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Investigating the Biological Properties of Tigecycline

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**A Thesis submitted to the University of Glasgow for the
Degree of Master of Science (by Research)**

In

Glasgow Dental School,
School of Medicine,
College of Medical, Veterinary and Life Sciences

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LIST OF CONTENTS

Acknowledgements	I
List of Tables	VII
List of Figures	VIII
List of Abbreviations	X
Author's Declaration	XII
Summary	XIII
Chapter 1: Introduction	1
1.1 Introduction to Cystic Fibrosis (CF)	1
1.1.2 Microbiology of the CF lung	2
1.2 CF an inflammatory disease of biofilm origin	4
1.2.1 Introduction to biofilms	4
1.2.2 Bacterial biofilms associated with CF	8
1.2.3 Quorum sensing within bacterial biofilms of the CF lung	9
1.2.4 Antimicrobial resistance in the CF lung	13
1.2.5 Summary	19
1.3 Inflammation and the involvement of inflammatory mediators	20
1.3.1 Introduction to inflammation	20
1.3.2 Inflammatory mediators	22
1.3.2.1 Introduction	22
1.3.2.2 Cytokines and chemokines	23

1.3.2.3	Matrix metalloproteinases	24
1.3.2.4	Summary	27
1.4	Tetracycline based antibiotic	27
1.4.1	Introduction to tetracyclines	27
1.4.2	Resistance to tetracyclines	29
1.4.3	Second generation tetracyclines	31
1.4.3.1	Doxycycline	31
1.4.3.2	Current <i>in vitro</i> research of subantimicrobial doses of doxycycline	32
1.4.3.2.1	Introduction	32
1.4.3.2.2	Current research	33
1.4.3.2.3	Summary	35
1.4.4	Third generation tetracyclines	35
1.4.4.1	Glycylcyclines	35
1.4.4.2	Tigecycline	36
1.4.4.3	Summary	42
1.5	General overview	43
1.6	Aims of the study	44
Chapter 2:	Materials and Methods	45
2.1	Antimicrobial properties of tigecycline	45
2.1.1	Preparation of tigecycline and storage	45

2.1.2	Bacterial strains and maintenance	45
2.1.3	Sensitivity testing of tigecycline	49
2.1.3.1	Standardisation of bacterial inoculum	49
2.1.3.2	Efflux pump inhibitor (EPI) preparation and maintenance	49
2.1.3.3	Sensitivity testing of oral and pulmonary planktonic bacteria to tigecycline \pm EPI	50
2.1.3.4	Sensitivity testing of oral and pulmonary planktonic bacteria to tigecycline \pm EPI	50
2.1.4.	A measurement of efflux pump activity of pulmonary bacteria using alanine β -naphthylamide (Ala-Nap) a fluorescent assay	52
2.1.5	Oral and pulmonary bacterial viability assays using 2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)	52
2.1.6	<i>Burkholderia</i> spp biomass assay	53
2.2	Immunomodulatory effects of tigecycline	54
2.2.1	Tigecycline purity assay	54
2.2.2	A549 Cell culture	55
2.2.3	Experimental procedures for A549 cell stimulation	56
2.2.4	OKF6/TERT2 Cell culture	57
2.2.5	Experimental procedures for OKF6-TERT2 cells	57
2.2.6	Tigecycline toxicity assay to A549 and OKF6/TERT2 cell lines	58
2.2.7	Gene expression for cytokines	58
2.2.7.1	RNA preparation from A549 and OKF6/TERT2 cells	58
2.2.7.2	DNase treatment of RNA samples	59
2.2.7.3	Quantification of nucleic acids	60
2.2.7.4	Reverse transcription	60

2.2.7.5	Real-time quantitative PCR (IL-6 and IL-8)	61
2.2.8	Cytokine assays using enzyme-linked immunosorbent assays (ELISA)	64
2.2.9	Experimental procedures of primary neutrophil cell line	66
2.2.9.1	Peripheral blood neutrophil preparation	66
2.2.9.2	Neutrophil cytokine production in response to tigecycline	66
2.2.9.3	IL-8 chemokine assay using ELISA	67
2.2.9.4	Matrix metalloproteinases using DuoSet ELISA	67
2.10	Data Analysis	68
Chapter 3 Results		70
3.1	Antimicrobial properties of tigecycline	70
3.1.1	Introduction	70
3.1.2	Effects of tigecycline against planktonic and sessile cells	70
3.1.2.1	<i>Burkholderia</i> spp	70
3.1.2.2	<i>Porphyromonas gingivalis</i> , <i>Fusobacterium nucleatum</i> and <i>Aggregatibacter actinomycetemcomitans</i> spp	72
3.1.3	Quantifying efflux pump activity	74
3.1.4	Effects of efflux pump inhibitor (MC-207,110) upon tigecycline sensitivity	77
3.1.4.1	<i>Burkholderia</i> spp	77
3.1.4.2	Effects of efflux pump inhibitor (MC-207,110) on tigecycline sensitivity to oral bacteria after 24 h	81
3.1.5	Biofilm disruptive effects of tigecycline on bacterial biofilms	86

3.2	Immunological properties of tigecycline	88
3.2.1	Introduction	88
3.2.2	Production of IL-8 derived from A549 cell line exposed to a range of LPS concentrations	89
3.2.3	Fluorescence-activated cell sorting in the analysis of Tigecycline purity	91
3.2.4	Tigecycline concentratin does not adversely affect A549 and OKF6/TERT2 cell lines	93
3.2.5	Immunomodulatory effects of tigecycline upon IL-6 production by a pulmonary and an oral epithelial cell line	95
3.2.6	Immunomodulatory effects of tigecycline upon IL-8 production by a pulmonary and an oral epithelial cell line	100
3.2.7	Immunomodulatory effects of tigecycline upon IL-8 And MMP-9 production by human neutrophils	105
Chapter 4:	Discussion	108
	Introduction	108
	Conclusion	121
Bibliography		124

LIST OF TABLES

Chapter 1: Introduction

- 1.1 General features of biofilm infections in humans compared with acute planktonic infections and superficial colonization/normal flora of the skin and mucosal membranes
- 1.2 The structure and substrate specificity of the three-component active efflux system in *P.aeruginosa*

Chapter 2: Material and Methods

- 2.1 Pulmonary strains
- 2.2 Oral strains
- 2.3 Polymerase chain reaction (PCR) reaction conditions
- 2.4 Primers used for analysis of pro-inflammatory response
- 2.5 Duoset ELISA solutions

Chapter 3: Results

- 3.1 Planktonic and sessile minimum inhibitory concentrations of *Burkholderia cepacia* spp to tigecycline
- 3.2 Planktonic and sessile minimum inhibitory concentrations of oral pathogens to tigecycline
- 3.3 Effects of a competitive substrate (MC,207-110) of efflux pumps on the sensitivity of *Burkholderia cepacia* spp to tigecycline
- 3.4 Effects of a competitive substrate (MC,207-110) of efflux pumps on the sensitivity of oral pathogens to tigecycline

LIST OF FIGURES

Chapter 1: Introduction

- 1.1 Scanning electron microscopy (SEM) images depicting Methicillin resistant *Staphylococcus aureus* (MRSA) isolates forming weak (a) adherent (b) and significantly established (c) biofilms.
- 1.2 SEM images depicting clinically important oral biofilms formed *in vitro*.
- 1.3 QS is accountable for virulence factors and diseased states in the CF lung.
- 1.4 *P. aeruginosa* QS regulatory system is associated with virulence factors in the CF lung.
- 1.5 Resistance mechanism of a bacterial biofilm.
- 1.6 Overview of *P. aeruginosa* efflux mediated resistance to tetracycline based antibiotics.
- 1.7 An example of inflammatory mediators involvement in bacterial tissue damage.
- 1.8: Chemical composition of the tetracycline class of antibiotics
- 1.9: Action of tigecycline within the bacterial ribosome.
- 1.10: Comparison between the chemical composition of minocycline and tigecycline

Chapter 3: Results

- 3.1 Quantification of efflux activity utilising Ala-nap uptake by differential strains of *Burkholderia* spp
- 3.2. Efflux pump inhibitor (MC-207,110) enhances sensitivity of planktonic *Burkholderia* spp. to tigecycline after 24 h exposure

- 3.3. Efflux pump inhibitor (MC-207,110) enhances sensitivity of sessile *Burkholderia* spp. to tigecycline after 24 h exposure
- 3.4. Efflux pump inhibitor (MC-207,110) enhances sensitivity of planktonic *Aggregatibacter actinomycetemcomitans* to tigecycline after 24 h exposure
- 3.5. Effects of efflux pump inhibitor (MC-207,10) on tigecycline sensitivity to sessile oral bacteria after 24 h
- 3.6. Tigecycline disrupts sessile *Burkholderia cepacia* spp. in a concentration dependant manner, which is improved with EPI
- 3.7. IL-8 protein expression from A549 cell line after exposure to varying ranges of LPS after 24 h
- 3.8. Analysis of CD86 and MHC II by FACS analysis when stimulated with tigecycline
- 3.9. LPS and tigecycline do not adversely affect the metabolic activity of A549 and OKF6/TERT2 cell lines after 24 h incubation.
- 3.10. IL-6 mRNA expression after exposure to varying concentrations of tigecycline \pm LPS after 4 and 24 h.
- 3.11. IL-6 protein expression after exposure to varying concentrations of tigecycline \pm LPS after 4 and 24 h
- 3.12. IL-8 mRNA expression after exposure to varying concentrations of tigecycline \pm LPS after 4 and 24 h
- 3.13. IL-8 protein expression after exposure to varying concentrations of tigecycline \pm LPS after 4 and 24 h
- 3.14. Immunomodulatory effects of tigecycline to IL-8 and MMP9 produced by human neutrophils in the presence of 1 mg/L PMA

ABBREVIATIONS

3O-C12:	N-3-oxododecanoyl homoserine lactone
Ab:	Antibody
Acyl-HSL:	Acyl-homoserine lactone
Ala-Nap:	Alanine β -naphthylamide
Bcc :	<i>Burkholderia cepacia complex</i>
BPE:	Bovine pituitary extract
BSA:	Bovine serum albumin
Calgary:	CG
C4-HSL:	N-butyryl- homoserine lactone
CF:	Cystic fibrosis
CFTR:	CF transmembrane conductance regulator
CLSI:	Clinical and Laboratory standard institute
COPD:	Chronic obstructive pulmonary disease
Ct:	Threshold cycle
ddH ₂ O:	Double distilled water
DMEM:	Dulbecco's modified Eagles medium
DPBS:	Dulbecco's phosphate buffered saline
ECACC:	European collection of cell cultures
ECM:	Extra cellular matrix
EGF:	epidermal growth factor
ELISA:	Enzyme-linked immunosorbent assay
EMMPIN:	Extracellular matrix metalloproteinase inducer
EPI:	Efflux pump inhibitor
FDA:	Food and drug administration
FITC:	Flurochrome fluorescein isothiocyanate
GAPDH:	Glyceraldehydes-3-phosphahet dehydrogenase
GCF:	Gingival crevicular fluid
IFN- γ :	Interferon gamma
IFNs:	Interferons
IL:	Interleukin
IL-1 β :	Interleukin-1 β
IL-6:	Interleukin-6
IL-8	Interleukin-8
K-SFM:	Keratinocyte serum-free medium
LB:	Luria broth
LPS:	Lipopolysaccharides
MAMPs:	microbe-associated molecular patterns
MC-207,110:	EPI L-Phe-L-Arg- β -naphthylamide
MCP-1:	Monocyte chemoattract protein-1

MHB:	Mueller hinton broth
MHC II:	Major histocompatibility complex II
MIC:	Minimal inhibitory concentration
MMP:	Matrix metalloproteinase
MRSA:	Methicillin resistant <i>Staphylococcus aureus</i>
MSSA:	Methicillin susceptible <i>Staphylococcus aureus</i>
NCTC:	National collection of type cultures
NTHi:	nontypeable <i>Haemophilus influenzae</i>
OKF6/TERT2:	Oral mucosal immortalised keratinocyte cell line
(ANOVA):	One-way analysis of variance
PAO1:	<i>P. aeruginosa</i> clinical and laboratory strain
PAMPS:	Pattern associated molecular patterns
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
PD1:	Protectin D1
PMA:	phorbol 12-myristate acetate
PMIC:	Planktonic minimal inhibitory concentration
PMN:	Polymorphonucleocytes
PPR:	Pattern recognition receptors
QS:	Quorum sensing
R _{FU} :	Relative fluorescent units
RND:	Resistance nodulation cell division
RT:	Room temperature
RT-PCR:	Real time polymerase chain reaction
RvE1:	Resolvin E1
SAM:	Schadler's anaerobic media
SDD:	Sub-antimicrobial doses doxycycline
SEB:	Staphylococcal enterotoxin B
SMIC:	Sessile minimal inhibitory concentration
SRP:	Scalling and root planning
TIMPs:	Tissue inhibitors of metalloproteinases
TLRs:	Toll-like receptors
TNF:	Tumour necrosis factor
TNF- α :	Tumour necrosis factor alpha
TSB:	Tryptic soy broth
XTT:	2,3 bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide

Declaration of originality

This thesis is the original work of the author.

Ryan Francis Quinn, July 2011

SUMMARY

Antibiotic resistance of many bacterial strains to current antibiotic treatment strategies are increasing. Bacterial biofilm related diseases displaying resistance to current antibiotics is an area of intense investigations. Failure to eradicate biofilm forming pathogenic microorganisms, coupled with an exacerbated host immune response results in extensive tissue damage and ultimately chronic inflammation within infected patients. Periodontitis and cystic fibrosis (CF) represent typical forms of chronic inflammatory disease

The aim of this study was to evaluate the efficacy of a newly developed glycylicline, tigecycline, against non-pseudomonal Gram-negative CF pathogens and oral pathogens commonly associated with inflammatory disease of the lung and oral cavity, respectively. Minimum inhibitory concentrations (MICs) of periodontal and pulmonary pathogens in planktonic growth phase and sessile biofilms were determined by serial doubling dilutions with tigecycline. Furthermore, planktonic MICs and sessile MICs exposed to tigecycline and a competitive efflux pump inhibitor MC-207,110 EPI were assessed by XTT assays. In addition the biomasses of sessile biofilms exposed to tigecycline and EPI were determined by crystal violet assay. This investigation demonstrated a significant improvement in the susceptibility of both planktonic and sessile cells to tigecycline following the addition of an EPI, indicating that the EPI enhanced sensitivity to antibiotic treatment of resistant bacterial strains. Furthermore, bacterial biofilm biomass

of the CF pathogen *Burkholderia cepacia* was reduced significantly by co treatments of tigecycline and EPI.

Finally, the immunomodulatory properties of tigecycline were evaluated using clinically relevant epithelial cell lines, A549 OKF6-TERT2 and a primary neutrophil cell line. Oral and pulmonary cell lines were co-inoculated with subinhibitory concentration of tigecycline and *Escherichia coli* derived lipopolysaccharide (LPS). The neutrophil cell line was co-inoculated with (phorbol 12-myristate acetate) PMA and tigecycline. Epithelial IL-6 and IL-8 levels were determined by ELISA and RT-qPCR, demonstrating only marginal down-regulation in some cases of these inflammatory mediators. However, tigecycline at subinhibitory concentrations did reduce the levels of IL-8 and MMP-9 synthesised by neutrophils in a dose dependent manner.

In conclusion, tigecycline alone is ineffective at killing and reducing biomass of mature biofilms associated with chronic inflammatory disease of the lungs and oral cavity, however the addition of a competitive efflux pump inhibitor decreased resistance of the bacterial biofilm to tigecycline. This would suggest a possible new chemotherapeutic use to treat patients who suffer from a chronic inflammatory disease. Furthermore, down-regulation of inflammatory mediators by subinhibitory concentration of tigecycline may indicate a potential use in the therapeutic management of both CF and periodontal disease, and other diseases of chronic inflammatory origin.

**Chapter 1:
Introduction**

1 Introduction

1.1 Introduction to Cystic Fibrosis

Cystic Fibrosis is the most common lethal autosomal recessive disease in the Caucasian population. CF sufferers currently have a life expectancy of approx 33 years, which has increased over the last 40 years when life expectancy was previously confined to early childhood (Ye *et al*, 2001). This increase in life expectancy is due to improvements in areas of infection awareness and antimicrobial treatment strategies (Chernish *et al*, 2003). Chronic infection and inflammation are the primary cause of morbidity and mortality in CF patients (Pilewski *et al*, 1999). CF is caused by a mutation in the CF transmembrane conductance regulator (CFTR) gene, located on chromosome 7q31 spanning approximately 230kb of genomic DNA containing 27 exons (Harris, 1992). This gene was first identified in 1989 by the groups of Lap-Chee Tsui (Toronto, Canada) and Francis Collins (Michigan, USA) (Harris, 1992). The mutation results in clinical manifestations including mucus plugging and obstruction of the respiratory and digestive tracts through over production of a dehydrated and thickened secretion (Serisier *et al*, 2009).

The commonest clinical symptom of CF, and most debilitating, is chronic obstructive lung disease. Deterioration of the CF lung starts in the upper lobes, which is in contrast to involvement of the lower lobes in pneumonia and bronchitis (Quinton, 1999). Mechanisms underlying the early acquisition of infection in CF are complex and incompletely understood (Davies *et al*, 2009). In general, immediately after birth, bacteria colonise and infect the airways taking advantage of the impaired mucociliary action and secretions produced

by the CF lung. This provides fertile grounds for bacterial growth and colonisation (Chernish *et al*, 2003).

1.1.2 Microbiology of the CF lung

Understanding the genetic trait that results in CF is just as important as identifying the bacteria that infect the CF lung (Hearst *et al*, 1995). In healthy individuals, inhaled bacteria are trapped by the mucus secretions that line the major airways. These bacteria are moved upward by mucociliary clearance and after swallowing are destroyed by stomach acids (Govan *et al*, 1996). In the CF lung, bacterial clearance from the airway is hampered by viscid airway secretions, resulting in bacterial colonisation, pulmonary damage and subsequently an exacerbated inflammatory response.

The most frequently reported respiratory pathogen in the CF lung is *Pseudomonas aeruginosa* (Govan *et al*, 1996). Greater than 93% of CF patients are reported to have *P. aeruginosa* infections (Son *et al*, 2007). *P. aeruginosa* has evolved a huge array of complex strategies to evade host defences to ensure its survival (Davies *et al*, 2009). Indeed, more than 90% of mortality associated with CF results from infections by *P. aeruginosa* (Fink *et al*, 2003). The phytopathogen *Burkholderia cepacia*, which is distantly related to *P. aeruginosa* was reported to cause infections in 3.5% of CF patients worldwide (McClellan *et al*, 2009).

Burkholder first identified *B. cepacia* in the 1950s identifying it as the bacterium commonly associated as the causative agent of soft onion rot,

which was later isolated as a virulent pathogen in CF sufferers in the 1980s (Tomlin *et al* 2005). By the early 1990s, multidrug resistant strains of *B. cepacia* emerged as a major infection control challenge for CF carers (Govan *et al*, 1996). CF patients colonized by *B. cepacia* experience a rapid decline in lung function compared to those colonised with *P. aeruginosa* (McClellan *et al*, 2009). Once *B. cepacia* has been established in the CF lung, it is rarely eradicated with infection ranging from maintaining stable respiratory functions to rapid and often fatal clinical decline (McClellan *et al*, 2009).

Once bacteria are established in the CF lung, host immune defences try to eradicate the infection. Pro-inflammatory mediators, such as Interleukin-8 (IL-8) and IL-6, expressed by respiratory epithelium recruit and activate inflammatory cells within the lungs. Influx of polymorphonucleocytes (PMNs) produces a wide range of antimicrobial factors aimed at killing the bacteria. However, this can inadvertently lead to pulmonary deterioration and epithelial necrosis (Fink *et al*, 2003). Failure to clear infection causes persistent neutrophil influx, leading to further damage to the lung. Overall, this indicates that successful treatment of CF requires in part halting the cycle of repetitive infections (Hassett *et al*, 2010).

To summarise, pathogenic bacteria that infect the respiratory tract early in the course of CF are rarely eradicated. This, coupled with an aggressive inflammatory response, is thought to be a key player of irreversible airway damage (Davies *et al*, 2009). Failure to eradicate these organisms are attributed to high-level multiple drug resistance, which is known to emerge

during treatment (Poole, 2001), and may be associated with intrinsic resistance (biofilms) or induced resistance (efflux pump activity). Therefore, it is important to understand the microenvironment of the CF lung to gain further insight into how successful treatment may be achieved. However, this is difficult given the biofilm nature of microorganisms within the CF lung.

1.2 CF an inflammatory disease if biofilm origin

1.2.1 Introduction to biofilms

Microbiologists have historically studied planktonic (free floating and homogeneous) cells in pure-culture. However, the link between sessile (surface attached and heterogeneous) cells with microbial pathogenesis in human infection is now a mainstream ideology (TomLin *et al*, 2005). Recent research reveals that a wide range of bacteria alternate between planktonic and sessile multicellular communities, commonly referred to as biofilms (Kvist *et al*, 2008). It is now estimated that up to 65% of microorganisms that cause human infections exist in biofilm communities, and this mode of growth is considered the prevailing lifestyle of microorganisms (Smith *et al*, 2008).

Biofilms are highly structured communities within a 3-dimensional architecture, characterised by interstitial voids, water channels and microorganisms that are surface-associated, and/or attached to one another and encapsulated within a self-produced protective extracellular matrix (ECM) (Costerton *et al*, 2003) (Figures 1.1 and 1.2). Microorganisms favour biofilm conditions as environmental stresses, such as nutrient deprivation, results in production of the viscous highly pro-inflammatory ECM that surrounds the

biofilm (Yoon *et al*, 2002; Worlitzsch *et al*, 2002). The ECM of *P. aeruginosa* is the result of a stress factor within the biofilm which promotes a mutation to over produce alginate, thereby offering protection from host immune responses and antimicrobial agents (Davies, 2002).

