers in infected animals using viable counts on linear scale were compared using a one-way analysis of variance (ANOVA) followed by a Newman-Keuls multiple comparisons test (PASW Statistics for Windows, version 17.0, Chicago, Illinois). A p value < 0.05 was considered to be a significant difference. When comparing AUC_{plasma} and AUC_{brain} between saline-treated and infected mice, a *z*-test was used to test significant differences as proposed by Bailer (4). A z value > 1.96 was considered to be a significant difference between groups.

Results

Bacterial burden in blood after inoculation of neutropenic and non-neutropenic mice

At 4 h after inoculating neutropenic mice with *P. aeruginosa* (5×10^5 CFU), bacteremia was relatively low (less than 4.65 Log_{10} CFU/mL of blood), and it was not until 8 h post-inoculation that bacteraemia appeared to be reproducibly established with blood levels of 5.05-5.38 Log_{10} CFU/mL (Fig. 1a). There was no significant difference in the level of bacteraemia between 8 and 13 h ($p > 0.05$). For this reason, all subsequent brain uptake experiments in neutropenic mice were conducted 8 h following bacterial inoculation or saline administration. When the same inoculum of *P. aeruginosa* was injected into non-neutropenic mice, no bacteremia was detectable, and therefore the inoculum was increased to approximately 5×10^7 CFU. As shown in Fig. 1b, bacteremia was minimal at 1 and 2 h post-inoculation (less than 2.95 Log_{10} CFU/mL), whereas at 4 h post-inoculation, blood levels reached a plateau of 4.90-5.48 Log_{10} CFU/mL which is similar to the level observed in

neutropenic mice (albeit at a lower inoculum). At 8 h post-inoculation, there was no significant difference in bacteremia relative to 4 h ($p > 0.05$), however, in order to keep a consistent time exposure between neutropenic and non-neutropenic studies, all brain uptake assessments in non-neutropenic mice were also undertaken at 8 h post-inoculation.

Brain uptake of colistin following systemic infection or LPS treatment

To ensure that *P. aeruginosa* inoculation or cyclophosphamide treatment did not interfere with HPLC analysis of colistin in plasma and brain homogenate, the HPLC methods developed previously (18) were re-validated using plasma and brain from bacteria-inoculated neutropenic mice. No changes in colistin chromatographic peak shapes or retention times were observed in colistin-spiked plasma or brain homogenate obtained from mice pretreated with cyclophosphamide and inoculated with bacteria. Using standard curve solutions of colistin prepared in plasma and brain homogenate from non-infected mice, the precision and accuracy values for QCs prepared in plasma and brain homogenate from infected mice were within acceptable ranges (as shown in Table 1). These results indicated that plasma and brain from non-infected mice were suitable to establish standard curves to calculate the concentrations of colistin in samples from infected animals.

All mice tolerated the current single subcutaneous dose of colistin with no observed toxicity over the experimental period. The plasma and brain concentrations of colistin in infected and non-infected neutropenic mice are shown in Fig. 2. Plasma concentrations of colistin were not significantly affected by inoculation with *P. aeruginosa*, with AUC_{plasma} values of infected and non-infected mice being 118.6 ± 11.6 and 108.3 ± 11.5 μg·h/mL, respectively ($z = 0.20$). Similarly, the brain homogenate concentrations of colistin were not different between infected and non-infected animals over the 4 h post-dose period with AUC_{brain} values of 2.74 ± 0.21 and 2.58 ± 0.28 μg·h/g, respectively ($z = 0.45$). Similarly,

when colistin was administered to non-neutropenic animals, there was no significant difference in the B:P ratio of colistin between infected and non-infected mice at 0.5 h post-administration (Fig. 3a), with B:P ratios of 0.043 ± 0.032 (infected mice) and 0.032 ± 0.011 (non-infected mice). Given that there was no difference in the brain uptake of colistin at this single time point, no further mice were dosed to obtain a concentration-time profile similar to that obtained for neutropenic mice (i.e. Fig 2). However, in mice treated with LPS from *S. enterica*, the B:P ratio of colistin at 0.5 h post-administration was 0.12 ± 0.053 (Fig. 3b), which was significantly higher than that of saline-treated mice (0.036 ± 0.011), confirming that when the BBB is perturbed, the brain uptake of colistin is indeed enhanced.