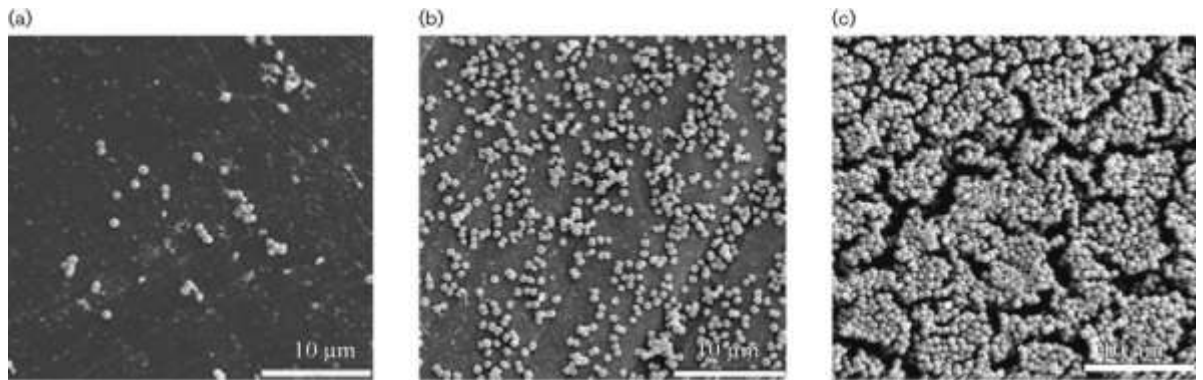


Figure 1.1 Scanning electron microscopy (SEM) images depicting MRSA isolates forming weak (a), adherent (b) and significantly established (c) biofilms. SEM images of three different stages of biofilm growth and attachment are depicted. (a) initial attachment and development of a bacterial biofilm, (b) increasing numbers of adhered bacteria are evident (c) a fully established microbial biofilm (Smith *et al*, 2008).

Biofilms can develop on either biotic or inert substrates from a single species or as a community derived from several species. Thereby, biofilms can be considered as complex cities of microbes that cooperatively interact in an altruistic manner (Hoffman *et al*, 2005). The advantages for microorganisms in forming a biofilm include protection from the environment, resistance to physical and chemical removal of cells, metabolic cooperation and a community based regulation of gene expression (Tote *et al*, 2009). Table 1.1 highlights the general features of biofilm infections in human host tissues.

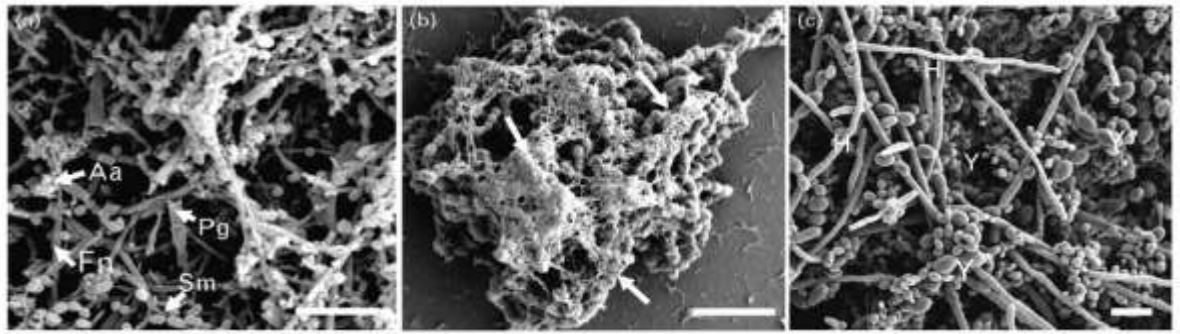


Figure 1.2 SEM images depicting clinically important oral biofilms formed *in vitro*. (a) Biofilm representative of subgingival periodontal plaque, consisting of *Streptococcus mitis* (Sm), *Fusobacterium nucleatum* (Fn), *Aggregatibacter actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg). (b) *Streptococcus mutans* biofilm. Polymeric matrix material (arrow) is evident surrounding the coccoid cells. (c) *Candida albicans* biofilm. With a combination of yeasts (Y) and hyphae (H) that form a lattice network to support the structure. The scale bar represents 10 μ m. (Ramage *et al*, 2010).

Table 1.1 General features of biofilm infections in humans compared with acute planktonic infections and superficial colonisation/normal flora of skin and mucosal membranes (Adapted from Hoiby *et al*, 2010).

Features of biofilm infections	Necessary condition for biofilm infection	Sufficient condition for biofilm infection	Also found in acute planktonic infection	Also found in colonisation/normal flora on skin and mucosal membranes
Aggregates of bacteria embedded in a self-produced polymer matrix	Yes	Yes	No	No / Yes
Tolerant to innate and adaptive immune responses	Yes	Yes	No	No / Yes
Inflammation	Yes	No	Yes	No
Biofilm-specific antigens	Seldom	Seldom	No	No
Antibody response	Yes (after some weeks)	No	Yes (after some weeks)	No
Chronic infection	Yes	Yes	No	No
Foreign-body associated infections	No	Yes	No (yes on first day of infection)	No
Localised infections	Yes	No	Yes	Yes
Focus for spreading or local exacerbation	Yes	No	Yes	Yes

1.2.2 Bacterial biofilms associated with CF

Bacteria that persist in lungs of CF patients preferentially survive as a biofilm in order to improve their potential for survival (Tote *et al*, 2009). The detection within CF sputum of quorum sensing molecules, which are used by *P. aeruginosa* for cell-to-cell communication *in vitro*, provides evidence that *P. aeruginosa* form biofilms in the CF lung (Moreau-Marquis *et al*, 2008). Inhaled bacteria in planktonic form initially attach to cell surface receptors on respiratory epithelial cells. In particular, respiratory cells within CF patients have an abundance of asialoGM1 receptors on their surface that *P. aeruginosa* pili and flagella attach to (Saiman *et al*, 2003). The CF lung provides an environment that allows conversion of this microorganism from a motile planktonic form to a mucoid sessile biofilm mode of growth (Kirov *et al*, 2007). This conversion in mode of *P. aeruginosa* growth is important as the microorganism often loses its associated polar flagellum during chronic airway disease. The absence of this structure enables the bacterium to become undetectable to the host immune system. As a consequence the host fails to initiate IL-8 production in response to the invading microorganism, thereby resulting in diminished neutrophil recruitment (Hassett *et al*, 2010). Furthermore, the non-mucoid *P. aeruginosa* phenotype is considered to be harmless as infections with this phenotype do not significantly alter lung function or antibody response (Pedersen *et al*, 1992).

P. aeruginosa biofilms within the CF lung live in stagnant microaerophilic mucus. It is understood that such biofilms favour anaerobic conditions, facilitating microbial growth to be 3-fold greater than microbes grown in

aerobic conditions (Yoon *et al*, 2002). Biofilms that form under these conditions leads to depleted oxygen levels in the lungs and are referred to as a chronic type II biofilm. Within these types of biofilm, bacteria are encapsulated within the thick and stagnant mucus lining the CF airway (Major *et al*, 2010). Many of these biofilm processes are controlled via cell-to-cell communication. *Stenotrophomonas maltophilia* is very often co-isolated with *P. aeruginosa* from CF patients. Pompilio *et al* (2010) demonstrated *S. maltophilia* was capable of adhering to and invading CF-derived IB3-1 cultured bronchial epithelial cells. Furthermore, they identified that *S. maltophilia* has the ability to form biofilms on either polystyrene or IB3-1 cultured epithelial cells. They also elucidated that *P. aeruginosa* standard laboratory and genetic reference strain PAO1 significantly improves *S. maltophilia* adhesiveness, indicating a potential synergistic relationship between these pathogens. The presence of *B. cepacia* pathogenic biofilms in CF patients elicits a robust neutrophilic response, resulting in an exacerbated production of pro-inflammatory mediators, such as elastase, reactive oxygen and reactive nitrogen. This neutrophilic response causes progressive deterioration of the airway epithelium and underlying tissue, which ultimately leads to the premature death of CF patients (Major *et al*, 2010).

1.2.3 Quorum sensing within bacterial biofilms of the CF lung

Key pathogenic factors, including virulence and resistance, are controlled within the CF lung via microbial communication. Bjarnsholt *et al* (2009) established that biofilm cells of *P. aeruginosa* recognise the presence of attracted PMNs and respond to this inflammatory response via cell-to-cell

contact with surrounding bacteria through signalling molecules. These are recognised to be quorum sensing (QS) molecules, which are widespread among different bacterial genera, emphasising the importance of coordinating bacterial behaviour as a group (Horswill *et al*, 2006).

QS regulation plays an important role in infections caused by opportunistic pathogens (Bjarnsholt *et al*, 2007). QS molecules induce the expression of genes in bacteria that are vital to biofilm formation, these include, virulence genes, surface motility and controlling antimicrobial resistance genes within the CF biofilm (Davies, 2002; Taga *et al*, 2003; Zhu *et al*, 2002). In bacterial communities QS systems can be viewed as a global regulatory system controlling the expression of numerous genes and phenotypes (Willcox *et al*, 2008). Bacteria can monitor population density via chemical communication allowing the bacteria to coordinate the behaviour of the group much like the characteristics observed in multicellular organisms (Taga *et al*, 2003). Bacterial cells are not physically aware of the presence of other bacteria, but can sense the concentration of signal molecules (Bjarnsholt *et al*, 2007). Sensing is achieved via the production of small signal molecules called autoinducers, which consist of 3 components: signal molecules, signal synthesis and signal receptors (Brackman *et al*, 2009). The acyl-homoserine lactone (acyl-HSL)-based signalling systems are the most well studied examples of QS with more than 70 Gram-negative bacterial species reported to use acyl-HSL based systems (Taga *et al*, 2003). Gram-positive QS bacteria usually use a modified oligopeptide two-component circuit as communication signals (Zhu *et al*, 2002).

Work conducted by Riedel *et al* (2002) provided evidence that both *P. aeruginosa* and *B. cepacia* form mixed biofilms in the lungs of CF patients and are capable of communicating with each other utilising the QS acyl-HSL thus contributing to the infection process in diseased states within the host (Figure 1.3).

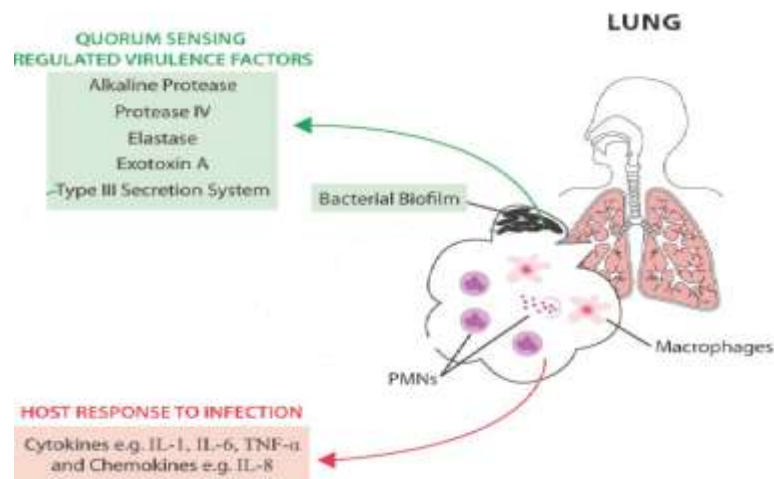


Figure 1.3 QS is accountable for virulence factors and diseased states in the CF lung.

QS regulated virulence factors (in green) are important mediators in infections of the lung and account for diseased state. Shown in red are the effects of QS AHL signal on the host response, indicating infiltration of PMNs and macrophages which damage host tissue by release of reactive oxygen species (Willcox *et al*, 2008).

QS in *P. aeruginosa* is well understood and is comprised of two acyl-AHL synthases, *LasI* and *RhlI*, that generate N-3-oxododecanoyl homoserine lactone (3O-C12) and N-butyryl-HSL (C4-HSL), respectively (Wilder *et al*, 2009). As these signals accumulate exponentially within the community they bind to their cognate transcriptional regulators *LasR* and *RhIR*, respectively, activating expression of target genes (Juhas *et al*, 2005) Therefore, *P.*

aeruginosa virulence factors including secreted toxins are under the control of the QS regulatory system (Winstanley *et al*, 2008). *P. aeruginosa* is dependent on the QS molecule 3O-C12 to drive expression of virulence genes which in turn induce pro-inflammatory cytokines such as IL-6 and IL-8 in airway epithelial cells (Figure 1.4) (Mayer *et al*, 2011).

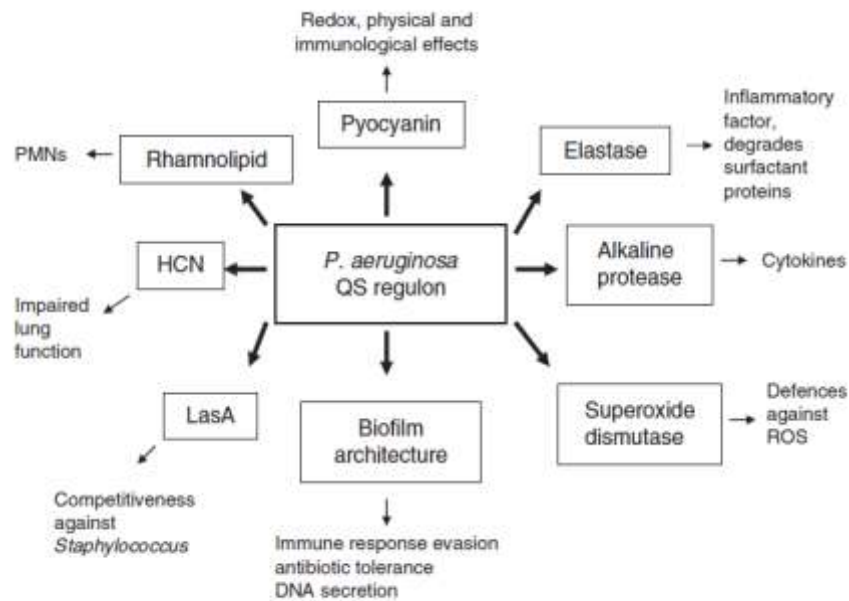


Figure 1.4 *P. aeruginosa* QS regulatory system is associated with virulence factors in the CF lung.

P. aeruginosa QS regulated virulence factors and their relevance to CF, indicating the intrinsic links between the QS regulon and biofilm formation, cytokine production, inflammatory factors, impaired lung function and recruitment of PMNs (Winstanley *et al*, 2008).

In conjunction to these findings, the *B. cepacia* QS system *cepIR* (a homologue of the *lasIR/rhlIR* system identified in *P. aeruginosa*) is known to control expression of the transcriptome and proteome associated with virulence factors and biofilm formation (TomLin *et al*, 2005). This group

demonstrated that *B. cepacia cepI* and *cepR* mutant biofilm biomasses were less than half of the observed value compared to the wild type strain K56-2. These findings indicate that both mutants were structurally impaired in both initial attachment stages and further biofilm development (TomLin *et al*, 2005). These investigations highlight the importance of QS in biofilm development and bacterial disease progression.

1.2.4 Antimicrobial resistance in CF lung

During chronic co-infection, *P. aeruginosa* and *B. cepacia* aggregate in mixed biofilms in the lungs of CF patients and synergistically enhance antibiotic resistance and persistent infection (Riedel *et al*, 2002). This resistance is attributed to the multicellular nature of biofilm communities, such as delayed penetration of the antibiotic into the biofilm, slower growth rates in some biofilms that undermines antibiotics that target cell growth, and altered microenvironments that antagonise the action of the antibiotic (Figure 1.5) (Kirov *et al*, 2007).

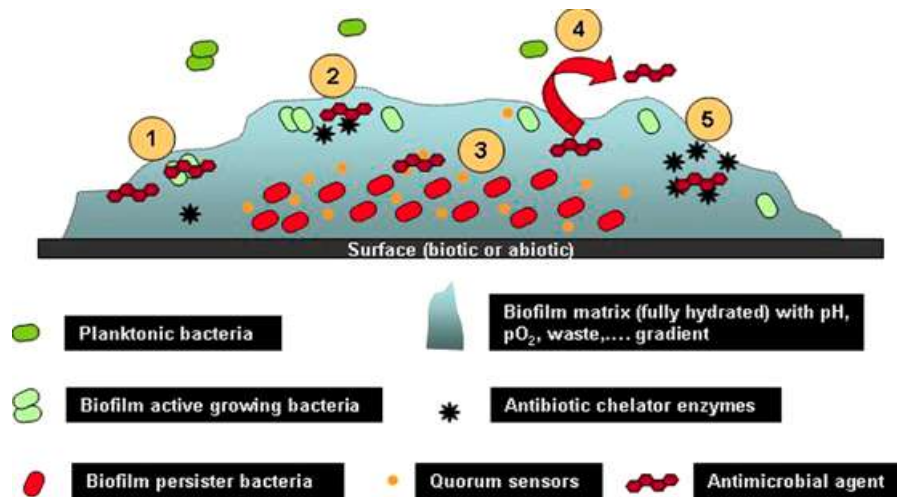


Figure 1.5 Resistance mechanism of a bacterial biofilm.

Proposed-biofilm associated resistance mechanisms: (1) Antimicrobial agents may fail to penetrate beyond the surface layers of the biofilm. Outer layers of biofilm cells absorb damage. Antimicrobial agents action may be impaired in areas of waste accumulation or altered environment (pH, pCO₂, pO₂, etc). (2) Antimicrobial agents may be trapped and destroyed by enzymes in the biofilm matrix. (3) Altered growth rate inside the biofilm. Antimicrobial agents may not be active against non-growing microorganisms (persister cells). (4) Expression of biofilm-specific resistance genes (efflux pumps). (5) Stress response to hostile environmental conditions (leading to an over-expression of antimicrobial agent-destroying enzymes) (Pozo and Patel, 2007).

In the biofilm community, mutations also occur that prove advantageous for the survival of bacteria (Zarantonellie *et al*, 2005). Alterations of drug targets or enzymatic inactivation of antimicrobial agents are well known mechanisms of antimicrobial drug resistance. Recently, active drug efflux has become increasingly important in understanding multidrug resistance (Hasdemir, 2007). Genes which regulate the expression of multidrug-efflux pumps are frequently mutated in *P. aeruginosa*. Indeed, inactivation of the negative regulator of the MexXY-OprM multidrug efflux pump increases resistance to aminoglycoside and other drugs (Feliziani *et al*, 2010).

Efflux pumps are responsible for the removal of intracellular toxic substances and metabolites in addition to excretion of signalling molecules into the environment to facilitate cell-to-cell communication (Kriengkauykiat *et al*, 2005). Resistance nodulation cell division (RND) type multidrug efflux proteins function with an outer membrane pore and a membrane fusion protein to pump out drugs. AcrAB-TolC of *Escherichia coli*, AcrAB homologue of *Salmonella enterica serovar typhimurium*, MexXY-OprM, MexC-MexD-OprJ, MexE-MexF-OprN, and MexX-MexY-OprM of *P. aeruginosa* and CeoAB-OpcM of *B. cepacia* are typical examples of such systems (Hasdemir, 2007, Chan *et al*, 2003). Table 1.2 summarises the structure and substrate activity of these efflux pumps.

Table 1.2 Structure and substrate specificity of the three-component active efflux system in *P. aeruginosa* (Adapted from Strateva *et al*, 2009,)

Cytoplasmic Membrane Pump	Periplasmic Linker	Outer Membrane Channel	Substrate
MexB	MexA	OprM	Quinolones, Macrolides, Tetracyclines, Lincomycin, Chloramphenicol, Novobiocin, β -lactams.
MexD	MexC	OprJ	Quinolones, Macrolides, Tetracyclines, Lincomycin, Chloramphenicol, Novobiocin, Penicillins.
MexF	MexE	OprN	Fluroquinolones, Carbapenems
MexY	MexX	OprM	Quinolones, Macrolides, Tetracyclines, Lincomycin, Chloramphenicol, Aminoglycosides, Penicillins.

In Gram-negative bacteria, most efflux pumps are three-component systems that span the inner and outer membranes and contain a periplasmic linker protein, which allows the other two components to come into contact with each other (Lomovskaya *et al*, 2001) (Figure 1.6).

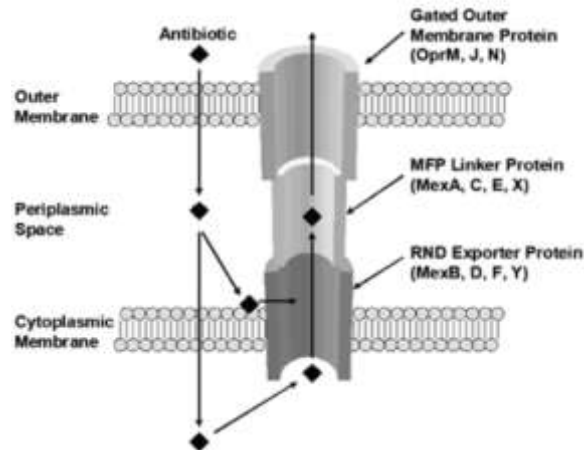


Figure 1.6 Overview of *P. aeruginosa* efflux mediated resistance to tetracycline based antibiotics.

Mechanism of action of efflux pumps against antibiotics. Structure and function of the MexAB-OprM and related efflux pumps of *P. aeruginosa*. Antibiotics can be captured from the periplasmic space, cytoplasmic membrane, and/or cytoplasmic space by MexB, D, F, or Y (RND exporter proteins). MexA, C, E, or X (MFP proteins) serve as conduits between the cytoplasmic and outer membranes. OprM, J, or N serves as the final step in removal of the antibiotic from the cell (Aeschlimann, 2003).

The four multidrug resistant RND efflux pumps of *P. aeruginosa* all confer various degrees of resistance to fluoroquinolones (Lomovskaya *et al*, 2001). Resistance is attributed to a reduced outer membrane permeability of *B. cepacia*, production of modifying enzymes and alteration of antibiotic targets (Gugliera *et al*, 2006). However, information relating to the contribution that drug efflux systems play in the drug resistance of *B. cepacia* is limited.