BBB integrity following systemic infection or LPS treatment

The B:P ratio of the BBB paracellular route marker, ^{14}C-sucrose, was determined in mice during bacteremia to confirm a lack of paracellular dysfunction, as suggested by the lack of difference in colistin brain uptake between non-infected and infected mice. In both neutropenic and non-neutropenic mice, the average B:P ratios of ^{14}C-sucrose were not significantly different between infected and non-infected mice (Fig. 4a), suggesting that even though bacteremia was present, the tight junctions at the BBB were still functionally intact. In contrast, a small but significant difference in ^{14}C-sucrose brain uptake was observed between saline- and LPS-treated mice with B:P ratios of 0.046 ± 0.0035 (LPS-treated) and 0.039 ± 0.0024 (saline-treated) (Fig. 4b).

Plasma cytokine levels following systemic infection or LPS treatment

Plasma concentrations of TNF-α, IL-1β and IL-6 in infected and non-infected non-neutropenic mice are shown in Fig. 5a. The values of all three cytokines were very low in

non-infected mice, with TNF-α and IL-1β levels being below the limit of quantitation of the assay (i.e. 8 pg/mL). After inoculation with *P. aeruginosa*, concentrations of cytokines were substantially higher with values of 774.1 ± 184.4 pg/mL for TNF-α, 110.7 ± 88.4 pg/mL for IL-1β and 1078.3 ± 235.5 pg/mL for IL-6, consistent with the pro-inflammatory mediators having been activated during systemic bacterial infection. Studies to measure the plasma cytokine levels in neutropenic mice were not conducted as results from non-neutropenic mice were more likely to resemble the clinical setting.

The levels of TNF-α, IL-1β and IL-6 were also determined in plasma of saline- and LPS-treated mice (Fig. 5b), given that *S. enterica* LPS induced BBB disruption therefore allowing for a better correlation to be made between BBB disruption and plasma cytokine levels. The plasma levels of these cytokines from LPS-treated mice were 84.8 ± 14.9 pg/mL for TNF-α, 9.3 pg/mL for IL-1β (SD value could not be calculated as concentrations of only two samples were above 8 pg/mL) and 916.5 ± 310.4 pg/mL for IL-6. The concentrations of cytokines in saline-treated mice were in all cases below the limit of quantitation of the assays. While statistical analysis could not be conducted, given that saline-treated mice exhibited non-quantifiable plasma cytokine levels, it was obvious that plasma concentrations of these three cytokines were higher after LPS treatment. In addition, the TNF-α plasma levels in infected mice were substantially higher than the corresponding levels in LPS-treated mice ($p < 0.05$), even though BBB disruption and enhanced colistin brain uptake was only observed in LPS-treated mice, suggesting a lack of correlation between BBB disruption and plasma levels of this cytokine.

Discussion

As a consequence of the development of antibiotic resistance, there has been increasing clinical use of the cationic polypeptide antibiotic colistin for the treatment of Gram-negative bacterial infections (31). Associated with this clinical use are reports of neurotoxicity, suggesting that colistin has the potential to permeate the BBB. We have demonstrated that colistin exhibits minimal penetration across the healthy BBB, however, has the ability to traverse the BBB during systemic inflammation due to LPS-mediated perturbation of the paracellular route (17). To reflect the clinical scenario more closely, the impact of systemic infection with *P. aeruginosa* on the brain uptake of colistin was assessed in a mouse model. The strain of *P. aeruginosa* selected to induce bacteremia (ATCC27853) was initially isolated from a patient and has been widely used to determine the antimicrobial activities of various antibiotics (15, 35), and therefore it was considered clinically relevant for this study.

It has been shown that inoculation of mice with *Escherichia coli* and *Streptococcus pneumoniae* at sepsis-inducing doses leads to BBB disruption, which was demonstrated by brain access of the impermeable horseradish peroxidise (38). Although a different bacterial strain, an increased BBB permeability and enhancement in colistin brain uptake was therefore expected following inoculation with *P. aeruginosa*. Interestingly, despite a high level of bacteremia similar to that achieved following inoculation with *E. coli* and *S. pneumoniae* (38), the plasma and brain concentrations of colistin did not differ between *P. aeruginosa*-infected and non-infected mice. Moreover, the B:P ratios of the BBB paracellular route marker, ^{14}C-sucrose, were not different between the two groups suggesting that the tight junctions were not disturbed following *P. aeruginosa* inoculation. However, administration of LPS from *S. enterica* led to an increase in the brain uptake of both colistin and ^{14}C-sucrose demonstrating that our model was able to discriminate between an intact and disturbed BBB,

suggesting the observations with *P. aeruginosa* were indicative of a negative impact on BBB dynamics. Although colistin is administered parenterally as its inactive pro-drug CMS (5), we did not assess the impact of bacterial infection on the brain uptake of CMS. Due to stability issues of CMS and its ability to convert to colistin *in vivo* (22, 23), it would be difficult to accurately quantify the brain uptake of CMS *in vivo* and furthermore, whether the brain uptake of CMS is altered during bacterial infection.

The lack of effect of *P. aeruginosa* on BBB integrity and colistin brain uptake was first assessed in neutropenic animals, whose immune system and ability to produce pro-inflammatory cytokines may be affected (9). Cytokines are pro-inflammatory mediators of the immune system that are released in order to respond to acute infection. These mediators are known to modify BBB permeability during neuroinflammatory disease states such as multiple sclerosis, meningitis and encephalitis and this is associated with their ability to affect the expression and function of inter-endothelial tight junction proteins including occludin and zonula occludens-1 (21, 30, 41). Various *in vitro* studies have demonstrated that pro-inflammatory mediators such as TNF-α, IL-1β and IL-6 are able to cause BBB paracellular dysfunction by altering the cytoskeleton structure of endothelial cells (10). It has also been demonstrated that direct administration of such cytokines *in vivo* could lead to BBB disruption (2, 36). Given the role of pro-inflammatory cytokines in mediating BBB disruption, and the altered inflammatory response of neutropenic mice, it was considered that the neutropenic state may have prevented the ability for bacteremia to induce BBB dyfsunction. For this reason, we then developed a bacterial infection model in non-neutropenic (i.e. non cyclophosphamide-treated) mice to determine whether the presence of neutrophils (and the subsequent altered release of pro-inflammatory cytokines) would lead to BBB dysfunction and enhanced colistin brain uptake. Such a scenario is also more likely to be representative of that observed in otherwise healthy patients who have contracted a bacterial infection. Not

surprisingly, a higher inoculum of *P. aeruginosa* was required to induce bacteremia in non-neutropenic mice. However, similar to that observed in neutropenic mice, no significant difference in the brain uptake of colistin or ^{14}C-sucrose was found between infected and non-infected mice suggesting the paracellular route was not disrupted in the infected mice. These studies suggested, that even in the presence of an intact immune system, bacteremia induced by *P. aeruginosa* was not able to induce BBB dysfunction.

To ensure that there was indeed an inflammatory response induced by bacteremia, and to examine possible relationships between inflammatory mediators and BBB disruption, the plasma levels of TNF-α, IL-1β and IL-6 were measured in infected and non-infected mice. For all three cytokines, the concentration in plasma was higher in the infected mice relative to non-infected mice. Despite the higher plasma cytokine levels, the BBB integrity was not affected during bacterial infection, suggesting that there was no clear correlation between plasma cytokine levels and BBB dysfunction, at least for the three cytokines examined. To further confirm this correlation, we measured the plasma cytokine levels in mice which we demonstrated did have a disturbed BBB (i.e. mice treated with LPS from *S. enterica*). It showed that even though we observed a significant increase in colistin and ^{14}C-sucrose brain uptake, plasma cytokine levels were actually lower than those observed following bacterial infection (where there was no BBB disruption). These data confirmed that for these three plasma cytokines, there did not appear to be a direct link between plasma levels and extent of BBB disruption.