Only a few multidrug efflux pumps have been described to date in some clinical isolates (Buroni *et al*, 2009). Investigations conducted by Wigfield *et al* (2002) discovered that the immuno-dominant antigens of *B. cepacia* are in part a multidrug efflux pump responsible for resistance to both nalidixic acid and tetracycline (Wigfield *et al* 2002).

Dean *et al* (2003) elucidated that resistance of *P. aeruginosa* to tetracycline antibiotics is a result of specific tetracycline-specific efflux pumps that differ from RND pumps. The investigations demonstrated that PAO1 laboratory strain exhibited reduced susceptibility to the tetracycline derivative tigecycline (Dean *et al*, 2003). Action of these efflux pumps were capable of being blocked using efflux pump inhibitors (EPIs), which are compounds that bind with the specific substrate pocket and inhibits efflux pump activity (Marquez, 2005). MC-207,110 is the first identified broad spectrum EPI that effectively inhibits all clinically relevant efflux pumps in Gram negative bacteria (Pages *et al*, 2005). Specifically, the MexAB-OprM and MexCD-OprJ efflux pumps of *P. aeruginosa* have been shown to be involved in biofilm mechanisms for resistance to the macrolide azithromycin (Gillis *et al*, 2005). Kvist *et al* (2005) demonstrated that *E.coli*, *P. aeruginosa*, *S. aureus* and *Klebsiella pneumonia* strains exposed to the EPIs thioridazine, 1-(1-naphthylmethyl)piperazine and Phe-Arg- β -naphthylamide, used independently or in combination, reduced biofilm biomass by 80% and 99% respectively. Whilst also reporting that these EPIs synergistically increased susceptibility to antibiotic treatments.

1.2.5 Summary

A plethora of Gram-positive and Gram-negative biofilm forming bacteria can cause direct destruction to host tissue and stimulate host cells to activate a wide range of inflammatory responses (Madianos *et al*, 2005). Production of cytokines and chemokines from damage of tissues attract inflammatory cells to the site of infection, which persists despite antibiotic treatments and innate and adaptive responses (Hoiby *et al*, 2010). It is important to understand these processes and what role inflammatory mediators play in the infection process.

1.3 Inflammation and the involvement of inflammatory mediators

1.3.1 Introduction to Inflammation

The innate immune system is the first line of host defence during an infection. It plays a key role in the early recognition and activation of pro-inflammatory responses to invading pathogens (Mogensen, 2009). This ancient system of microbial recognition activates host defences via germ-line encoded receptors that recognise structural components of microorganisms (Dempsey *et al*, 2003). These microbial structures are termed pathogen associated molecular patterns (PAMPs) and/or microbe-associated molecular patterns (MAMPs), which are recognised by the hosts pattern recognition receptors (PRR) (Akira *et al*, 2006). Specifically the family of PRR includes Toll-like receptors (TLRs), bacterial lipopolysaccharide (LPS) acts as a PAMP for TLR4, the bacterial cell constituent peptidoglycan is recognised by TLR3 and bacterial flagellum is recognised by TLR5 (Cigana *et al*, 2009). The TLRs that recognise these molecular patterns during infection instigate the host's inflammatory response, including the production of key inflammatory cytokines and chemokines (Havashi *et al*, 2011). Immune reactions of the innate system involve cytokine-dependent resistance to infection from pathogens. Production of cytokines such as tumour necrosis factor (TNF), interferons (IFNs), interleukins and chemokines combined with major histocompatibility complex (MHC) independent killing are essential to combat invading pathogens (Olszewska, 2005). Inflammatory cytokines, produced by infected damaged tissue, aid in recruitment of cells of the innate immune system, primarily macrophages and neutrophils (Esche *et al*, 2005). Acute inflammation is the initial response of the body to harmful stimuli and is achieved by increased movement of plasma and leukocytes from blood into injured tissues.

Movement of cells is attributed to an influx of inflammatory cytokines which co-ordinate the acute phase of inflammation (Dempsey *et al*, 2003). This involves the local vascular system, the immune system and various cells within the injured tissue. Activated inflammatory cells, including, macrophages and neutrophils produce pro-inflammatory mediators, which stimulate the production of matrix metalloproteinase's (MMPs) (Alpagot *et al*, 2006). Neutrophils release antimicrobial oxidants and proteases and dead neutrophils release DNA, which can directly damage tissue causing an additional increase in pro-inflammatory cytokines (Hassett *et al*, 2010) (Figure 1.7)

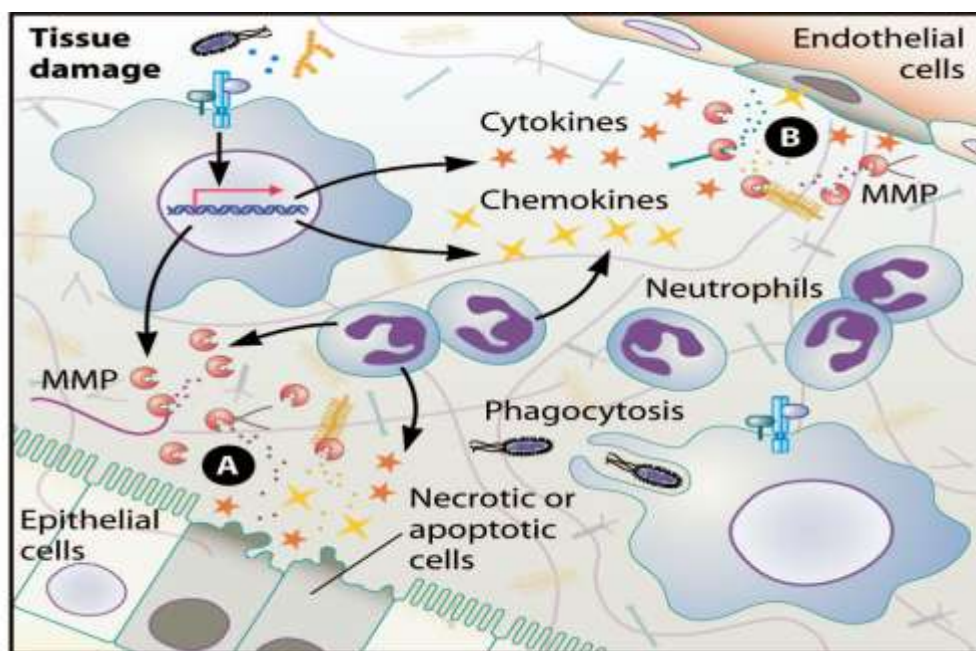


Figure 1.7 An example of inflammatory mediators involved in bacterial tissue damage.

Macrophages and neutrophils are attracted to the site of infection and eliminate the pathogen by releasing a substantial amount of toxic products, such as reactive oxygen species, cytokines and MMPs. Finally, pathogens are phagocytosed and tissue repair is initialised (Vanlaere, *et al*, 2009).

It is widely agreed that resolution of inflammation is an active process rather than a passive decay of pro-inflammatory signals (Van Dyke, 2008). Recent research (Schwab *et al*, 2007) has identified biochemical pathways that are activated during inflammation, thus, pointing to an active biochemical resolution (Bhatavadekar *et al*, 2009). Resolvin E1 (RvE1) and protectin D1 (PD1) are mediators produced during the resolution stages of inflammation and termed resolution agonists (Schwab *et al*, 2007). Schwab *et al* (2007) demonstrated that both compounds at low (nanograms) concentration had the ability to regulate the effects of leukocyte infiltration and furthermore to stimulate macrophage phagocytosis of apoptotic PMNs, therefore concluding that this active process of resolution promotes return of tissue homeostasis (Serhan *et al*, 2007).

1.3.2 Inflammatory mediators

1.3.2.1 Introduction

Inflammation is a well-characterised immune response of innate immunity, which is an integral part of defence to reduce infection within the host. Inflammation can be divided into three phases: an acute phase, immune response and a chronic phase (Salvie *et al*, 2005). These three inflammatory phases are tightly regulated by specific chemokines and cytokines due to the risk of mass tissue damage by reactive oxygen species produced by inflammatory cells. The importance of these inflammatory mediators and inflammatory cells in relation to the chronic inflammatory process will be discussed.

1.3.2.2 Cytokines and chemokines

Cytokines and chemokines are important non-matrix targets of the inflammatory response. Chemokines are a family of chemotactic cytokines, which attract leukocyte populations through interaction with specific receptors expressed on host cells (Garlet *et al*, 2003a). Chemokines produced by airway epithelial cells have an important role in the regulation of key inflammatory processes within the lungs (Krakauer *et al*, 2002). Modulation of chemokine function can occur through several mechanisms, for example up-regulation or inactivation by tissue proteases, including MMPs (Tosi, 2005).

Pro-inflammatory mediators such as IL-8 and IL-6, expressed by respiratory epithelium, recruit and activate inflammatory cells in the lungs. IL-8 acts a chemoattractant factor for PMNs and can be found in gingival crevicular fluid (GCF) in patients with periodontal disease (Garlet *et al*, 2003b). Endothelial cells, fibroblasts and pulmonary epithelial cells are inherently linked with neutrophil mediated influx at sites of infection (Standiford *et al* 1990). PMNs produce a wide range of antimicrobial factors that have the ability in some cases to eradicate bacteria, however, these molecules can inadvertently lead to pulmonary deterioration and epithelial necrosis (Fink *et al*, 2003). Standiford *et al* (1990) demonstrated that LPS stimulated A549 cells posses effector activity associated with the inflammatory process via generation of IL-8. Work conducted by Krakauer *et al* (2002) demonstrated synthesis and expression of IL-6 and IL-8 from the A549 epithelial cell line. Furthermore, the biological activity and efficacy of IL-8 is unregulated by 10-fold when processed MMP-9, therefore resulting in a more potent

chemoattractant (Lint *et al*, 2007). IL-6 is associated with many inflammatory disorders, in particular, within bone biology. IL-6 has been shown to mediate metabolic bone disease promoting osteoclast formation resulting in the degradation of bone structures as seen in patients with periodontal disease (Kirkwood *et al*, 2003, Vernillo *et al*, 1994). A study conducted by Kirkwood *et al* (2003) demonstrated that an analog of the tetracycline derivative doxycycline termed CMT-8 (chemically modified tetracycline-8) decreased IL-6 mRNA stability in a dose dependent manner. This was achieved by inhibiting IL-1 beta-induced IL-6 expression in MC3T3-cell line at the post transcriptional level, which is inherently important in gene regulation (Patil *et al*, 2004). Therefore, indicating a potential role of doxycycline to positively mediate metabolic bone diseases mediated by the cytokine IL-6. Furthermore Cazalis *et al* (2009) also demonstrated the potential therapeutic effects of tetracycline, doxycycline and CMT-3 on the production of pro-inflammatory mediators IL-1 beta, IL-6 and IL-8 in an ex vivo human whole blood assay stimulated with LPS derived from the periodontal pathogen *P. gingivalis*. The observed effects of this study demonstrated that tetracycline, doxycycline and CMT-3 showed positive reductions of the level of all LPS induced cytokines. Therefore, reducing the key pro-inflammatory mediators associated with, in particular, periodontal disease, but encompassing chronic inflammatory disease.

1.3.2.3 Matrix Metalloproteinase's (MMPs)

MMPs are a family of zinc-dependent endopeptidases capable of degrading many extracellular matrix proteins by catalysing the breakdown of proteins on

the cell plasma membrane or within the extracellular matrix (Preshaw *et al*, 2004a). MMPs are involved in the cleavage of cell surface receptors, the release of apoptotic ligands, and chemokine/cytokine activation and inactivation (Lint *et al*, 2007). To demonstrate the clinical relevance of MMPs, MMP mutant mouse strains have been generated. These have identified the importance of MMPs towards cell proliferation, differentiation, adhesion and inflammation (Le *et al*, 2007).

Currently, there are a total of 25 MMPs that have been characterised (Lint *et al*, 2007). The identification of new non-matrix MMP substrates involved in inflammation highlights the diverse role of MMPs. These enzymes can enhance leukocyte invasion and regulate inflammatory activity of serine proteases, cytokines and chemokines. These host-derived enzymes play an important role in degradation of the extracellular matrix (Emingil *et al*, 2006a). Both MMP-2 and MMP-9 have been found to favour anti- or pro-inflammatory action respectively (Le *et al*, 2007).

MMPs are important for creating cellular environments required during development and morphogenesis. Under normal physiological conditions, activities of MMPs are precisely regulated at the transcriptional level (Visse *et al*, 2003). However, MMP host-derived enzymes produced by cells in response to microbial virulence factors, in particular MMP-2 and MMP-9, are known to be induced by bacterial LPS (Albert *et al*, 2003). MMPs are crucial for a normal immune response and are involved in neutrophil influx following LPS exposure. This forms part of the early phase of the host response to

LPS. However, extensive release of these proteinases leads to severe tissue damage (Vanlaere *et al*, 2009).

During periodontal and lung inflammation, MMPs are under the control of several regulatory mechanisms. Up-regulation is controlled by inducers termed extracellular matrix metalloproteinase inducer (EMMPRIN). Conversely, inhibition is governed by inhibitors termed tissue inhibitors of metalloproteinases (TIMPs) (Emingil *et al*, 2006a). These molecules are synthesised and secreted by most connective tissue cells and macrophages (Guignabert *et al*, 2005).

Current evidence in the literature demonstrated by Wong *et al* (2009) identified lung MMP-2, 7, 10, 12, 14, TIMP-1 and 4 to be involved in the pathogenesis of chronic obstructive pulmonary disease (COPD) and asthma (Wong *et al*, 2009). Increased serum expression of MMPs are directly associated with bacterial-induced lung inflammation and attributed to impaired lung function (Roderfeld *et al*, 2009). Expression of MMP-1, 2, 3, 7, 8, 9, 12, 13, and TIMP-1 have been characterised in sputum and bronchoalveolar lavage of patients with CF (Roderfeld *et al*, 2009). This evidence highlights the importance of these mediators in the inflammatory process and progression of diseased states within the host.

There is increasing evidence that fragments of extracellular matrix proteins play an important role in inflammatory cell recruitment to the lung in animal models of airway inflammation (Gaggar *et al*, 2008). Key information to

understand activities of these proteins in different inflammatory airway disease could aid the development of more selective therapeutics for diseases associated with inflammatory processes (Wong *et al*, 2009).

1.3.2.4 Summary

In summary, bacterial biofilms are primarily responsible for inflammation within host tissue. However, it is the inflammatory response to bacterial species that cause most damage in chronic inflammatory disease. A well orchestrated host inflammatory response has the capability to eliminate the microbial challenge. An acute inflammatory response that is resolved in a timely manner prevents tissue destruction. However, failure to return tissue to homeostasis results in neutrophil mediated destruction and chronic inflammation (Van Dyke, 2007). Chronic inflammation leads to a progressive shift in the type of cells present at the site of inflammation, characterised by simultaneous destruction and healing of the tissue from the inflammatory process.

1.4 Tetracycline based antibiotics

1.4.1 Introduction to tetracyclines

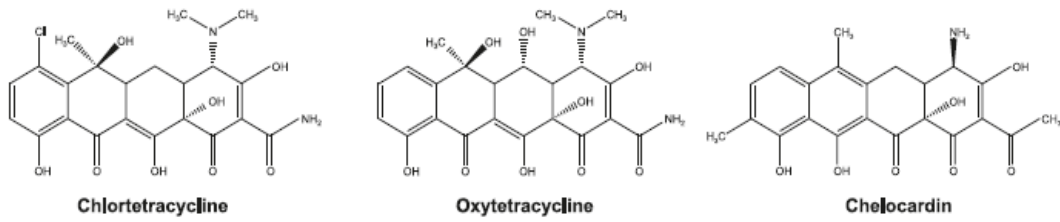
Current research has identified that tetracycline related compounds can be beneficial towards the treatment of several chronic inflammatory diseases, including periodontal disease and cystic fibrosis (Raza *et al*, 2006). The emergence of these benefits has resulted in a wider understanding of the physiological, cellular and molecular mechanisms of the inflammatory

response, and how these responses may be better controlled and managed by antibiotic treatment (Rempe *et al*, 2007). These studies will now be discussed.

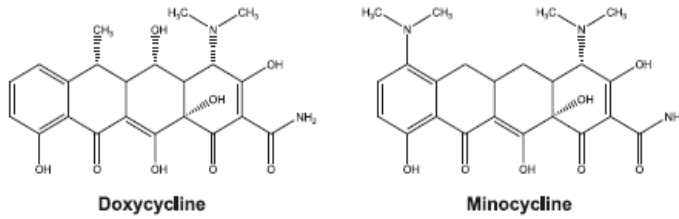
Tetracycline compounds irreversibly bind to the 30S ribosomal subunit through an interaction with 16S rRNA, which prevents docking of aminoacylated tRNA. Tetracyclines were discovered in the 1940s by Benjamin Duggar as a bioactive substance produced by the Gram positive bacterium *Streptomyces* (Zakeri *et al*, 2008) that exhibit activity against a wide range of microorganisms (Garrison *et al*, 2009). Tetracyclines are inexpensive broad-spectrum bacteriostatic antibiotics that have been used to treat a vast array of diseases caused by Gram-positive and Gram-negative organisms (Roberts, 2003).

Recent investigations have demonstrated tetracyclines to be effective in the treatment of a number of inflammatory diseases including periodontitis and a vast array of central nervous system pathologies (Dunston *et al*, 2011). Tetracyclines include tetracycline, chlortetracycline, oxytetracycline, demeclocycline, methacycline, doxycycline, minocycline, and tigecycline (Aminov *et al*, 2004). Figure 1.8 highlights the chemical structures of this diverse group of antibiotics. In addition to their antibacterial activity, tetracyclines have a number of non-antimicrobial effects shared by most tetracycline compounds. These include anti-oxidant, anti-apoptotic, anti-metastatic and anti-inflammatory effects (Dunston *et al*, 2011).

Natural Products



Second Generation



Third Generation

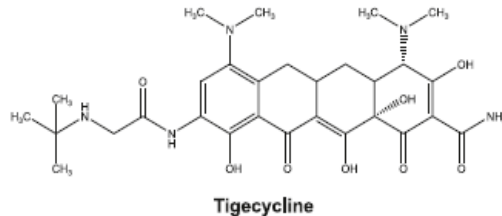


Figure 1.8 Chemical composition of the tetracycline class of antibiotics.

Chemical structures of three different generations of tetracyclines: from the natural products obtained from the bacterium *Streptomyces* to second and third generation semi-synthetic antibiotics of minocycline, doxycycline and tigecycline (Zakeri *et al*, 2008).

1.4.2 Resistance to tetracyclines

The indiscriminate use of this class of drug has resulted in widespread resistance among many clinical isolates of bacteria. As a consequence, there has been a reduction in number of tetracycline-based antibiotics used to treat infections (Aminov *et al*, 2004). Resistance to tetracycline based antibiotics is attributed to tetracycline resistant bacteria that carry 1 of the 36 different

required tetracycline resistant genes (*tet* genes), that are often associated with movable elements such as plasmids, transposons, conjugative transposons, and integrons (Roberts, 2003). Thus, resistance to tetracycline antibiotics can, therefore, be transferred to human pathogens and opportunistic bacteria during infection via horizontal gene transfer.

Lactobacillus sakei resistance to tetracyclines is attributed to two functional genes. Ammor *et al* (2008) demonstrated that tetracycline resistance is conferred via a transposon associated *tet(M)* gene, which codes for a ribosomal protection protein and a plasmid carrying *tet(L)* gene coding for a tetracycline efflux pump. Their investigations highlighted that *tet* genes increased the MIC of tetracycline on from <32 mg/L to >256 mg/L (Ammor *et al* 2008). Plasmids are emerging as important elements for resistance. Blanco *et al* (2005) described three separated *tet* genes located on small plasmids in *Actinobacillus pleuropneumoniae* that conferred resistance to tetracyclines, a *tet(B)* gene was also evident to confer resistance to tetracycline, doxycycline and minocycline (Blanco *et al*, 2005).

Furthermore, it has also been highlighted that mutations to bacterial efflux pumps can result in resistance to tetracyclines in *Neisseria gonorrhoeae*, *Helicobacter pylori* and *Mycobacterium avium* (Roberts, 2003). Work conducted by Zarantonelli *et al* (1999) demonstrated that a 4-fold increase in resistance to tetracycline resulting from of a one base pair deletion in the *mtrR* gene which codes for the bacteria *mtrCDE* efflux pump (Zarantonellie *et al*,

2005). To overcome these common resistance mechanisms, improvements of the classic chemical composition were designed and will be discussed.

1.4.3 Second generation tetracyclines

1.4.3.1 Doxycycline

A derivative of tetracycline is the semi-synthetic drug doxycycline. Doxycycline was invented and clinically developed in the early 1960s by Pfizer Inc. and is marketed under the brand name Vibramycin (Chopra *et al*, 2001). Doxycycline exhibits broad-spectrum antibacterial activity that interferes with prokaryotic protein synthesis at the ribosome level (Rempe *et al*, 2007). Doxycycline is commonly prescribed at 100 mg – 200 mg/day demonstrating bacteriostatic effects for systemic infections (Golub *et al* 2001). Multiple infectious diseases can be treated with doxycycline including brucellosis, pasteurellosis, borreliosis, rickettsioses, trepanomatosis, cholera, leptospirosis, Q fever, pulmonary and urinary infections associated with chlamydia and mycoplasma, gonococchia, and anthrax (Aupee *et al*, 2009).