There are various reasons as to why there appeared to be differences between the BBB-disrupting effects of our two treatment paradigms. Firstly, it is important to note that bacterial infection with *P. aeruginos*a led to a larger pro-inflammatory response (relative to *S. enterica* LPS administration), yet the BBB disrupting effects of LPS appeared greater. This suggests that there may be a direct effect of LPS species on the BBB, independent of release

of plasma cytokines, as has been suggested elsewhere (16). Alternatively, given the levels of cytokines released in these studies were well below those previously shown to induce BBB disruption both *in vitro* and *in vivo* (10-12, 36, 38), it is possible that a more representative correlation may be observed if higher bacterial inocula of *P. aeruginosa* were administered. Indeed, the levels of TNF-α and IL-6 released in patients with non-bacterial induced acute pancreatitis (where enhanced BBB permeability was detected) were substantially higher than the levels observed in our studies following *P. aeruginosa* infection (14). It should also be noted that we only measured the levels of IL-1β, IL-6 and TNF-α and it is possible that other cytokines such as IFN-γ or IL-10 may also play an important role in BBB disruption (32). It also possible that *P. aeruginosa* ATCC 27853 may be less virulent (and BBB disruptive) than other *P. aeruginosa* strains or other clinically-relevant bacterial strains. The impact of such strains on BBB disruption, and on the brain uptake of colistin (and other therapeutic agents), warrants further investigation. Nevertheless, we have demonstrated that the brain uptake of colistin does not appear to be substantially affected during bacteremia induced with this clinically-relevant strain. We have also confirmed that such an enhancement in the brain uptake of colistin is possible following administration of LPS from *S. enterica*, which suggests that the CNS exposure of this antibiotic may be increased during infection with other bacterial strains.

Conclusions

These studies have demonstrated that, despite increasing the plasma concentrations of three pro-inflammatory cytokines, induction of bacteremia by *P. aeruginosa* did not alter the BBB paracellular route or the ability of colistin to enter the brain parenchyma following systemic administration. Furthermore, a direct relationship between plasma levels of TNF-α, IL-1β and IL-6 and BBB disruption was not evident *in vivo*, in contrast to that which has been suggested from *in vitro* studies.

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559 **FIGURE LEGENDS**

560

561 **Figure 1.** Bacteremia (CFU/mL) at various time points following intramuscular
562 administration of *P. aeruginosa* to (a) neutropenic Swiss Outbred mice and (b) non-
563 neutropenic Swiss Outbred mice. Data are presented as mean ± SEM (n = 4). * indicates a *p*
564 value < 0.05, using a one-way ANOVA.

565 **Figure 2.** Plasma (μg/mL) and brain (μg/g) concentrations of colistin after subcutaneous
566 administration of colistin sulfate (40 mg/kg) to neutropenic Swiss Outbred mice 8 h after
567 inoculation with *P. aeruginosa* (~5 ×105 CFU) or saline. The data indicate plasma (▲) and
568 brain (Δ) from infected mice and plasma (●) and brain (○) from non-infected mice, and are
569 presented as mean ± SEM (n = 4).

570 **Figure 3.** Brain:plasma **(**B:P) ratios of colistin following administration to (a) non-
571 neutropenic Swiss Outbred mice inoculated with saline or *P. aeruginosa* (~5 ×107 CFU) and
572 (b) Swiss Outbred mice administered saline or LPS from *S. enterica* (3 mg/kg at 0, 6 and 24
573 h). Data are presented as mean ± SEM (n = 4). * indicates $p < 0.05$ relative to saline-
574 administered mice, using a Student's *t*-test.