LaPlante *et al* (2008) demonstrated the pharmacodynamic abilities of doxycycline in an *in vitro* murine model infected with methicillin resistant *S. aureus* (MRSA). Three strains of MRSA were evaluated that displayed inducible macrolide lincosamide-streptogramin type B resistance (W2, R2507, R2529). All three strains were susceptible to doxycycline with MICs of 0.25 / 4 and 4 mg/L respectively, and also reduced biofilm biomass of respective strains over a 72 h time course (LaPlanet *et al*, 2008). Furthermore, El-Azizi (2007) demonstrated that combination therapy of doxycycline with

amphotericin B improved killing activity *in vitro* against *Candida parapsilosis*, *C. krusei* and *C. glabrata* biofilms by 18.94%, 24.52% and 29.15%, respectively.

However, bacterial resistance to antibiotics is common. The *in vitro* activity of minocycline and doxycycline were compared by Canton *et al* (2003) against epidemiologically defined CF *S. maltophilia* isolates. These studies highlighted that doxycycline was less active against these strains than minocycline with an MIC₉₀ of 8 mg/L compared to minocyclines MIC₉₀ of 1 mg/L (Canton *et al*, 2003). This resistance is due in part to the overuse of doxycycline to treat bacterial infections associated with CF.

Doxycycline exhibits a variety of additional and less well-known properties when used at sub-antimicrobial doses doxycycline (SDD). Among these are separate and distinct anti-inflammatory properties that can be used to treat a range of inflammatory diseases associated with an influx of monocytes (Raza *et al*, 2006). The effects of doxycycline at doses below clinical prescription concentrations will be discussed to evaluate what role they may play in inflammatory disease caused by pathogenic bacteria.

1.4.3.2 Current *in vitro* research of subantimicrobial doses of doxycycline

1.4.3.2.1 Introduction

Golub *et al* (1983) initially reported the secondary beneficial properties of the tetracycline compound, minocycline, independent of its antimicrobial

properties (Golub *et al*, 1983). These initial findings were followed up, where it was demonstrated that a reduction in GCF collagenase activity was directly related to the sub-antimicrobial dose of oral administered minocycline given at 40 mg, significantly below the 200 mg dose commonly prescribed (Golub *et al*, 1985). Golub termed this new emerging concept of managing periodontitis as host modulation therapy. Current literature describes the use of SDD (20 mg Periostat®) in the treatment of inflammatory disorders associated with periodontal diseases (Choi *et al*, 2004; Golub *et al*, 2001; Emingil *et al*, 2004; Preshaw *et al*, 2004a; Thomas *et al*, 1998). These current works, although not exhaustive, will be discussed to highlight the significant importance of these secondary benefits.

1.4.3.2.2 Current research

Caton *et al* (2001) demonstrated that an oral dose of SDD given twice daily in combination with scaling and root planning (SRP) over a 6 month period resulted in clinical improvement of chronic periodontitis patients, and did not result in antimicrobial resistance to the compound. Work conducted in 2001 by Golub *et al* identified that patients receiving SDD twice daily for 12 weeks had a significant reduction in the destructive actions of collagenases commonly found in GCF of patients with chronic periodontitis. These investigations also demonstrated that the oral pathogens *Actinomyces* and *Fusobacterium* spp detected in subgingival plaque samples did not develop resistance to doxycycline and all isolates remained susceptible to 3 mg/L dose of doxycycline (Golub *et al* 2001).

Emingil *et al* (2004) demonstrated that SDD combined with periodontal SRP can reduce MMP-8 levels to 50% lower than placebo group patients suffering from periodontal disease and only receiving SRP over a 6 month period. Similar findings were also demonstrated by Choi *et al* (2004), identifying that SDD in conjunction with a SRP program led to reduction in MMP-8, MMP-9 and IL-6 synergistically over 120 days when compared to a placebo control group. This resulted in lower levels of inflammation at sites of previous disease (Choi *et al*, 2004). Preshaw *et al* (2004b) also demonstrated that SDD was of clinical benefit in the treatment of chronic periodontitis by enhancing the efficacy of SRP. The overall outcome of these and associated findings has resulted in the SDD at 20mg (Periostat®) being approved by the United States Food and Drug Administration (FDA) as an effective host modulatory drug for the treatment of periodontitis (Caton and Ryan 2011).

Raza *et al* (2005) investigated the use of SDD and demonstrated a significant reduction in the key inflammatory cytokine, monocyte chemoattractant protein-1 (MCP-1) in human lung epithelial cells. Thus, indicating a potential role for SDD as a new anti-inflammatory therapeutic option for individuals with chronic lung disease. Gu *et al* (2009) demonstrated doxycycline concentrations of 0.1, 1 and 10µM reduced inflammatory mediators associated with chronic inflammation. The group reported that doxycycline concentrations of 0.1, 1 and 10µM reduced tumour necrosis factor- α (TNF- α) and MMP-9 levels by 46%, 52% and 71%, and 18%, 20% and 41%, respectively. Moreover, Guignabert *et al* (2005), showed that pre-treatment with doxycycline suppressed production of MMP-9 by infiltrating macrophages, and also

partially inhibited recruitment of inflammatory cells at the site of tissue damage, thereby almost normalising tissue histology of inflamed trachea. These findings were echoed by Lee *et al* (2004), who established that SDD was effective at reducing airway inflammation, airway hyper-responsiveness, and expression of MMP-9 mRNA and protein in a murine model of asthma.

1.4.3.2.3 Summary

In addition to its potent antibacterial effects, doxycycline possesses significant immunomodulatory properties that has important effects upon inflammatory mediators associated with oral and pulmonary inflammation. However, bacterial resistance to tetracyclines is well established. Therefore, developments of the tetracycline antibiotics have led to new classes that are capable of overcoming bacterial resistance mechanisms. This new class will now be discussed.

1.4.4 Third generation tetracyclines

1.4.4.1 Glycylcycline

The use of tetracyclines has been limited in recent years because of the emergence of resistant microorganisms that are capable of efflux and ribosomal protection mechanisms (Zhanel, *et al* 2004). Such microorganisms include *P. aeruginosa*, *B. cepacia*, *E.coli* and *Salmonella enterica serovar typhimurium* (Chan *et al*, 2007; Hasdemir, 2007; Feliziani *et al*, 2010; Wigfield *et al*, 2002). To overcome this growing concern, there has been an expansion of antibiotic derivatives by synthetic improvements producing analogues that overcome current resistance mechanisms (Magalhaes da silva *et al*, 2010).

Such developments led to the discovery in 1993 of the third generation of tetracycline antibiotics called glycylicyclines (Pankey, 2005). Glycylicyclines are currently the only derivative that exhibit broad antibacterial activity comparable to early tetracyclines. They demonstrate potent activity against both Gram-positive and -negative bacteria, including strains that carry efflux and ribosome protection (Sum *et al*, 1998).

1.4.4.2 Tigecycline

The most developed glycylicycline to date is 9-tert-butyl-glycylamido derivative tigecycline (Zhanel, *et al* 2004). Tigecycline (Tygacil®) is a glycylicycline containing semi-synthetic derivatives of minocycline, exhibiting an expanded broad spectrum of *in vitro* activity (Peterson 2008a). Furthermore, it is an effective treatment for community-acquired pneumonia, intra-abdominal, skin and skin structure infections (Rubino *et al*, 2010). Glycylicyclines were specifically designed to overcome tetracycline resistance mediated by ribosomal protection proteins and tetracycline-specific efflux pumps (Olson *et al*, 2006). This is achieved by tigecycline, which binds reversibly to a helical region (H34) on the 30S subunit of the bacterial ribosome, thereby blocking entry of aminoacyl transfer RNA into the A binding site of the ribosome (Figure 1.9) (Doan *et al*, 2006).

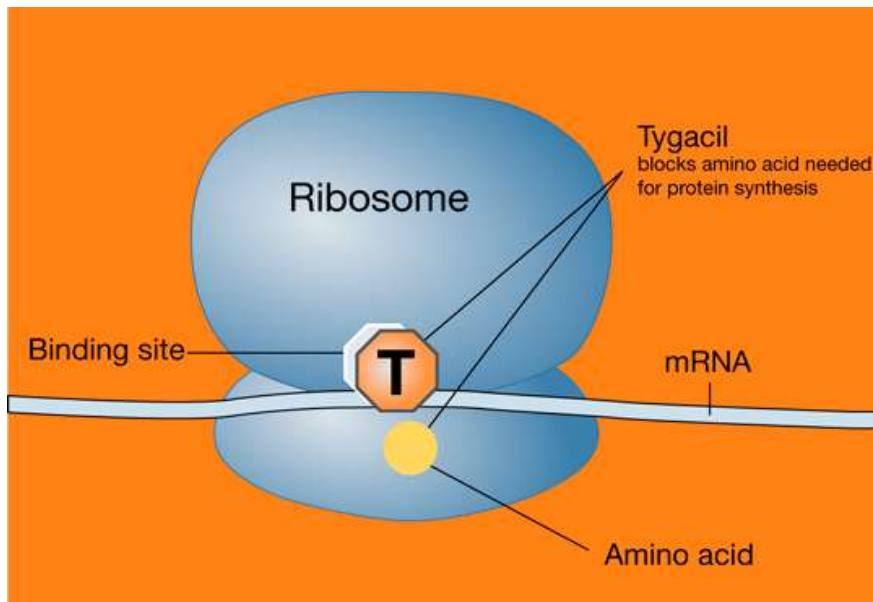


Figure 1.9 Action of tigecycline within the bacterial ribosome. Schematically depicts the interaction of a tigecycline (Tygacil) molecule interrupting the growing chain of amino acids required for bacterial cell function (www.tygacil.co.uk)

Subsequently, this process inhibits protein synthesis by preventing incorporation of amino acid residues into the elongated peptide chain. This action is the result of the addition of a modified glycyllamido moiety to the ninth position of the D ring (Figure 1.10) aiding in both efflux and ribosomal resistance (Peterson, 2008). Work conducted by Olson *et al* (2006) demonstrated inhibition of protein synthesis with an *in vitro* translation assay. This determined the nature of the interaction of tigecycline with the A site of the 16S rRNA of the ribosome. These experiments demonstrated that tigecycline inhibits protein synthesis in an *in vitro* assay system, binding to the ribosome 5-fold and >100-fold greater affinity than minocycline and tetracycline, respectively (Olson *et al*, 2006).

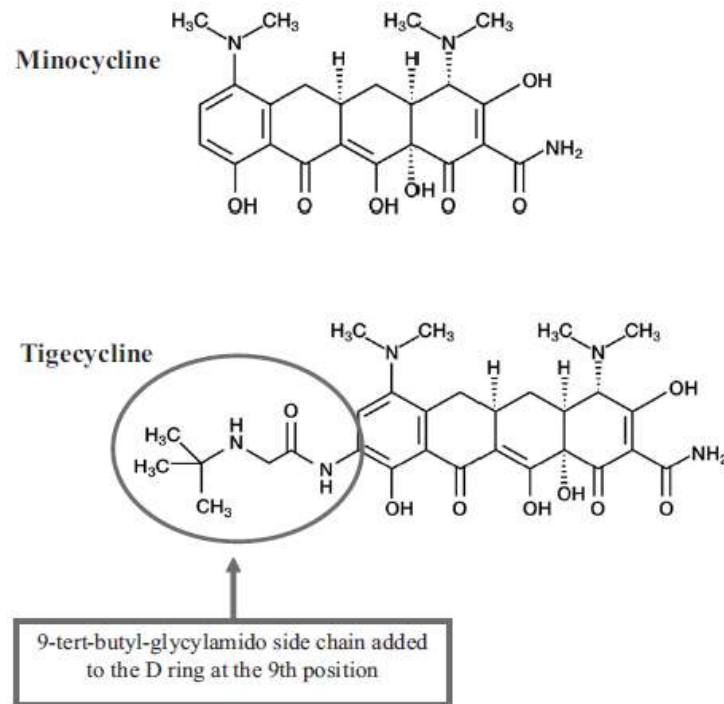


Figure 1.10 Comparison between the chemical composition of minocycline and tigecycline. The chemical structure of minocycline and its derivative tigecycline. The modification of minocycline is clearly seen by the addition of the bulky 9-tert-butyl-glycylamido side chain at position 9 on the D ring. This addition provides tigecycline with expanded microbiological and therapeutic benefits (Peterson, 2008).

The therapeutic benefits of tigecycline have been demonstrated *in vivo* by Murphy *et al* (2000) where cardiac titers of vancomycin-susceptible *Enterococcus faecalis* were reduced in a rat model of experimental endocarditis. Tigecycline is also known to be more active *in vitro* against MRSA and vancomycin-susceptible and resistant *E. faecalis* (Murphy *et al*, 2000). The wide ranging effects of tigecycline also include potent activity against methicillin susceptible *S. aureus* (MSSA), methicillin susceptible and resistant *S. epidermidis*, penicillin susceptible and resistant *Streptococcus pneumoniae* (Petersen *et al*, 2002; Pankey, 2005; Vouillamoz *et al*, 2008).

These findings were mirrored in part by Petersen *et al* (2002), demonstrating that tigecycline was three times more effective than vancomycin in an *in vivo* model of MRSA infection.

In relation to bacterial biofilm forming pathogens commonly associated with pulmonary diseases, tigecycline offers improved activity to many current CF therapeutic agents. *Burkholderia* spp display varying MIC values to tigecycline owing to the action of drug efflux systems, MIC₅₀ and MIC₉₀ values of 0.5 - 4 and 4 - 32 mg/L (Livermore *et al* 2008). These MIC values are lower than those compared to minocycline. Tigecycline appears to be more efficacious against *S. maltophilia* with MIC₅₀ and MIC₉₀ of 0.5 - 1 and 2 mg/L respectively and an MIC range of 0.06 - 4 mg/L (Livermore *et al* 2008). *P. aeruginosa* exhibits high levels of resistance to tigecycline with an MIC₅₀ and MIC₉₀ of 8 and ≥32, respectively, which is lower than minocycline MIC₅₀ and MIC₉₀ of ≥32 (Garrison *et al*, 2009). Tigecycline has potent activity against Gram-positive organisms, demonstrating an MIC₅₀ and MIC₉₀ for MSSA of 0.12 and 0.25 mg/L, MRSA isolates have a slightly higher MIC₅₀ and MIC₉₀ of 0.12 and 0.5 mg/L (Norskov-Lauritsen *et al*, 2009).

Smith *et al* (2009a) demonstrated the bacteriostatic activity of tigecycline against MRSA biofilm isolates recording a MIC of 0.06 mg/L, however, 45% of biofilm associated bacterial cells survived after treatment with tigecycline. Earlier findings by the group demonstrated that a subinhibitory dose of tigecycline (0.24 mg/L) significantly altered expression (18.6%) of biofilm forming MRSA genome Smith *et al* (2008). A plethora of genes encoding

ribosomal proteins and virulence factors were upregulated. However, genes encoding biofilm-associated proteins, capsule synthesis enzymes and genes encoding toxic shock syndrome toxin 1 were significantly reduced (Smith *et al* 2009b). It was concluded that at low doses tigecycline may promote advantageous benefits to modulate virulence factors associated with *S. aureus*. These data are of significant importance as biofilm structures are difficult to eradicate and can display susceptibilities towards antimicrobials 10 – 1000 times less than planktonic cells (Smith *et al* 2008).

Furthermore, minocycline and doxycycline exert biological effects independent of their antimicrobial activity, which include inhibition of pro-inflammatory mediators and prevention of pathological tissue destruction (Amin *et al*, 1996). Doxycycline and tigecycline share commonality of their therapeutic uses and both are inhibitors of MMPs (Skulason *et al*, 2009). In a murine model, Salvatore *et al* (2009) demonstrated that six days of tigecycline treatment significantly reduced lung inflammation, pulmonary pro-inflammatory cytokines and chemokines. It was reported that tigecycline at 0.2 mg in 0.2 mL per mouse on the first day of treatment followed by 0.1 mg in 0.2 mL per mouse for a further 5 days reduced bronchoalveolar lavage *Mycoplasma pneumonia* cultures from 6 Log₁₀ CFU/mL to approximately 4.5 Log₁₀ CFU/mL. This was coupled with a significant reduction of Interleukin-1 β (IL-1 β), Interleukin-12 (IL-12) and Interferon gamma (IFN- γ) (Salvatore *et al*, 2009). The *in vitro* modulatory effect of tigecycline was also investigated by Saliba *et al* (2009), here tigecycline exhibited anti-inflammatory properties independent of its antimicrobial effects. Tigecycline at subinhibitory concentrations demonstrated

a reduction of TNF- α , IL-1 β and IL-6 derived from staphylococcal enterotoxin B (SEB) and staphylococcal toxic shock syndrome toxin-1 stimulated human peripheral blood mononuclear cells (Saliba *et al*, 2009). These findings provide evidence that subinhibitory concentrations of tigecycline can modulate the inflammatory process through its inhibitory effects on pro-inflammatory cytokines.

Of interest, these properties of immunomodulation are evident in other antibiotic compounds. It is well known that subinhibitory concentrations of antibiotics may be beneficial for bacterial communities, by facilitating increased expression of virulence genes and enhancing biofilm formation (Davies *et al*, 2006). These advantageous properties to bacterial biofilm formation were demonstrated by Hoffman *et al* (2005). They demonstrated that a subinhibitory concentration of aminoglycoside antibiotic induced biofilm formation by *P. aeruginosa* and *E. coli* thereby conferring biofilm-specific aminoglycoside resistance. Nevertheless, subinhibitory concentrations are also capable of decreasing virulence factors of bacterial communities and immunomodulate inflammatory responses (Ryan *et al*, 2008). Indeed, the semi-synthetic macrolide azithromycin at subinhibitory concentrations decreases biofilm formation of highly resistant clinical nontypeable *Haemophilus influenzae* (NTHi) isolates commonly associated with early airway infections in CF (Starner *et al*, 2008).

1.4.4.3 Summary

Current antibiotics produced by a range of microorganisms, in particular the actinomycetes and fungi, are now fast becoming outdated and less effective (Pelaez, 2006). To overcome this problem, new antibiotic classes have been developed by chemical adaptations of original natural products. Research and development of antibiotics has led to the production of semi-synthetic compounds and truly synthetic antibiotics. These classes, specifically the tetracyclines, have demonstrated in some instances secondary beneficial characteristics. Such benefits encompass inhibition of extracellular matrix degradation, reduced inflammatory pulmonary cytokines and chemokines associated with chronic inflammatory lung disease and advantageously modulating pro-inflammatory cytokines and MMPs associated with chronic periodontitis (Gu, *et al* 2009, Salvatore *et al*, 2009 and Choi *et al*, 2004). It is apparent that tetracyclines, such as tigecycline, may play a dual role both in terms of their antibacterial activity and in relation to modulating deleterious immune responses.

1.5 General overview

Bacterial pathogens that utilise multidrug resistant efflux pumps, as part of antibiotic resistance, are of key importance to bacterial disease progression and will be investigated. These investigations will examine the role of efflux pumps in planktonic and sessile bacterial cells and incorporate a competitive efflux pump inhibitor to determine susceptibility to tigecycline. The results of these investigations may provide insight to present new opportunities to effectively treat bacterial related infections.

Currently, there is limited information within the literature surrounding the beneficial effects of tigecycline on inflammatory mediators. In particular, the effects of tigecycline upon IL-6, IL-8 and specific MMPs produced during chronic inflammation by immune cells and host tissue is not well established. It is of current interest to investigate if subinhibitory concentrations of tigecycline could beneficially modulate the production of IL-6, IL-8 and MMPs derived from clinically relevant human cell lines. Thus, determining if tigecycline could mirror current *in vitro* research associated with SDD.

1.6 Aims of the study

The aim of this study was two-fold: Initially investigate the efficacy of tigecycline against important Gram-negative CF and periodontal pathogens, and to examine if clinically relevant cell lines exposed to tigecycline possess any immunomodulatory properties.

The study will therefore investigate the following:

1. Microbiological capacity of tigecycline

To examine the effects of tigecycline on inhibition and killing of planktonic and sessile bacteria associated with CF and periodontitis. Examine the role of efflux pumps associated with antibiotic resistance using tigecycline. Incorporate efflux pump inhibitors to determine if efflux pumps are employed during infection.

1a) Anti-biofilm activity

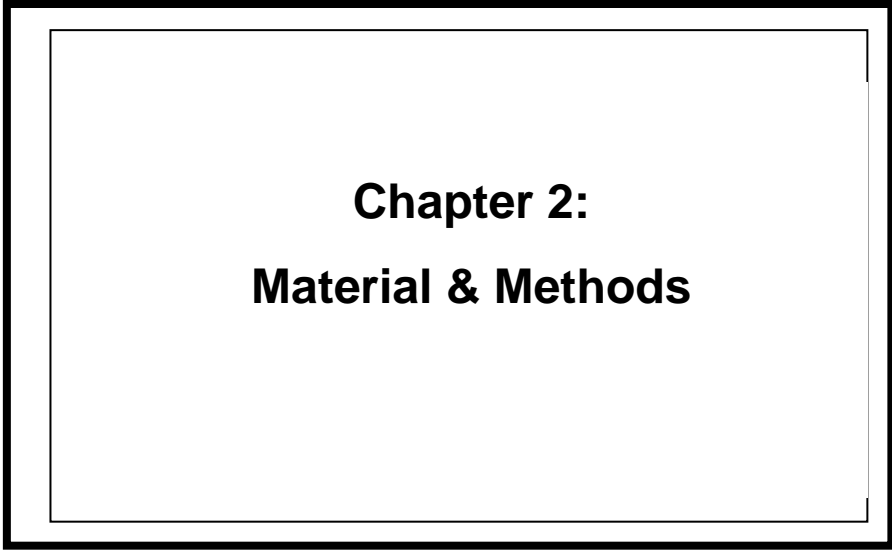
1b) Role of efflux pumps in CF and periodontal pathogens

2. Immunomodulatory capacity of tigecycline

Examine the immunomodulatory capacity of subantimicrobial concentrations of tigecycline using clinically relevant cell lines by:

2a) The Investigation of the effects of subantimicrobial doses of tigecycline on IL-6 and IL-8 production from pulmonary derived A549 and oral derived OKF6/TERT2 cell lines.

2b) The Investigation of the effects of these concentrations on neutrophil derived IL-8 and MMP-9 release.



**Chapter 2:
Material & Methods**

2 Material and methods

2.1 Antimicrobial properties of tigecycline

2.1.1 Preparation of tigecycline and storage

Tigecycline white powder was kindly gifted by Wyeth Pharmaceuticals (Princeton, New Jersey, USA) and used in all antimicrobial and immunological experiments. Tigecycline was prepared by adding 10 mg of powder into 10mL of sterile doubled distilled water (ddH₂O [Oxoid, UK]) and sterilized using a 0.22µL filter (Millipore, UK). This 1 mg/mL solution was stored at -80°C, and a new stock solution prepared on a weekly basis. Tigecycline was used at varying concentrations dependent upon each experimental parameter.

2.1.2 Bacterial strains and maintenance

The bacterial strains used throughout these experiments were comprised of both oral and pulmonary pathogens. The *Burkholderia* strains were isolated from children with cystic fibrosis at the Royal Hospital for Sick Children (Yorkhill Division), Glasgow, UK (courtesy of Dr Craig Williams) and from Foothills Hospital, Calgary, Canada (courtesy of Dr Douglas Storey) (Table 2.1). The oral bacteria used in these experiments are all associated with chronic inflammatory diseases of the periodontium (*Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans*) were obtained from culture collections within Glasgow Dental Hospital, Glasgow UK (courtesy of Prof Andrew Smith) (Table 2.2). All strains were stored routinely in Microbead tubes (Prolab diagnostics, UK) at -80°C until required.

Pulmonary clinical isolates were maintained and incubated at 37°C (Laboratory Thermal Equipment, UK) in either Luria broth (LB) (Sigma, Aldrich, UK) or LB agar. Cation supplemented Mueller Hinton Broth (MHB) (Fluka, UK) was used throughout all susceptibility testing assays. For the oral clinical isolates, *A. actinomycetemcomitans* were maintained and grown in 5% CO₂ (Binder, USA) at 37°C in tryptic soy broth (TSB) (Oxoid, UK) supplemented with 0.8% glucose (Fisher Scientific, UK) and 0.6% yeast extract (Oxoid, UK). TSB was used throughout all susceptibility testing assays. *P. gingivalis* and *F. nucleatum* spp were maintained and grown in an anaerobic chamber (85% N₂, 10% CO₂, 5% H₂, Don Whitley Scientific, MK3 anaerobic work station, Bradford, UK) on blood agar at 37°C. Blood agar was prepared on site using Columbia media (Oxoid, UK) supplemented with 5% v/v lysed horse blood (E&O Laboratories Limited, Scotland) and 1.5% w/v bacteriological agar (Bioconnections, UK). Schadlers anaerobic media (SAM) (Oxoid, UK) was used throughout all susceptibility testing assays.

Table 2.1 Pulmonary strains. *Burkholderia* spp used in the following experiments comprised 17 strains of *Burkholderia cepacia complex*: 9 strains from Yorkhill (YK), 6 from Calgary (CG) and two type strains (K56-2 and American Type Culture Collection ATCC 117765). Three strains of *Burkholderia gladioli* were obtained from Yorkhill.

<i>Burkholderia cepacia complex</i> (genomovar)	<i>Burkholderia gladioli</i>
YH06.79061A (II)	YH07.14759A
YH06.70610 (II)	YH08.56940A
YH08.29412A(II)	YH09.6793
YH07.24721A (II)	
K 56-2 (III)	
CG13862-1 (III)	
ATCC 117765 (III)	
YH07.37324A (III)	
YH08.32475A (III)	
CG16192 (III)	
CGJ415 (III)	
CGJ2315 (III)	
CG1394 (III)	
CG6433 (III)	
YH05.56937A (IV)	
YH07.24721B (V)	
YH07.59399B (V)	

Table 2.2 Oral strains. The oral isolates used in the following experiments comprised of 5 strains of *P. gingivalis*, including 1 national collection of type cultures (NCTC) strain; 4 *F. nucleatum*, including 1 NCTC strain; and 3 strains *A. actinomycetemcomitans*, including 2 NCTC strains. All strains were obtained from Glasgow Dental Hospital culture collection.

<i>Porphyromonas gingivalis</i>
W50
2111
2114
2098
11834 (NCTC)
<i>Fusobacterium nucleatum</i>
494
10562 (NCTC)
01/105 (1)
01/105 (3)
<i>Aggregatibacter actinomycetemcomitans</i>
10981 (NCTC)
10982 (NCTC)
9707

2.1.3 Sensitivity testing of tigecycline

2.1.3.1 Standardisation of bacterial inoculum

Pulmonary isolates and controls (K-56-2 and ATCC 117765) were grown in 10mL of LB media in an orbital shaker (IKA®, KS4000i control, Germany) at 37°C overnight. Cells were then centrifuged, washed with sterile phosphate buffered saline (PBS) and resuspended in 10 mL sterile PBS. Oral isolates and controls (11835 NCTC, 10562 NCTC and 10981 NCTC) were grown on either blood agar plates (*A. actinomycetemcomitans*) or on fastidious anaerobic agar (Oxoid, UK) (*P. gingivalis* and *F. nucleatum*) for 48 h in anaerobic incubator at 37°C and in 5% CO₂, respectively. A loopful of each strain was placed in 10mL of respective media and placed back in the anaerobic chamber at 5% CO₂ at 37°C for a further 48 h. Cells were standardised to an optical density at 550nm (OD_{550nm}) of 0.20 (equivalent to 1 x 10⁸ CFU/mL) using a spectrophotometer (Fisher Scientific, Colorimeter model 45, USA). Cells were subsequently diluted 1:100 in 10mL of respective media to provide approximately 1 x 10⁶ cells per mL, which was then used for sensitivity testing.

2.1.3.2 Efflux Pump Inhibitor preparation and maintenance

EPI L-Phe-L-Arg-β-naphthylamide (MC-207,110) (Sigma, UK) was used for all susceptibility testing at a concentration of 64 mg/L in each experiment. EPI was prepared by adding 10 mg of MC-207,110 powder to 10mL of sterile ddH₂O, which was subsequently sterilised through a 0.22μL filter.

2.1.3.3 Sensitivity testing of oral and pulmonary planktonic bacteria to tigecycline ± EPI

Cells were standardised as described in Section 2.1.3.1. The MIC of tigecycline was determined by standard Clinical and Laboratory Standard Institute (CLSI) broth microdilution methodology in 96-well round bottom plates (Costar, UK). This technique was employed to highlight the effect of different concentrations of MC-207,110 on the MIC of tigecycline. Tigecycline was tested at a range from 0 to 128 mg/L alone and in combination with MC-207,110. *Burkholderia* isolates were exposed to serial doubling dilutions of tigecycline ± MC-207,110 at 64 mg/L sealed with parafilm (Pechiney, plastic packaging, USA) and incubated for 24 h at 37°C. *P. gingivalis* and *F. nucleatum* spp was incubated for 48 h in an anaerobic chamber at 37°C. The methodology was identical for the *A. actinomycetemcomitans* spp, however plates were placed in an aerobic incubator with 5% CO₂ at 37°C for 48 h. The MICs were determined based upon inhibition of growth when visualised by looking at the bottom of the plate with the naked eye.

2.1.3.4 Sensitivity testing of oral and pulmonary sessile bacteria to tigecycline ± EPI

Burkholderia spp isolates were prepared and standardised as described in Section 2.1.3.1. Biofilm formation was based on methodology adapted from Ceri, *et al* (1999). Two hundred microlitres of standardised bacterial cells was added to wells of a 96-well NUC peg plate (NUC, Denmark) with column 12 serving as the negative control with MHB media only. Inoculated plates were

sealed with parafilm and placed in an incubator on a rocking platform (Grant bio, UK) at 37°C for 72 h to allow culture media to wash over the pegs and form a mature biofilm. After 72 h, adhered biofilms were visible on pegs inserted into the culture media.

P. gingivalis, *F. nucleatum* and *A. actinomycetemcomitans* isolates were prepared and standardised as described in Section 2.1.3.1. Two hundred microlitres of standardised bacterial cells were added to each well of a 96 well flat bottom plate (Costar) with column 12 serving as the negative control containing TSB media only for *A. actinomycetemcomitans* and Schadlers media only for *P. gingivalis* and *F. nucleatum*. Inoculated plates were sealed with parafilm and placed in an anaerobic chamber at 37°C for 48 h to allow a mature biofilm to form on the bottom of the plate. After 48 h, adhered biofilms were visible on the base of each well and washed gently with sterile PBS before each experiment. The methodology was identical for the *A. actinomycetemcomitans* spp, however they were grown in an aerobic incubator with 5% CO₂ at 37°C for 48 h.

Susceptibility of *Burkholderia* spp to tigecycline ± EPI was assayed using a 96 peg plate and a 96-well flat bottom plate (NUC, UK). *B. cepacia* species (spp) was exposed to serial doubling dilutions of tigecycline ± MC-207,110 at 64 mg/L sealed with parafilm and incubated at 37°C atmosphere for 24 h. After inoculation, plates were incubated at 37°C atmosphere for 24 h. The MICs were determined based upon inhibition of growth when visualised by looking at the relative biomass on each peg. Subsequent biomass and viability

assays were conducted as described in section 2.1.5 and 2.1.6. XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carbox-anilide [Sigma, UK]) was prepared in media associated with each specific bacterial strain.

2.1.4 A measurement of efflux pump activity of pulmonary bacteria using alanine β -naphthylamide (Ala-Nap) a fluorescent assay.

An Ala-Nap fluorescent assay was performed to assess efflux pump activity, as previously described by Lomovskaya *et al* (2001). Ala-Nap is enzymatically cleaved inside the cells to produce highly fluorescent β -naphthylamine, therefore, the higher the fluorescence the lower the efflux pump activity, as described by Rajendran *et al* (2011). YHBG3, YHBG12 and YHBCC4 isolates were washed and standardised as described in section 2.1.3.1 to 5×10^5 cells in buffer solution (K_2HPO_4 [50mM], $MgSO_4$ [1mM], and glucose [0.4%]) at pH 7.0, and dispensed into black flat-bottomed microtitre plate (Costar 3603, Corning, NY). The reaction was initiated by the addition of Ala-Nap at a final concentration of 128 mg/L. Fluorescence was quantified at 30s intervals for 1 h at 37°C using a fluorescent plate reader (FLUOstar OPTIMA, BMG Labtech, Aylesbury, Buckinghamshire, UK) with an excitation wavelength of 320nm and emission of 460nm.

2.1.5 Oral and pulmonary bacteria viability assay

XTT (Sigma) was prepared in 50mL freshly prepared bacterial media to produce a saturated 50 mg/L solution of XTT. The XTT solution was filtered through a 0.22 μ m filter, before using XTT for the reduction assay. Prior to each assay, menadione (10 mM in acetone) was added to a final

concentration of 1 μ M. One hundred microlitres of XTT/menadione solution was added to each biofilm in a 96-well microtitre plate, and to the control wells to measure background XTT-reduction levels. One hundred microlitres of XTT was added to the pre-washed biofilms, for both treated and control samples and incubated at 37°C for 3 h. Eighty microlitres of supernatant was transferred by pipetting from the XTT treated 96-well microtitre plate to a fresh 96-well microtitre plate to measure colour change in a plate reader (FLUOstar, Omega) at 490 nm.

2.1.6 *Burkholderia* spp biomass assay

Biomass assays were performed to calculate the density of *Burkholderia* spp biofilms following treatment with tigecycline \pm EPI. Remaining supernatant in wells were discarded after performing the XTT assay and washed with PBS until the biofilm became clear. Biofilm was air dried in an incubator at 37°C for 1 h. One hundred microlitres of 0.5% w/v crystal violet solution was added to each well and left at room temperature (RT) for 5 min. The crystal violet dye solution was then discarded by pipetting and residual dye washed off under gentle running tap water. One hundred microlitres of 100% ethanol was added to each well. Ethanol was mixed in each well by pipetting with the remaining crystal violet dye still adherent to the biofilm. Eighty microlitres of the ethanol from each well was transferred to a clean plate to record the biomass reading. This colour change was measured in a plate reader (FLUOstar, Omega) at 570 nm.

2.2 Immunomodulatory effects of tigecycline

2.2.1 Tigecycline purity assay

Bone marrow derived dendritic cells were generated from C57BL/6 mice and maintained in GI 1640 media (Sigma, UK) supplemented with 1% pen strep (Gibco, UK), 10% foetal calf serum (Gibco, UK) and 1% L-glutamine (Gibco, UK). Tigecycline was prepared as described in section 2.1.2 and standardised to 0.1, 0.4 and 1.6 mg/L in GI supplemented media.

The cell concentration was standardised to 1×10^5 cells/mL in 20mL of supplemented GI. One microlitre of cell media was added to each well of a 6 well cell culture plate (Costar, Corning Incorporated, NY, USA). Wells 1 to 3 were inoculated with 0.1, 0.4 and 1.6 mg/L of tigecycline, well 4 with 50 mg/L of *E.coli* derived LPS and well 5 was used as control of supplemented GI media only. Inoculated cells were incubated in a humidified atmosphere for 24 h at 37°C at 5% CO₂.

Cell media was pipetted out and adherent cells were scraped from each well and transferred into a 15mL falcon tube. Supernatant was spun for 5 min at 340 g and supernatant discarded. Cells were resuspended in 500µL FCR block (Miltenyi biotech, Germany) and incubated at 4°C for 15 min. One hundred microlitres of supernatant from each falcon tube was transferred into fresh FACS tubes (Becton Dickinson, NJ, USA). One hundred microlitres of each antibody (Ab) was added to each falcon tube at each separate concentration (Ab used were Anti-CD86 (IGE-2A-FITC (Becton Dickinson, NJ, USA), anti-MHC II (Rat IGG-2A) FITC (Becton Dickinson, NJ, USA) and ISO

(Becton Dickinson, NJ, USA) only as a control.) Ab was left to bind to dendritic cells for 30 min in the dark at 4°C. Cells were subsequently washed twice in FACS buffer (2.5%FCS, PBS, 0.02% azide) and spun at 340 g for 5 min between each wash and supernatant discarded. Cells were resuspended in 300µL of FACS buffer and flow cytometry was carried out on a FACScanto (Becton Dickinson, NJ, USA). Flow cytometry data was then analysed using FLOJO software (Tree Star, USA)

2.2.2 A549 Cell culture

A549 human lung carcinoma cells were obtained from the European Collection of Cell Cultures (ECACC). A549 cells were maintained in Dulbecco's Modified Eagles Medium (DMEM) (Sigma) containing 10% foetal bovine serum (Sigma, UK), supplemented with 200mM L-Glutamine, 100mg/L penicillin and 100mg/L streptomycin (Sigma). Cells were seeded at 1×10^6 cells/mL in 75cm² cell culture flasks (Greiner Bio-one, Gloucestershire, UK) in an incubator with a humidified atmosphere at 37°C and 5% CO₂ (C&M scientific, Scotland). Culture media was changed every two days and cells split 1:10 during each passage at 1×10^6 cells/mL.

Cells were washed with 10mL of prewarmed Dulbecco's phosphate buffered saline (DPBS) (Sigma) and passaged at 90% confluence, with 0.5% trypsin EDTA (Sigma) for 5 min at 37°C in 5% CO₂. Once detached, 15mL of DMEM was added to flasks to inactivate the effects of trypsin. The cells were standardized to a final concentration of 8×10^5 cells in 20 mL DMEM in 75cm² flasks. Cells were then incubated in 5% CO₂ at 37°C in a humidified

atmosphere and experimental procedures undertaken on cells between passage 9 and 16. All cell culture work was carried out in a sterile environment in a Microflow cabinet (Ervicecare, Hampshire, UK).

2.2.3 Experimental procedures for A549 cell stimulation

A549 cells were prepared as described above to 80-100% confluence, washed and trypsinised. Cells were then transferred to a bijoux tube and centrifuged for 5 min at 10,000 g. Supernatant was discarded and cells washed with 10mL DPBS for 3 min to wash residual DMEM media from cells. Cells were resuspended in 2mL of DMEM and gently mixed by pipetting.

Twenty microlitres of the resuspended cell media was placed in a 1.5mL centrifuge tube with 10µL Trypan Blue solution (Sigma, UK) and mixed. 10µL of cell media was suspended on a Naeuber haemocytometer and viable cells counted. Cell culture media was standardised to 1×10^5 cells/mL in 20mL of DMEM. One millilitre of cell media was added to each well of a 24 well cell culture plate (Costar, Corning Incorporated, NY, USA) and incubated for 4 h and 24 h at 37°C in 5% CO₂.

Following overnight incubation, cell supernatant was removed by pipetting. Cells were stimulated in duplicate, with 500µL of media serving as a control. Experimental wells contained a total of 500µL of media and tigecycline at 0.1, 0.4 or 1.6 mg/L ± *E. coli* derived LPS (Sigma) prepared to a working concentration of 50 mg/L. LPS in media served as a second control.

2.2.4 OKF6/TERT2 Cell culture

OKF6/TERT2 cells were provided by the Rheinwald laboratory (Brigham and Woman's Hospital, Boston). Cells were cultured in keratinocyte serum-free medium (K-SFM [37010-022] Invitrogen, UK) supplemented with 100 IU penicillin, 100 mg/L streptomycin, 25 mg/L bovine pituitary extract (BPE), 0.2 ng/mL epidermal growth factor (EGF) and 0.3 mM CaCl₂ (0.4 mM total Ca⁺⁺). BPE and EGF were filter-sterilised through a 0.2 µm filter. Cells were seeded at 5 x 10³/mL in a 75 cm² flask. At approximately 30% confluence, media was changed to 50% K-SFM, 25% DMEM, 25% Ham's F12 and, supplemented as above with penicillin/streptomycin, 1.5 mM L-glutamine, BPE and EGF. All cell culture work was carried out in a sterile environment in a Microflow cabinet (Ervicecare, Hampshire, UK). Cells were passaged at approximately 90% confluence, using 0.05% trypsin/ EDTA. For cytokine assays, cells were cultured in defined-KSFM (10744-019, Invitrogen)

2.2.5 Experimental procedures for OKF6/TERT2 cells

Cells were standardised as described in Section 2.2.4, 1mL of cell media was added to each well of a 24-well cell culture plate (Costar, Corning Incorporated, NY, USA) and incubated for 4 h and 24 h at 37°C in 5% CO₂.

Following overnight incubation cell supernatant was removed by pipetting. Cells were stimulated in duplicate, with 500µL of media serving as a control. Experimental wells contained a total of 500µL of media and tigecycline at 0.1, 0.4 or 1.6 mg/L ± *E. coli* derived LPS (Sigma, UK) prepared to a working concentration of 50 mg/L. LPS in media served as a second control.

2.2.6 Tigecycline toxicity assay to A549 and OKF6/TERT2 cell lines

A549 and OKF6/TERT2 cells were standardised and prepared for assays as described in sections 2.2.2 and 2.2.4. Each cell line was stimulated with LPS \pm tigecycline as described in section 2.2.3 and 2.2.5. XTT was prepared initially as outlined in section 2.1.5

To produce a saturated 50 mg/L solution of XTT for each cell line, 50mL of cell media specific to each cell line was used. Two hundred and fifty microlitres of prepared XTT was added to each well after each cell line had been stimulated for 24 h. XTT was incubated at 37°C for 3 h. Eighty microlitres of supernatant was transferred by pipetting from the XTT treated wells to a fresh 96-well microtitre plate to measure colour change in a plate reader at 490 nm.

2.2.7 Gene expression for cytokines

2.2.7.1 RNA preparation from A549 and OKF6/TERT2 cells

A549 and OKF6/TERT2 gene expression was measured by quantitative RT-PCR. A549 and OKF6/TERT2 cells were treated with differing concentration of tigecycline and a constant amount of LPS as described above. After the 24 h time point cells were removed from incubation and each well supernatant was removed and stored in cryovials at -80°C for subsequent ELISA analysis.

RNA was extracted from each well using RNeasy Mini Kit (Qiagen LTD, Crawley, West Sussex, UK) following manufacture's guidelines. In brief,

350 μ L of Buffer RLT (Qiagen) was added to each well for direct lysis of cellular monolayer. Lysate was collected from each well and placed in a 1.5 mL microcentrifuge tube. Lysate was vortexed for 10s and pipetted to ensure visible clumps were not present. Three hundred and fifty microlitres of 70% ethanol was added to homogenize the lysate and mixed by pipetting. Seven hundred microlitres of sample was transferred to an RNeasy spin column (Qiagen), placed in a 2mL collection tube (Qiagen), and centrifuged for 15s at 10,000g. Seven hundred microlitres of Buffer RW1 (Qiagen) was added to the RNeasy spin column and centrifuged for 15s at 10,000g to wash the spin column membrane and flow through discarded. Five hundred microlitres of Buffer RPE (Qiagen) was added to the spin column, centrifuged for 15s at 10,000g to wash the spin column membrane and flow through discarded. Five hundred microlitres of Buffer RPE was added to the spin column and centrifuged for 2s at 10,000g to wash the spin column membrane and flow through discarded. The RNeasy spin column was placed in a new 1.5mL collection tube with the addition of 50 μ L RNase-free water (Qiagen) directly to the spin column membrane and centrifuged for 1 min at 10,000g to elute the RNA. Finally, to ensure a high concentration of RNA the flow through was added back into the spin column membrane and centrifuged again for 1 min at 10,000g.

2.2.7.2 DNase treatment of RNA samples

To ensure RNA was clear from DNA contamination, 15 μ L of RNA was heated to 37°C for 30 min with the addition of 2 μ L of 10x Buffer (Promega, UK) and 2 μ L of DNase (Promega, UK). Finally, 2 μ L of stop solution (Promega, UK)

was added to each sample and this was incubated for a further 10 min at 65°C. The 'clean' RNA was collected and quantified using a spectrophotometer, described below.