575 **Figure 4.** Brain:plasma (B:P) ratios of ^{14}C-sucrose following administration to (a)
576 neutropenic or non-neutropenic Swiss Outbred mice inoculated with saline or *P. aeruginosa*
577 (~5 x 10^5 to 5 x 10^7 CFU/thigh) and (b) Swiss Outbred mice administered saline or LPS from
578 *S. enterica* (3 mg/kg at 0, 6 and 24 h). Data are presented as mean ± SEM (n = 6) and *
579 indicates a significant ($p < 0.05$) difference between saline and LPS-treated mice, using a
580 Student's *t*-test.

581 **Figure 5.** Plasma concentration (pg/mL) of TNF-α, IL-1β and IL-6 from non-neutropenic
582 mice (a) 8 h after inoculation with saline or *P. aeruginosa* (~5 × 107 CFU) and (b) 4 h after

583 last dose of LPS (*S. enterica*) or saline. Data are presented as mean ± SEM (n=4). * indicates
584 $p < 0.05$ between bacteria-inoculated and saline-treated animals using a Student's *t*-test, +
585 indicates that plasma levels were below 8 pg/mL and # indicates n = 2 (as the remaining
586 replicates were below 8 pg/mL).

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602 **TABLE LEGEND**

603 **Table 1.** Precision and accuracy values of the quality control (QC) samples prepared in
604 plasma and brain homogenate obtained from infected neutropenic mice and compared to a
605 standard curve prepared in matrices obtained from non-infected mice.

Target colistin conc. in matrices from infected mice	Mean ± SD Measured colistin concentration (µg/mL or µg/g)	Precision (%)	Accuracy (%)
Plasma (µg/mL, n = 4)			
1.25	1.26 ± 0.044	3.2	100.8
10.00	10.9 ± 1.46	13.4	109.2
Brain (µg/g, n = 4)			
0.19	0.184 ± 0.025	13.4	96.8
3.00	3.17 ± 0.084	2.6	105.7

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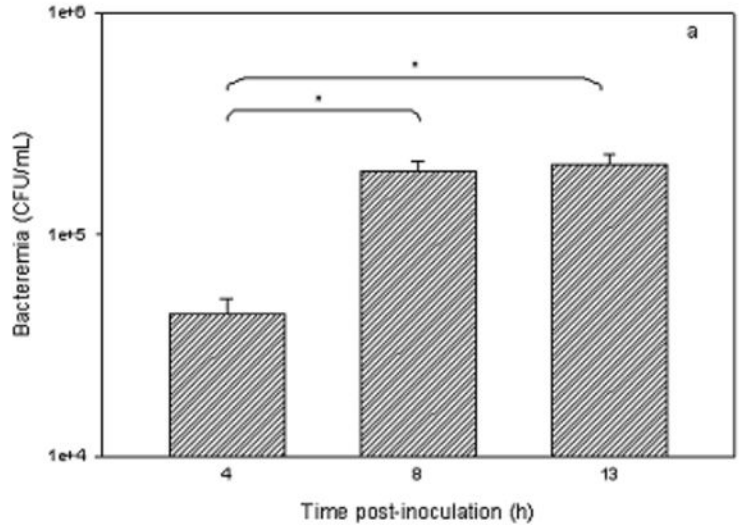