2.2.7.3 Quantification of nucleic acids

Purified RNA (1.5µL) was quantified in a NanoDrop™ ND-1000 spectrophotometer (Labtech International, Ringmer, East Sussex UK). The reference was established with sterile filtered, UV-treated ddH₂O prior to RNA quantification. Total RNA samples were quantified accordingly and recorded as ng/µL. Samples with a 260/280 nm ratio of 1.7 to 1.8 were deemed to be of high enough quality for subsequent quantitative PCR reactions. RNA samples were stored at -70°C for subsequent analysis.

2.2.7.4 Reverse transcription

cDNA was prepared using superscript II reverse transcriptase (Invitrogen, Paisley, UK) as follows: 100ng RNA was incubated in RNase-free water (Invitrogen) with 0.5µg Oligo(dT) (Invitrogen) and 10mM dNTP mixture at 65°C for 5 min, quick chilled, then reverse transcribed using 100U superscript II RT at 42°C for 50 min in the presence of the manufacturer's buffer (50mM Tris.HCL (pH 8.3), 75mM KCl, 3 mM MgCl₂) with 0.01M DTT and 1µL (40 units) RNase OUT Ribonuclease inhibitor (Invitrogen) in a total volume of 25µL. Finally, the superscript was inactivated by heating to 70°C for 15 min. In addition, 'no RT' controls were performed in the absence of Superscript. The cDNA was used immediately in PCR or stored at -20°C.

2.2.7.5 Real-time quantitative PCR (IL-6 and IL-8)

1 in 20 dilutions of cDNA samples were prepared and 2 μ L of this was PCR-amplified using 200nM primers (0.5 μ L forward and reverse primers at 10 μ M) (Table 2.3) with 12.5 μ L SYBR green master mix (Invitrogen) and 9.5 μ L of DNase/RNase free water (Qiagen, UK) to a total volume of 25 μ L per reaction. Reactions were performed in duplicate on 0.2mL optical tube strips (Agilent technologies) using the Mx3000P QPCR machine (Agilent technologies).

A standard dissociation curve protocol was included after 40 amplification cycles to confirm that only one product was made. Threshold cycle (Ct) was determined for all samples after carefully selecting appropriate threshold. Amplification plots were looked at in the logarithmic scale and threshold selected to be above any baseline fluorescence. In the region where the amplification was exponential (steep curve) and where the majority of replicates gave very similar Ct values (i.e. where duplication amplification plots were parallel).

Expression levels of IL-6 and IL-8 were measured in relation to the house keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The thermal profile was optimised for all primers (Tables 2.4). No RT controls were included to check for DNA contamination and no template controls, with RNase free water (Qiagen), were run to rule out other contamination problems.

Table 2.3 Primers used for analysis of pro-inflammatory mediators in this study

Primers and associated primer sequences used during all pro-inflammatory investigations.

Primer	Sequence	Reference
IL-6 Forward	3' CAATCTGGATTCAATGAGGAGAC 5'	Girault <i>et al</i> , 2002.
IL-6 Reverse	3' CTCTGGCTTGTTCTTCACTACTC 5'	
IL-8 Forward	3' TTAGCACTCCTTGGCAAAC 5'	Li <i>et al</i> , 2007.
IL-8 Reverse	3' CAGAGACAGCAGAGCACACAA 5'	
GAPDH Inner 1	3' CAAGGCTGAGAACGGGAAG 5'	McKimmie <i>et al</i> , 2008.
GAPDH Inner 2	3' GGTGGTGAAGACGCCAGT 5'	

Table 2.4 PCR reaction conditions

PCR reaction conditions to analyse expression of IL-6 and IL-8 from A549 and OKF6/TERT2 cells. The total time for complete run ~ 142 min and consisted of 3 phases.

	Temperature (°C)	Time
Denaturing (1 cycle)	50	2 min
	95	10 min
Amplification (40 cycles)	denature 95	30 s
	anneal 56	30 s
	extend 72	30 s
Extend (1 cycle)	72	10 min

2.2.8 Cytokine assays using enzyme-linked immunosorbent assays (ELISA)

Cytokines concentrations in culture supernatants from A549 and OKF6 cell experiments were measured by sandwich ELISA using human IL-6 and human IL-8 Cytoset™ (Biosource, USA) in accordance with manufacturer's instructions. Table 2.5 indicates solutions used in all assays. Assays were optimised and validated prior to use. Immulon 4 HBX flat-bottom 96-well microtitre plates (Fisher Scientific, UK) were coated with IL-6 or IL-8 capture antibody (4 µg/ml in PBS), which were sealed and incubated overnight at 4°C. Subsequent incubations were carried out at room temperature. Two hundred microlitres of blocking buffer was added to each well for 1 h. After the step described above and subsequent steps below, microtitre plates were washed 5 times with wash buffer. A standard curve consisting of 7 doubling dilutions (range 15.63 - 1000 pg/ml) was prepared to calculate concentrations of IL-6 and IL-8 in the samples. One hundred microlitres of standards and samples were added in duplicate and incubated for 2 h, biotinylated IL-6 or IL-8 detection antibody (20 ng/ml in cell culture media + 0.1% BSA +0.05% Tween®20) was added and incubated for 2 h. Streptavidin-horseradish peroxidase was added for 20 min and protected from light. Plates were developed with 100µL 3,3',5,5'-tetra-methylbenzidine (TMB, R&D Systems, UK). Blue colour was allowed to develop in the dark. The final colour change was measured at an absorbance of 560 nm. Cytokine concentrations were calculated using a 4-parameter curve fit. Graphpad Prism, version 4.0 for Windows (Graphpad Software, CA, USA) and was used for statistical analysis to determine significant changes, p values less than 0.05

Table 2.5 Duoset ELISA solutions

ELISA solutions for IL-6, IL-8 and MMP-9 as described in section 2.2.8

Solutions	Mixture
PBS	PBS tablets (Sigma) (1 per 100mL)
Wash Buffer	0.05% Tween 20 in PBS, pH 7.2 – 7.4 (Sigma)
Reagent Diluent	1% bovine serum albumin (BSA fraction V) (Sigma) in PBS pH 7.2 – 7.4
Substrate Solution	1:1 mixture of Colour Reagent A (H ₂ O ₂) and Colour Reagent B (TMB)

2.2.9 Experimental procedures of primary neutrophil cell cultures

2.2.9.1 Peripheral blood neutrophil preparation

Twenty milliliters venous blood was collected in the presence of 250U/mL sodium heparin. Twenty milliliters of Polymorphprep™ (Fresenius, Norway) was added to 4 15mL falcon tubes. Five milliliters of blood was carefully layered by Pasteur pipetting ensuring the blood did not mix with the Polymorphprep™. Each falcon tube containing the cells and Polymorphprep™ was centrifuged at 500 x g in a swing bucket rotor for 30 min at RT. Plasma and mononuclear cells were removed initially from the interface. Neutrophils were harvested from the lower subsequent band. An equal amount of PBS (5mL) was mixed with sterile water (5mL) and neutrophils added to the media and centrifuged at 400 x g in a swing bucket rotor for 10 min. Supernatant was discarded and pellet resuspended in 5mL ammonium chloride lysis buffer (eBioscience, UK) to remove any residual contamination of the neutrophils. Neutrophils were centrifuged at 400 x g for 10 min and subsequently washed 3 times in RPMI by centrifuging at 330 x g for 5 min at RT.

2.2.9.2 Neutrophil cytokine production in response to Tigecycline

Neutrophils were added to a 96-well flat bottom plate (Costar), at 2×10^5 cells/mL in 100ul complete GI 1640 supplemented with 10% fetal calf serum (Sigma, UK). Cells were co-stimulated in triplicate, with 1 mg/L (PMA) and tigecycline at 0.1, 1 and 10 and 100 mg/L respectively for 24 h at 37°C.

2.2.9.3 IL-8 chemokine assay using ELISA

IL-8 within coculture supernatants from neutrophil cell experiments were measured by sandwich ELISA using human IL-8 Cytoset™ (Biosource, USA) in accordance with manufacturer's instructions, and carried out as described in section 2.2.8.

2.2.9.4 Matrix metalloproteinase 9 assay using DuoSet ELISA

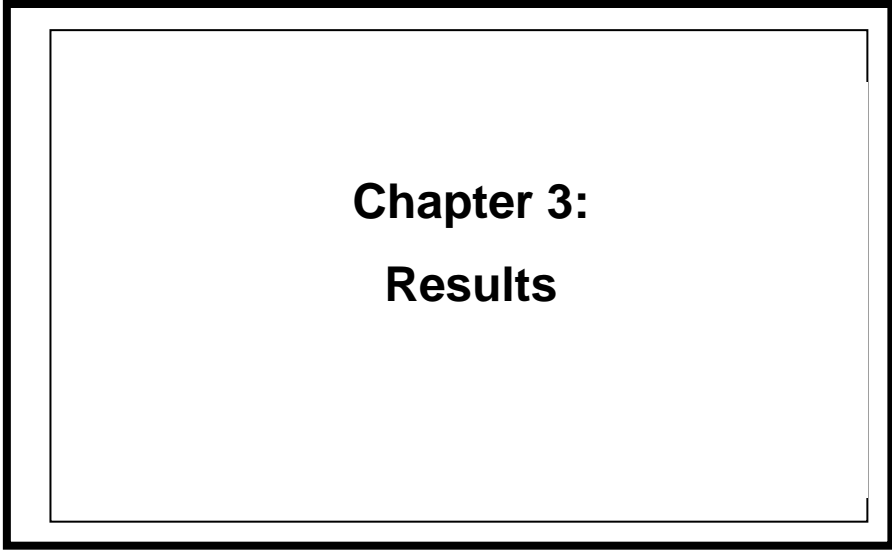
MMP-9 within coculture supernatants from neutrophil cell experiments was measured by duoSet ELISA using human total MMP-9 (R&D systems, USA). Initially the assay was optimised and validated prior to use.

Immulon 4 HBX flat-bottom 96-well microtitre plates (Fisher Scientific) were coated with 100µL of capture antibody diluted to working concentration in PBS, sealed and incubated overnight at RT. Each well was aspirated and washed in 400µL of wash buffer 3 times. One hundred microlitres of reagent diluent was added to each well to block the plate and incubated for 1h at RT. Wells were washed as described above. One hundred microlitres of standards prepared in reagent diluent and samples were added to wells in duplicate, and incubated for 2h at RT. Plates were washed again as described above. One hundred microlitres of detection antibody (4ug/ml) diluted to working concentration in reagent diluent was added to all wells and incubated for 2 h at RT. Wells were washed as described above. One hundred microlitres of the working dilution of streptavidin-horseradish peroxidase was added to each well and incubated in the dark at RT for 20 min. Wells were washed as described above. The final colour change was measured after addition of

TMB substrate at an absorbance of 560 nm. A standard curve was prepared to calculate concentration of MMP-9 in samples (range 31.15 -2000 pg/ml) Cytokine concentrations were calculated using a 4-parameter curve fit. Graphpad Prism, version 4.0 for Windows (Graphpad Software, CA, USA) and was used for statistical analysis to determine significant changes, p values less than 0.05

2.2.10 Data analysis

All data and statistical analysis for all microbiological and immunological assay were performed using Graphpad Prism, version 4.0 for Windows (Graphpad Software, CA, USA) to determine significant changes, p values less than 0.05. Non-parametric Mann-Whitney tests were employed for all microbiological results and the One-way analysis of variance (ANOVA) tests were performed to compare control samples with all treatments of the A549 and OKF6/TERT2 cell lines.



**Chapter 3:
Results**

3 Results

3.1 Antimicrobial properties of tigecycline

3.1.1 Introduction

These experiments aimed to evaluate the activity of tigecycline against bacterial pathogens associated with both oral and respiratory tract infections. This included *Burkholderia* spp, which are associated with chronic inflammatory disease of the lung, and *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* spp., which are associated with chronic inflammatory diseases of the periodontium. In addition it was intended to evaluate the role of multidrug efflux pumps in the sensitivity of tigecycline against these different bacterial pathogens.

3.1.2 Effects of tigecycline against planktonic and sessile cells

3.1.2.1 *Burkholderia* spp.

These experiments evaluated the activity of tigecycline against *Burkholderia* spp. grown both planktonically and as sessile biofilms (Table 3.1). The planktonic MIC (PMIC) for the *B. cepacia* complex ranged from 8 to 32 mg/L, with an MIC₅₀ of 16 mg/L. The PMIC of tested *B. gladioli* strains ranged from 4 to 8 mg/L. The sessile MIC (SMIC) for the *B. cepacia* complex ranged from 32 to 64 mg/L, with an MIC₅₀ of 64 mg/L. The SMIC of the *B. gladioli* tested was 32 mg/L.

Table 3.1 Planktonic and sessile minimum inhibitory concentrations of *Burkholderia cepacia* spp. to tigecycline

Strain	Tigecycline MIC (mg/L)	
	PLANKTONIC	SESSILE
<i>Burkholderia cepacia</i>		
K 56-2 (III)	32	32
ATCC 117765 (III)	32	64
YHBCC1 (II)	32	64
YHBCC2 (II)	32	64
YHBCC3 (II)	32	32
YHBCC4 (II)	16	32
YHBCC5 (III)	16	64
YHBCC6 (III)	32	64
YHBCC7 (III)	32	64
YHBCC8 (III)	32	64
YHBCC9 (III)	16	32
YHBCC10 (III)	32	64
YHBCC11 (III)	16	32
YHBCC12 (III)	32	64
YHBCC13 (IV)	8	32
YHBCC14 (V)	16	64
YHBCC15 (V)	16	64
<i>Burkholderia gladioli</i>		
YHBG1	8	32
YHBG2	4	16
YHBG3	8	32

3.1.2.2 *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggregatibacter actinomycetemcomitans* spp.

These experiments evaluated the activity of tigecycline against *P. gingivalis*, *F. nucleatum* and *A. actinomycetemcomitans* grown both planktonically and as sessile biofilms in triplicate (n=3) with respective controls. The PMIC for the *P. gingivalis* and *F. nucleatum* remained below the minimum concentration tested (≤ 0.25 mg/L). The PMIC of the *A. actinomycetemcomitans* tested was 8 mg/L (Table 3.2).

The SMIC of *P. gingivalis* increased from the PMIC and ranged from 2 mg/L to 64 mg/L, with an MIC₅₀ of 4 mg/L. *F. nucleatum* resistance to tigecycline increased dramatically in sessile form from the PMIC with a range of 64 mg/L to 128 mg/L with an MIC₅₀ of 128 mg/L. This significant increase in resistance was also evident with *A. actinomycetemcomitans* having a SMIC of 128 mg/L for all strains.

Table 3.2. Planktonic and sessile minimum inhibitory concentrations of oral pathogens to tigecycline

Strain	Tigecycline MIC (mg/L)	
	PLANKTONIC	SESSILE
<i>Porphyromonas gingivalis</i>		
W50	≤0.25	2
2111	≤0.25	4
2114	≤0.25	4
2098	≤0.25	64
11834	≤0.25	32
<i>Fusobacterium nucleatum</i>		
494	≤0.25	64
10562	≤0.25	≥128
01/105 (1)	≤0.25	≥128
01/105 (3)	≤0.25	≥128
<i>Aggregatibacter actinomycetemcomitans</i>		
10981	8	≥128
10982	8	≥128
9709	8	≥128

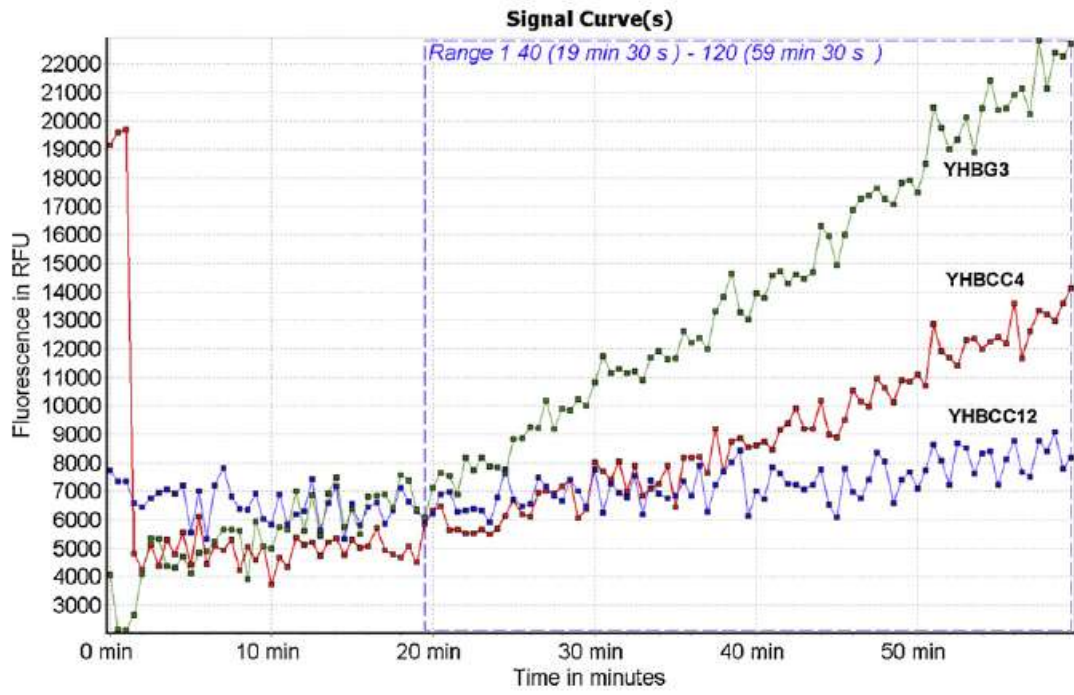
3.1.3 Quantifying efflux pump activity

Expression of drug efflux pumps is a common resistance mechanism in Gram positive and negative bacteria leading to increased levels of drug resistance in a vast range of bacterial species. These experiments were designed to evaluate the constitutive expression of efflux activity using a fluorescent based uptake assay system as described in section 2.1.4. Analysis of β -naphthylamine production by 3 strains of *Burkholderia* demonstrated differential efflux activity carried out in triplicate (n=3). Figure 3.1A shows a linear increase in fluorescence as a function of time over 60 min, owing to intracellular hydrolysis of Ala-Nap. This data is summarised in a histogram in Figure 3.1B. The rate of cleavage of Ala-Nap was highest in YHBG3 (MIC 8 mg/L) compared with YHBCC4 (MIC 16 mg/L) and YHBCC12 (MIC 32 mg/L). Both results indicate that the lower the MIC the higher the efflux activity for a particular strain.

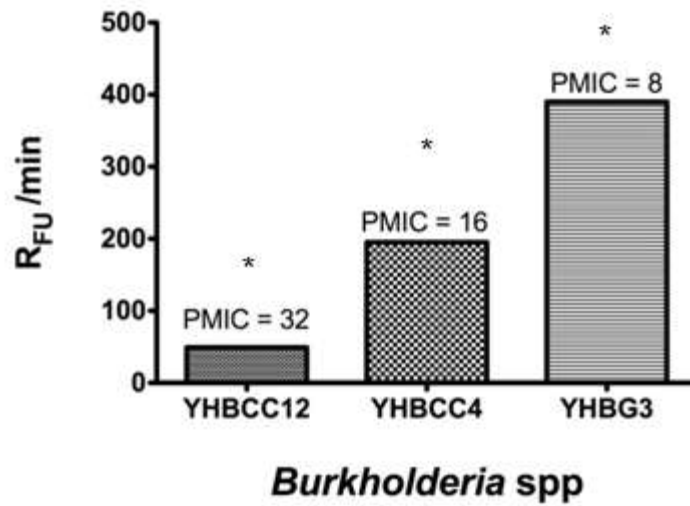
Figure 3.1 Quantification of efflux activity utilising Ala-nap uptake by differential strains of *Burkholderia* spp.

The *Burkholderia* spp evaluated by serial dilutions of tigecycline displayed a PMIC range of 8 – 32 mg/L (Table 3.1). (A) Ala-Nap (128 mg/L) uptake of selected strains YHBG3, YHBCC4 and YHBCC12 shows an increase in fluorescence is depicted owing to intracellular hydrolysis of Ala-Nap. The greater fluorescence was quantified in the case of YHBG3 (*Burkholderia gladioli*), with a MIC of 8 mg/L, indicating low efflux activity. In the case of YHBCC4 (MIC = 16mg/L) and YHBCC12 (MIC = 32mg/L), less fluorescence was quantified owing to high efflux activity. (B) Bar graph demonstrating relative fluorescent units (R_{FU}) for each strain. A significant increase in efflux activity was associated with an increase in MIC ($p < 0.005$).

A



B



3.1.4 Effects of efflux pump inhibitor (MC-207,110) upon tigecycline sensitivity

These experiments were designed to evaluate the contribution of efflux pumps to tigecycline sensitivity. An EPI, which acts as a competitive substrate inhibitor, was used in combination with planktonic and sessile cells and the MIC's determined as described in section 2.1.3.3. All experiments were carried out in triplicate (n=3) with no treated cells representing the controls

3.1.4.1 *Burkholderia* spp.

The PMIC for the *B. cepacia complex* treated with tigecycline ranged from <0.125 to 1 mg/L, with an MIC₅₀ of 1 mg/L (Table 3.1). In comparison to those treated with only tigecycline in the presence of EPI tigecycline demonstrated a significant increase in sensitivity ($p < 0.001$), representing approximately a 37-fold change (Figure 3.2A). The PMIC of the *B. gladioli* tested ranged from <0.125 to 0.25 mg/L. Planktonic *B. gladioli* treated with tigecycline in the presence of EPI also demonstrated significantly increased sensitivity ($p < 0.01$), with a 32-fold change compared to tigecycline alone (Figure 3.1B).

The SMIC for the *B. cepacia complex* ranged from 0.5 to 4 mg/L, with an MIC₅₀ of 2 mg/L (Table 3.1). In the sessile form, sensitivity was also significantly increased ($p < 0.0001$) in the presence of EPI, representing a 26-fold change in comparison to tigecycline alone (Figure 3.3A). The SMIC of the *B. gladioli* tested was 2 mg/L with a 4-fold change when exposed to EPI and significantly increased sensitivity ($p < 0.001$) (Figure 3.2B).