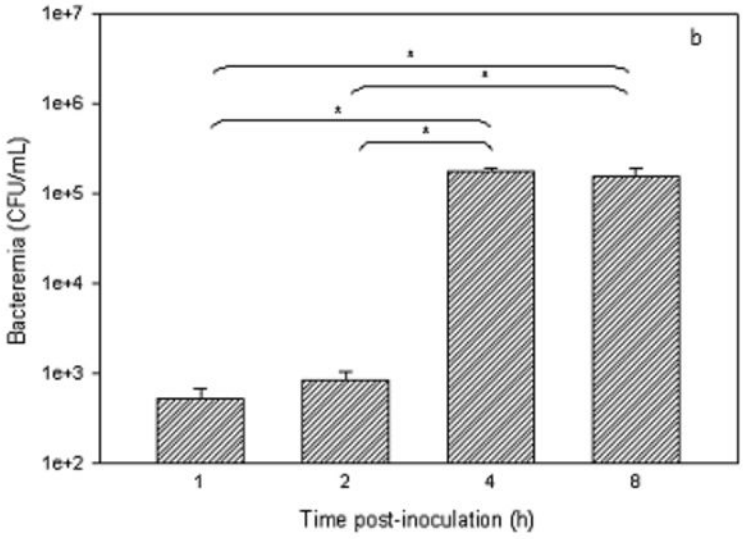

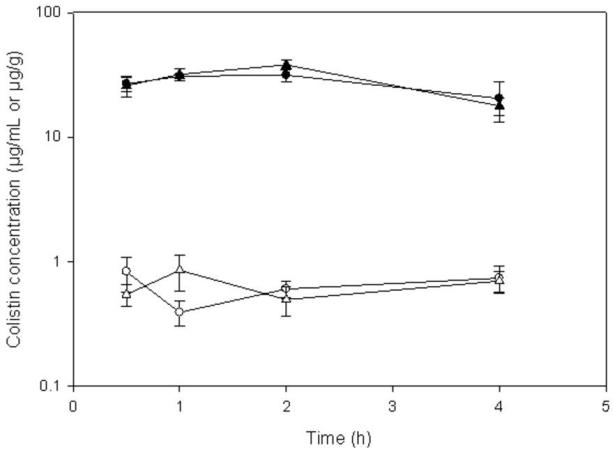

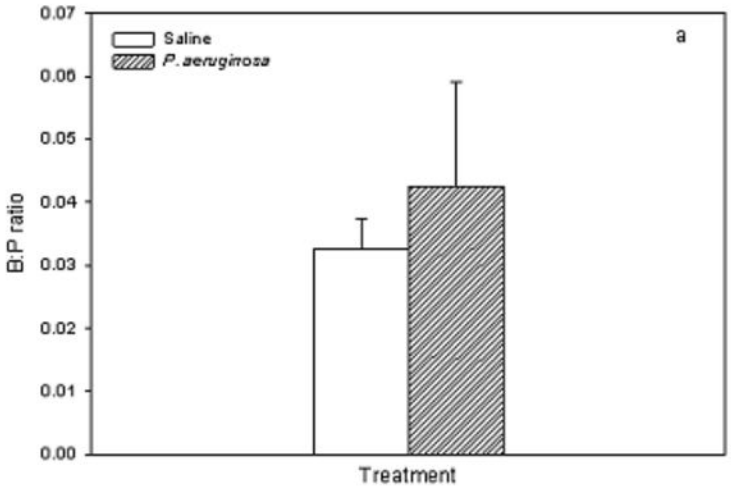

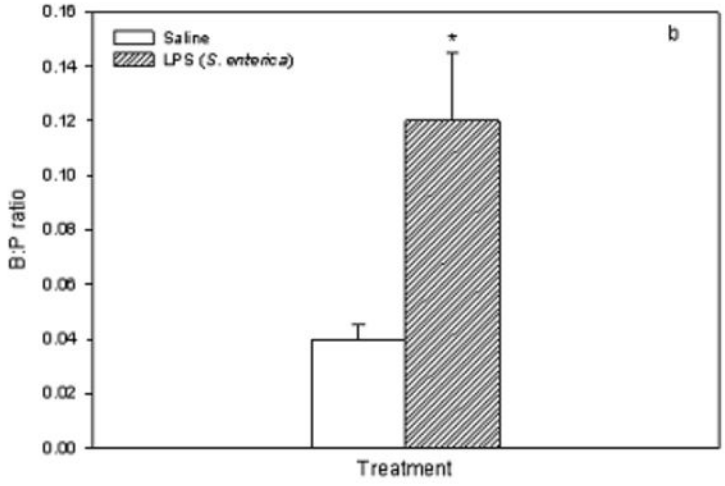