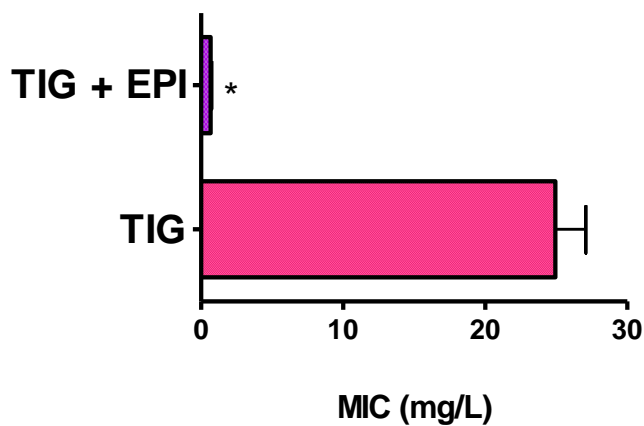
Table 3.3. Effects of a competitive substrate (MC-207,110) of efflux pumps on the sensitivity of *Burkholderia cepacia* sp. to tigecycline

Strain	Planktonic tigecycline MIC (mg/L)		Sessile tigecycline MIC (mg/L)	
	MC-207,110 (64 mg/L)	Fold- change	MC-207,110 (64 mg/L)	Fold- change
<i>Burkholderia cepacia</i> complex (genomovar)				
K 56-2 (III)	0.5	64	1	32
ATCC 117765 (III)	1	32	0.5	128
YHBCC1 (II)	1	32	1	64
YHBCC2 (II)	1	32	1	64
YHBCC3 (II)	0.5	64	1	32
YHBCC4 (II)	0.5	32	1	32
YHBCC5 (III)	0.25	64	1	64
YHBCC6 (III)	1	32	2	32
YHBCC7 (III)	1	32	2	32
YHBCC8 (III)	1	32	2	32
YHBCC9 (III)	0.25	64	4	8
YHBCC10 (III)	1	32	2	32
YHBCC11 (III)	0.5	32	1	32
YHBCC12 (III)	<0.125	>128	2	32
YHBCC13 (IV)	0.5	16	2	16
YHBCC14 (V)	0.25	64	2	32
YHBCC15 (V)	1	16	4	16
<i>Burkholderia gladioli</i>				
YHBG1	0.25	32	2	16
YHBG2	0.25	16	2	8
YHBG3	<0.125	>128	2	16

Figure 3.2. Efflux pump inhibitor (MC-207,110) enhances sensitivity of planktonic *Burkholderia* spp. to tigecycline after 24 h exposure.

Planktonic (A) *B. cepacia* and (B) *B. gladioli* were exposed to serial doubling dilutions of tigecycline \pm MC-207,110 and incubated for 24 h at 37°C. The data represent the mean and standard deviation of three experiments (n=3) performed in triplicate. The MIC was determined based on inhibition of growth when visualised in a 96 well plate. A significant reduction in tigecycline concentration inhibited growth of planktonic *B. cepacia* in the presence of MC-207,110 (64 mg/L) by approximately 37-fold ($p < 0.001$), and a significant reduction in tigecycline concentration inhibited growth of planktonic *B. gladioli* in the presence of MC-207,110 (64 mg/L) by 32-fold ($p < 0.01$).

A



B

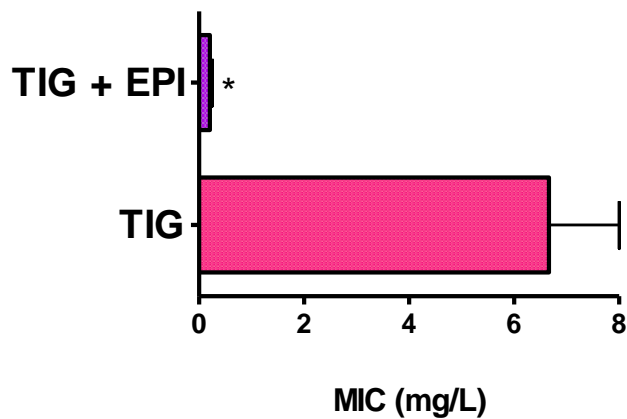
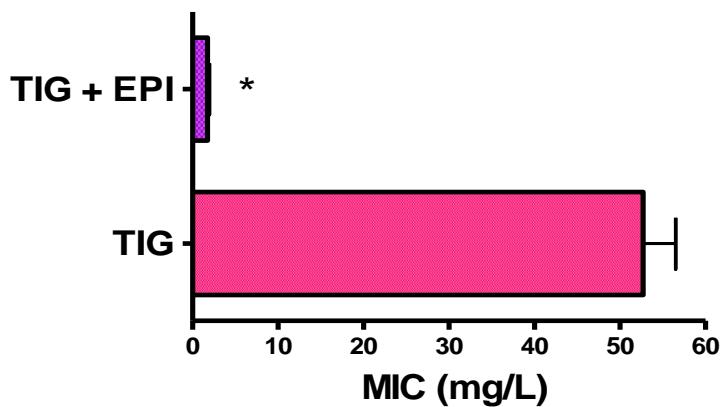
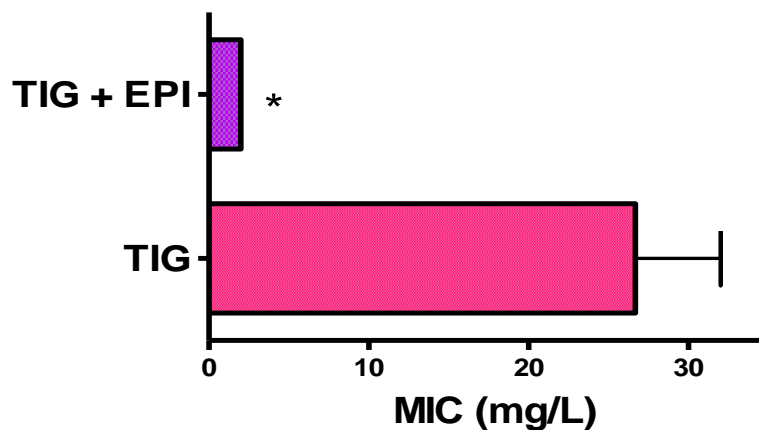


Figure 3.3. Efflux pump inhibitor (MC-207,110) enhances sensitivity of sessile *Burkholderia* spp. to tigecycline after 24 h exposure. Sessile (A) *B. cepacia* and (B) *Burkholderia gladioli* were exposed to serial doubling dilutions of tigecycline \pm MC-207,110 and incubated for 24 h at 37°C. The data represent the mean and standard deviation of three experiments (n=3) performed in triplicate. The sessile MIC was determined based on metabolic reduction within a 96 well plate. A significant reduction in tigecycline concentration inhibited growth of sessile *B. cepacia* in the presence of MC-207,110 (64 mg/L) by approximately 26-fold ($p < 0.0001$), and a significant reduction in tigecycline concentration inhibited growth of planktonic *B. gladioli* in the presence of MC-207,110 (64 mg/L) by 14-fold ($p < 0.001$).

A



B



3.1.4.2 Effects of efflux pump inhibitor (MC-207,10) upon tigecycline sensitivity to oral bacteria after 24 h.

The PMIC of *P. gingivalis* and *F. nucleatum* remained below detectable levels ≤ 0.25 mg/L (Table 3.4.) In comparison to planktonic *A. actinomycetemcomitans* treated only with tigecycline, there was a significant ($p < 0.0001$) increase in sensitivity in the presence of EPI, representing approximately an 8-fold change and an MIC of 1 mg/L (Figure 3.4).

SMIC of *P. gingivalis* ranged from ≤ 0.25 to 8 mg/L with an MIC₅₀ of 1 mg/L (Figure 3.5) A slight reduction in sensitivity to tigecycline is demonstrated by the addition of MC-207,110 (64 mg/L) by approximately 2-fold ($p < 0.05$). A significant reduction in tigecycline concentration inhibited growth of sessile *A. actinomycetemcomitans* in the presence of MC-207,110 (64 mg/L) by 5-fold ($p < 0.001$), with a SMIC between 8 and 32 mg/L and a MIC₅₀ of 8 mg/L. In contrast to these results, a small reduction of tigecycline concentration is observed to inhibit growth of *F. nucleatum* in the presence of MC-207,110 (64 mg/L), with a SMIC ranging between 16 and ≥ 128 mg/L, which are not statistically different. All experiments were carried out in triplicate ($n=3$), controls were represented by bacterial cells treated with media only,

Table 3.4. Effects of a competitive substrate (MC-207,110) of efflux pumps on the sensitivity of oral pathogens to tigecycline

Strain	Planktonic tigecycline MIC (mg/L)		Sessile tigecycline MIC (mg/L)	
	MC-207,110 (64 mg/L)	Fold- change	MC-207,110 (64 mg/L)	Fold- change
<i>Porphyromonas gingivalis</i>				
W50	≤0.25	n/a	1	1
2111	≤0.25	n/a	1	4
2114	≤0.25	n/a	≤0.25	4
2098	≤0.25	n/a	8	8
11834	≤0.25	n/a	4	8
<i>Fusobacterium nucleatum</i>				
494	≤0.25	n/a	16	4
10562	≤0.25	n/a	≥128	0
01/105 (1)	≤0.25	n/a	≥128	0
01/105 (3)	≤0.25	n/a	≥128	0
<i>Aggregatibacter actinomycetemcomitans</i>				
10981	1	8	32	4
10982	1	8	32	4
9709	1	8	8	16

n/a – none applicable, no fold change observed.

Figure 3.4 Efflux pump inhibitor (MC-207,110) enhances sensitivity of planktonic *Aggregatibacter actinomycetemcomitans* to tigecycline after 24 h exposure. *A. actinomycetemcomitans* was exposed to serial doubling dilutions of tigecycline \pm MC-207,110 and incubated for 24 h at 37°C. The data represent the mean and standard deviation of three experiments (n=3) performed in triplicate. The MIC was determined based on inhibition of growth when visualised in a 96 well plate. A significant reduction in tigecycline was required to inhibit growth of planktonic *A. actinomycetemcomitans* in the presence of MC-207,110 (64 mg/L) by 8-fold ($p < 0.0001$).

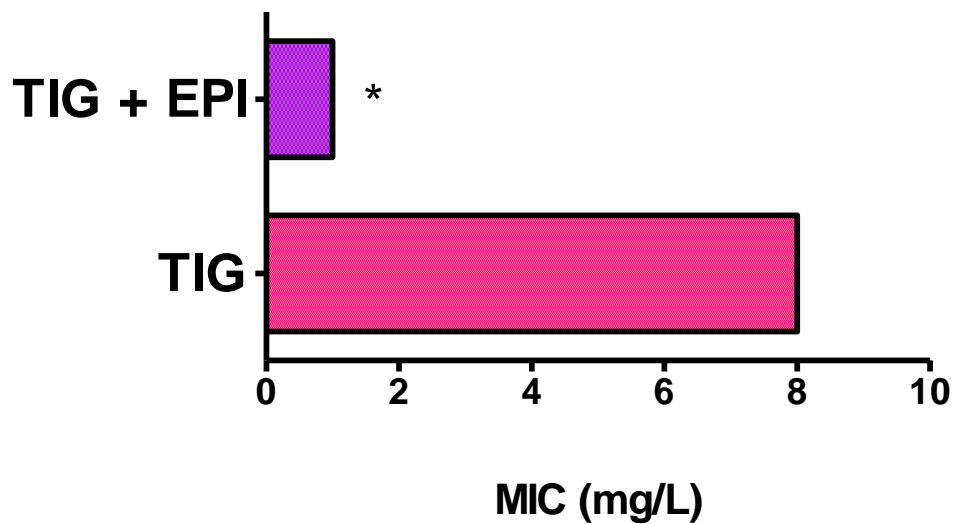
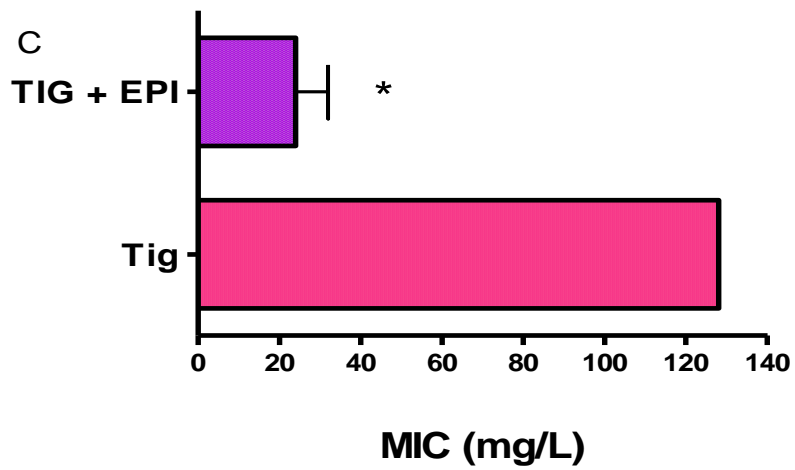
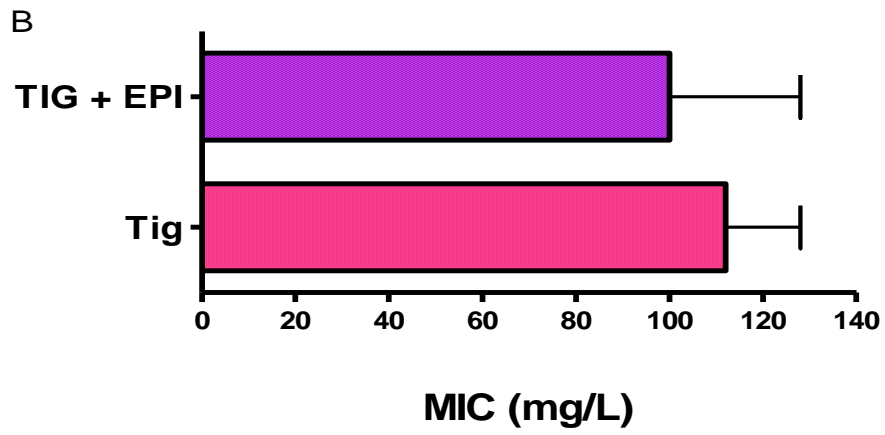
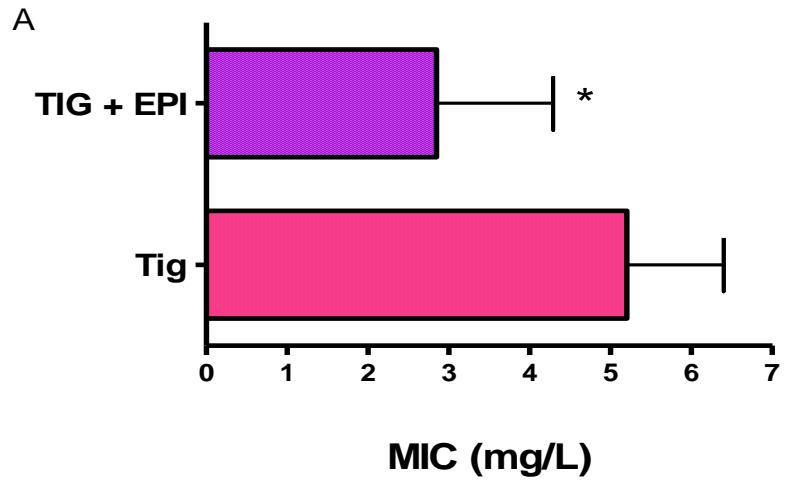


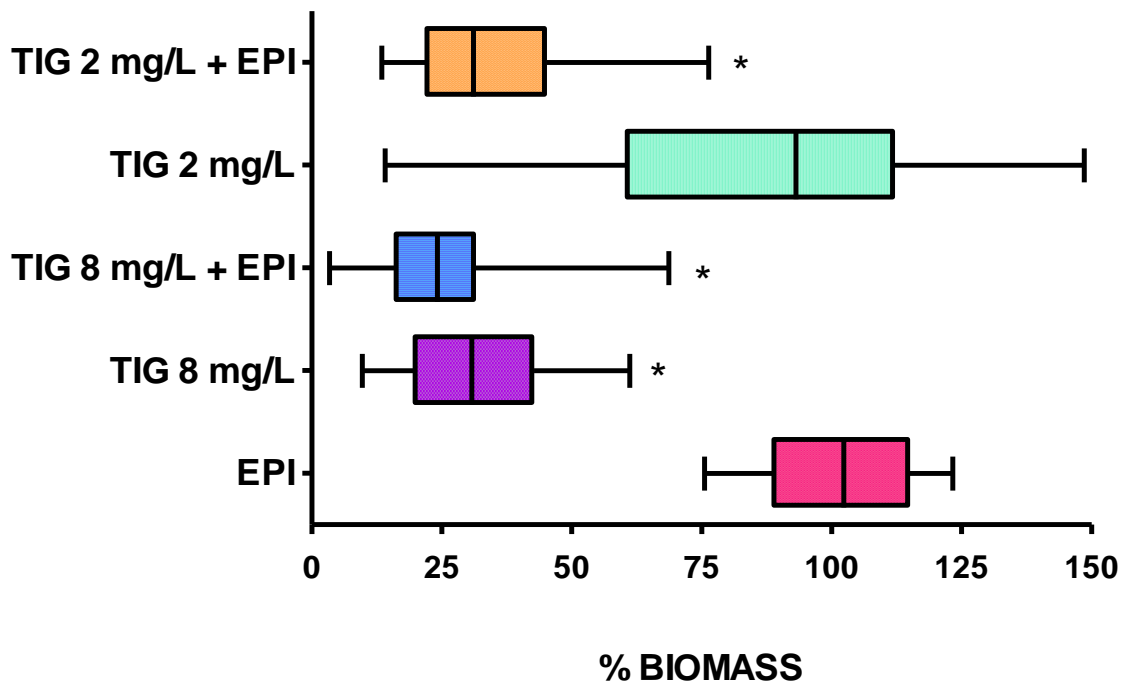
Figure 3.5 Effects of efflux pump inhibitor (MC-207,10) on tigecycline sensitivity to sessile oral bacteria after 24 h. Sessile (A) *P. gingivalis* (B) *F. nucleatum* and (C) *A. actinomycetemcomitans* were exposed to serial doubling dilutions of tigecycline \pm MC-207,110 and incubated for 24 h at 37°C. The data represent the mean and standard deviation of three experiments (n=3) performed in triplicate. The sessile MIC was determined based on metabolic reduction within 96-well plate. A slight reduction in tigecycline concentration inhibited growth of sessile *P. gingivalis* in the presence of MC-207,110 (64 mg/L) by approximately 2-fold ($p < 0.05$), and a significant reduction in tigecycline concentration inhibited growth of sessile and *A. actinomycetemcomitans* in the presence of MC-207,110 (64 mg/L) by 5-fold ($p < 0.001$). In contrast to these results, a small reduction of tigecycline concentration is observed inhibiting growth of *F. nucleatum* in the presence of MC-207,110 (64 mg/L), this data was not statistically different.



3.1.5 Biofilm disruptive effects of tigecycline on bacterial biofilms.

This experiment was designed to determine whether tigecycline had an impact on biofilm stability, and whether homeostatic control of the biofilm through inhibition of efflux mediated detoxification in combination with tigecycline had any detrimental effects on sessile *Burkholderia* spp biofilm. Strains were grown as described in section 2.1.3.4. EPI alone did not affect the biomass of the *Burkholderia* spp as shown by the biomass >100% (Figure 3.5). In comparison to EPI alone, tigecycline 8 mg/L ± EPI results in significant reduction of biomass ($p < 0.0001$). Tigecycline at 2 mg/L did not show any significant reduction of the biomass, as many strains exhibited >100% biomass. However, 2 mg/L in the presence of EPI resulted in a significant difference in comparison to EPI alone ($p < 0.0001$).

Figure 3.6 Tigecycline disrupts sessile *Burkholderia cepacia* spp. in a concentration dependant manner, which is improved with EPI . 17 strains of *B. cepacia* spp were exposed to serial doubling dilutions of tigecycline \pm MC-207,110 and MC-207,110 (64 mg/L) incubated for 24 h at 37°C. The data are shown as box and whisker plots and represent the median and quartiles of six experiments (n=6). A significant reduction in tigecycline was required to inhibit growth of sessile *Burkholderia* spp in the presence of MC-207,110 (64 mg/L). 8 mg/L and 2 mg/L represent the MIC₅₀ of the sessile forms of *Burkholderia* spp \pm MC-207,110. ($p < 0.0001$).



3.2 Immunological properties of tigecycline

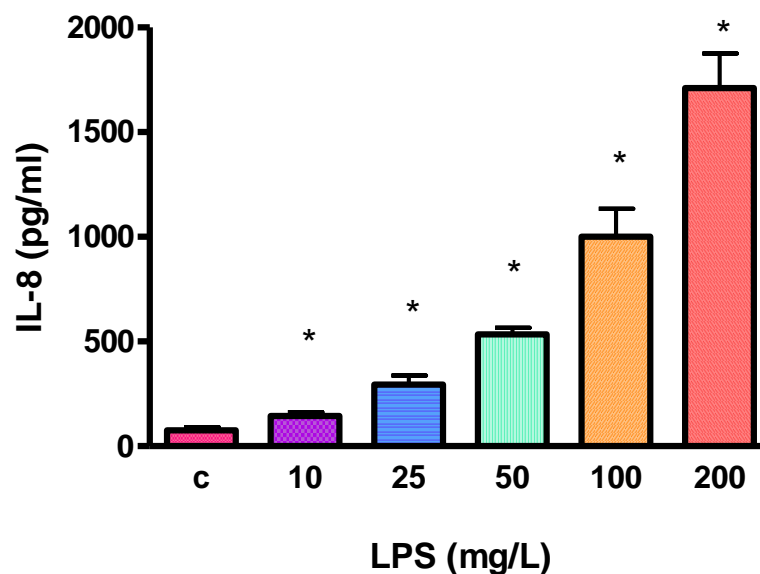
3.2.1 Introduction

These experiments were designed to evaluate the effects of tigecycline on two inflammatory mediators IL-6 and IL-8, which was achieved by co-stimulating several cell lines in the presence of tigecycline, as described in section 2.2.3, 2.2.5 and 2.2.9. qRT-PCR and ELISAs were performed to quantify inflammatory outputs to several key cytokines and chemokines. The purity of tigecycline provided was assayed by FACS (Figure 3.8) to ensure that no contaminants were present in the drug that could adversely affect the results of subsequent cellular work, as described in section 2.2.1. To determine if any of these concentrations of tigecycline were toxic to the cell lines an XTT assay was carried (Figure 3.9) out as described in section 2.2.6 to ensure results were valid and accurate.