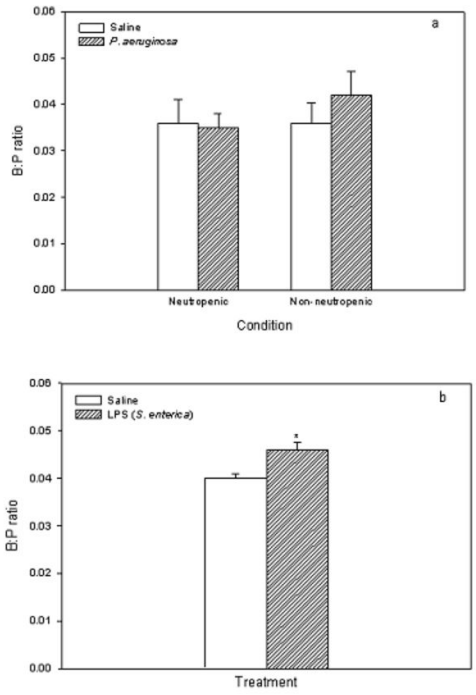

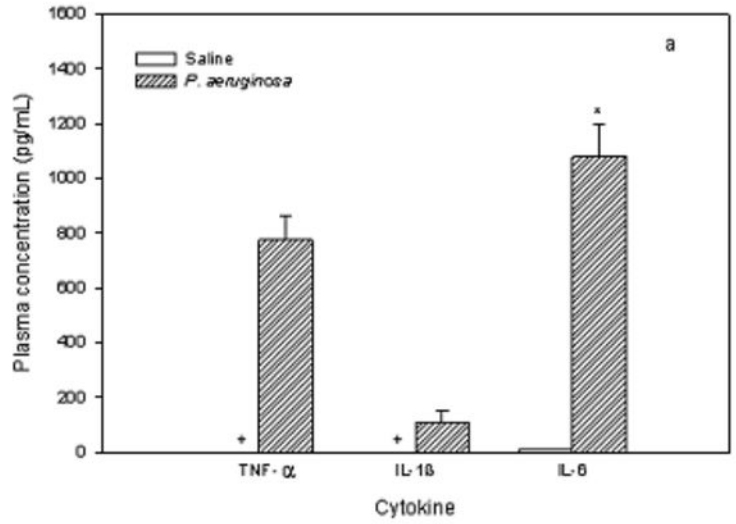

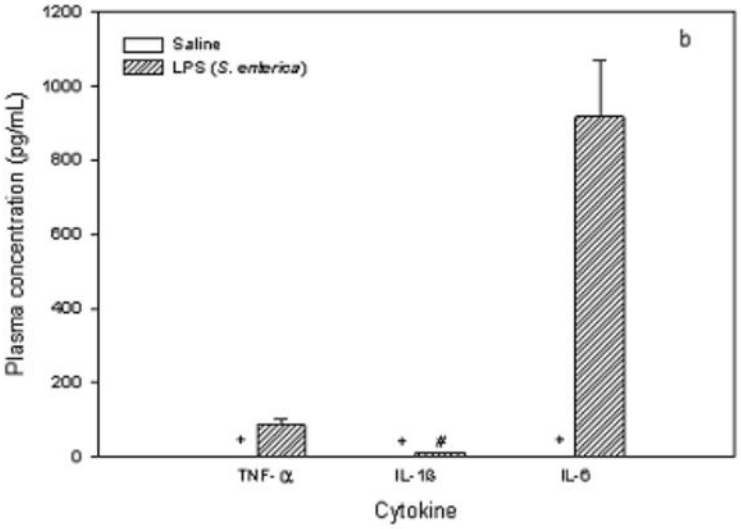

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