3.2.2 Production of IL-8 derived from A549 cell line exposed to a range of LPS concentrations.

This experiment was conducted to determine the relative amount of LPS to be used throughout all cellular investigations. The A549 cell line was prepared and maintained as described in section 2.2.2, and ELISAs carried out as described in section 2.2.8. The assay was carried out in duplicate and represent the mean and standard deviation of three experiments (n=3) and all data analysis was performed as described in section 2.2.10. The data summarised in Figure 3.7 demonstrated that an increase in bacterial LPS resulted in a significant increase of IL-8 expression ($P < 0.05$)

Figure 3.7 IL-8 protein expression from A549 cell line after exposure to varying ranges of LPS after 24 h. A549 cell line was stimulated with 0 – 200 mg/L of LPS for 24 h in microaerophilic conditions (5% CO₂) supernatant was harvested and the concentration of IL-8 determined for each sample quantified using ELISA. Experiment was carried out in duplicate and represent the mean and standard deviation of three experiments (n=3). An increase of LPS concentration resulted in an increase of IL-8 expression from the A549 cell line. Stimulation with 200 mg/L LPS resulted in the highest levels of IL-8 production, 10 mg/L LPS represent the lowest production levels of IL-8. Each concentration increase of LPS (~ double) resulted in approximately a 2-fold increase of IL-8 expression.

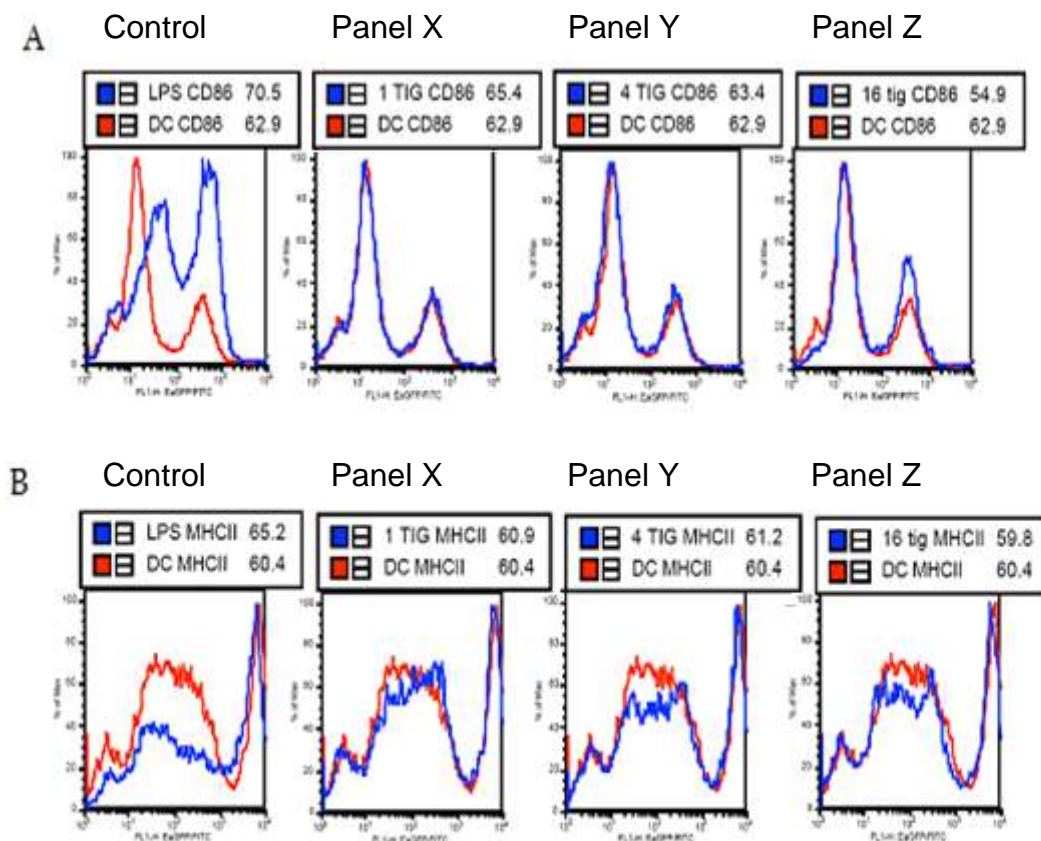


3.2.3 Fluorescence-activated cell sorting in the analysis of tigecycline purity

The experiment was designed to determine if the tigecycline powder provided was free of endotoxin that could adversely affect the results of cellular experiments. The dendritic cells were generated from bone marrow cells of C57BL/6 mice which were grown within the GBRC at Glasgow University by Mr John Butcher on my behalf. Dendritic cells were stimulated with LPS showed an upregulation of both CD86 (Figure 3.8A) and MHC II (Figure 3.8B) markers on the cell surface. When stimulated with varying ranges of tigecycline, no upregulation of either marker was evident, therefore it was concluded that tigecycline powder was pure and free of LPS contaminants.

Figure 3.8 Analysis of CD86 and MHC II by FACS analysis when stimulated with tigecycline

Dendritic cells were grown overnight, antibodies were conjugated to the fluochrome fluorescein isothiocyanate (FITC) to allow for specific binding to dendritic cell markers (A) CD86 and (B) MHC II followed by subsequent stimulation with LPS 50 mg/L and 0.1 mg/L (Panel X), 0.4 mg/L (Panel Y) and 1.6 mg/L (Panel Z) of tigecycline and incubated in microaerophilic conditions (5% CO₂) at 37°C for 24 h. (A) CD86 and (B) MHC II controls were both up regulated when dendritic cells were stimulated with LPS (far left graphs for A and B). The blue lines on the graphs represent the dendritic cells stimulated with LPS compared to the red line which represent the control set of dendritic cells that were not stimulated. The right shift in fluorescence of B equates to increased cell numbers detected by the antibodies.

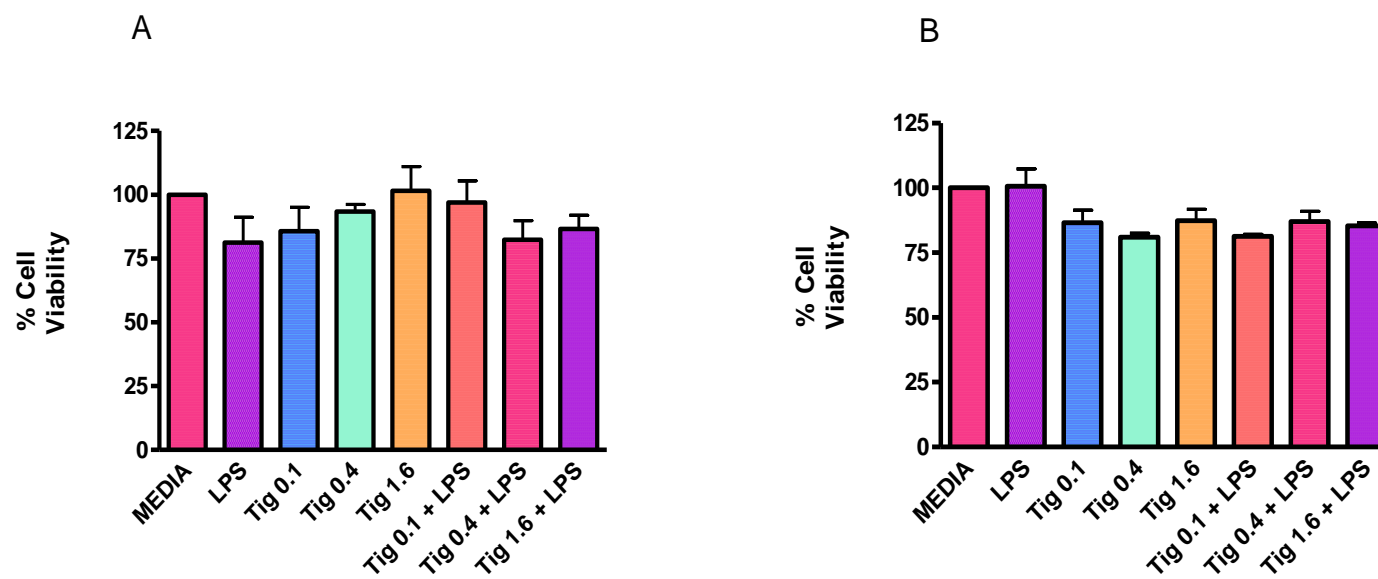


3.2.4 Tigecycline concentration does not adversely affect A549 and OKF6/TERT2 cell lines.

These experiments were conducted to determine if exposure to tigecycline elicited a detrimental effect on the metabolic activity of each cell line. A549 and OKF6/TERT2 cells were cultured as described in section 2.2.2 and 2.2.4 and the experiment conducted as described in section 2.2.6. Treatment of A549 (Figure 3.8A) and OKF6/TERT2 (Figure 3.8B) with LPS and tigecycline did not result in any significant reduction in metabolic activity after 24 h.

Figure 3.9 LPS and tigecycline do not adversely affect the metabolic activity of A549 and OKF6/TERT2 cell lines after 24 h incubation.

(A) A549 and (B) OKF6/TERT2 cell lines were stimulated with 0.1 mg/L, 0.4 mg/L and 1.6 mg/L of tigecycline, DMEM and defined-KSFM media alone served as a negative controls for A549 and OKF6/TERT2 cells respectively, LPS (50 mg/L) serving as a positive control. The experiments were all carried out in duplicate and represent the mean and standard deviation of three experiments (n=3). There was variability between both cell lines, however there was no significant reduction of metabolic activity associated with either cell line when stimulated with LPS and varying ranges of tigecycline.



3.2.5 Immunomodulatory effects of tigecycline upon IL-6 production by a pulmonary and oral epithelial cell line.

These experiments were designed to evaluate the immunomodulatory activity of tigecycline against the synthesis of pro-inflammatory cytokine IL-6 by the pulmonary cell line (A, B) A549 and (C, D) oral cell line OKF6/TERT (Figure 3.10 – 3.11). Cells were stimulated with tigecycline at 0.1 mg/L, 0.4 mg/L and 1.6 mg/L \pm 50 mg/L of *E.coli* derived LPS for (A, C) 4 h and (B, D) 24 h. To ensure reliability all experiments were carried out in duplicate on three separate occasions, after 4 and 24 h cell supernatant were stored for analysis with ELISA and genomic material harvested for analysis by RT-qPCR.

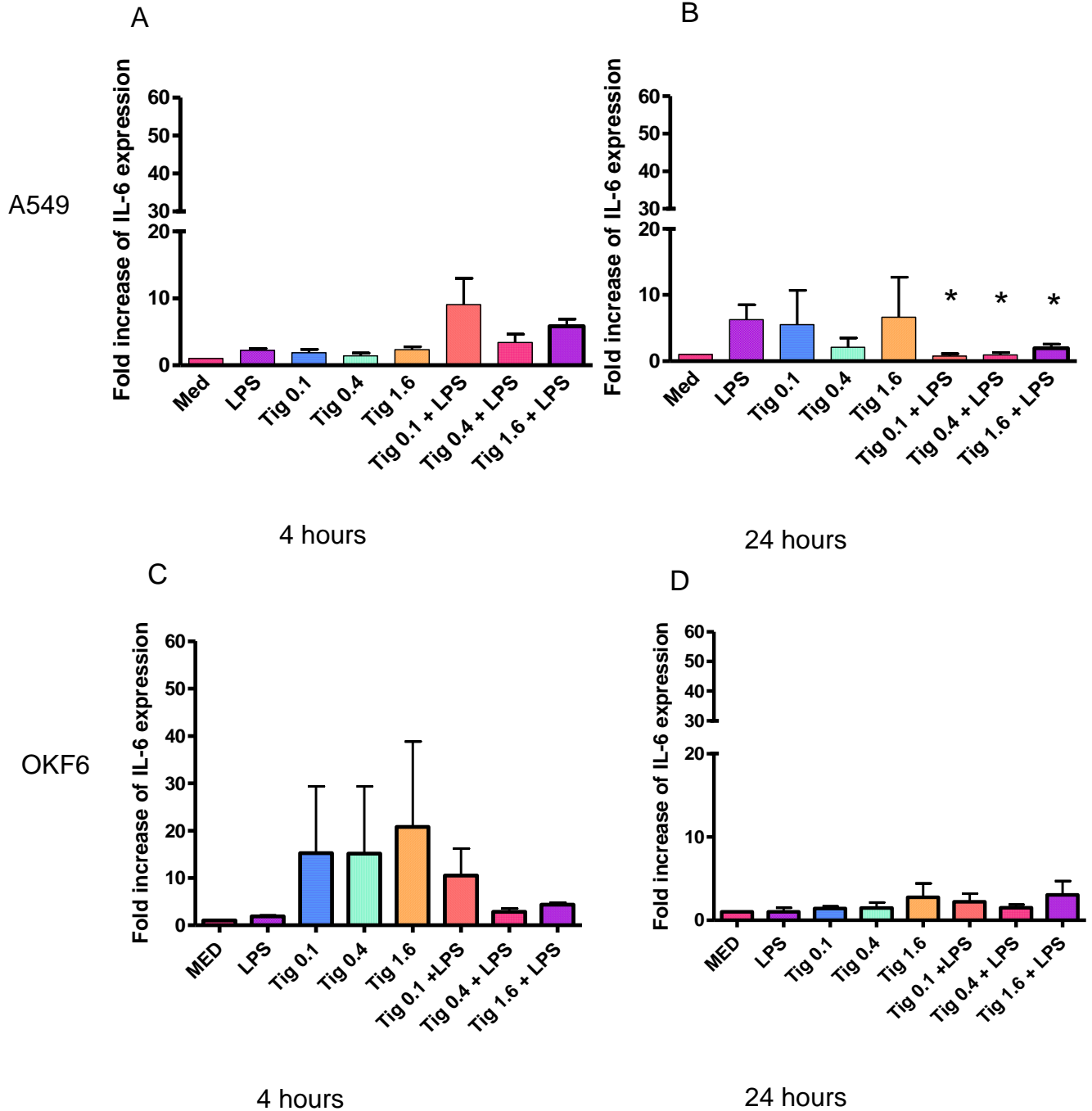
Figure 3.10 IL-6 mRNA expression after exposure to varying concentrations of tigecycline ± LPS after 4 and 24 h.

Figure 3.10(A). After 4 h tigecycline treated A549 cells expressed approximately the same levels of IL-6 when compared to the media control, in contrast, tigecycline + LPS (0.1 / 1.6 mg/L) induced an 8-fold and 5-fold increase in IL-6 expression respectively when compared to the LPS control. This data was not significantly different.

Figure 3.10(B). At the 24 h time point IL-6 expression of tigecycline treated A549 cells showed similar levels of expression to the LPS control, with exception to tigecycline 0.4 mg/L. Tigecycline + LPS treated A549 cells demonstrated a marked decrease in IL-6 expression at all concentrations. Resulting in a significant 4-fold reduction compared to the 4 h time point and significantly different when compared to the LPS control ($p < 0.05$) The decrease in the levels of expression were similar to the media control, with exception to tigecycline 1.6 mg/L + LPS treated cells.

Figure 3.10(C). OKF6 cells stimulated with tigecycline at 0.1 and 0.4 mg/L both increased expression of IL-6 transcripts by 14-fold, those cell treated with tigecycline at 1.6 mg/L producing a 20-fold increase in expression when compared to the media control. When the cell-line was co-stimulated with tigecycline 0.1, 0.4 and 1.6 mg/L + LPS, lower levels of IL-6 expression were observed when compared to the tigecycline (only) treated cells. This data was not statistically different.

Figure 3.10(D). IL-6 expression after 24 h was reduced considerably when compared to the 4 h time point for all treatments with tigecycline alone. All three concentrations caused almost a 6-fold reduction in expression. Tigecycline 0.1 mg/L + LPS also caused a reduction by 3-fold. Expression levels of IL-6 in all cells treated were not significantly different to the media control.



Experiment were carried out in duplicate and represent the mean and standard deviation of three experiments (n=3).

Figure 3.11 IL-6 protein expression after exposure to varying ranges of tigecycline ± LPS after 4 AND 24 h.

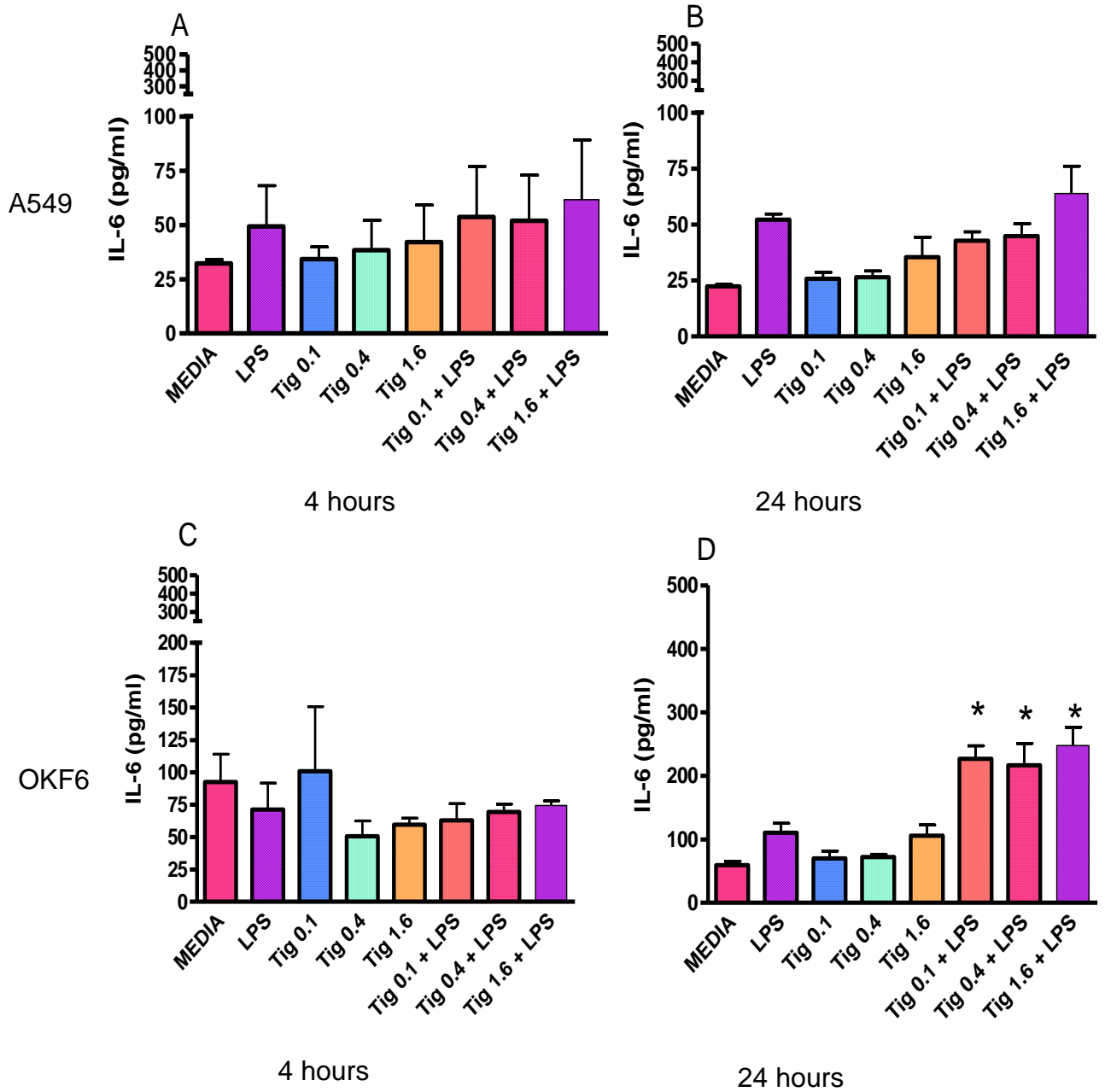
Figure 3.11(A). IL-6 protein expression levels after 4 h from A549 cell line resulted in low levels of expression for all experimental parameters. Cells treated with tigecycline at 0.1 - 1.6 mg/L represented a dose dependent increase of IL-6 which was lower than the LPS control. Stimulation of cells with tigecycline 0.1 mg/L and 0.4 mg/L + LPS resulted in similar levels of expression as the LPS control. Cell treated with tigecycline 1.6 mg/L + LPS appeared to have higher levels of expression of IL-6 when compared to the LPS control. This data was not significantly different.

Figure 3.11(B). After 24 h of stimulation with tigecycline 0.1 - 1.6 mg/L the cells had decreased levels of IL-6 expression. This data was also the case for those cells treated with tigecycline 0.1 - 0.4 mg/L + LPS. However, cells treated with tigecycline 1.6 mg/L + LPS appeared to have higher levels of expression of IL-6 when compared to the LPS control. This data was not significantly different.

Figure 3.11(C). After 4 h IL-6 protein expression by the OKF6 cell-line treated with tigecycline at 0.1 mg/L displayed expression levels similar to the media control, but higher than that of the LPS-stimulated cells control. Cells treated with tigecycline at 0.4 mg/L and 1.6 mg/L expressed similar levels of IL-6, however, they expressed lower levels of IL-6 when compared to the LPS and media controls. Cells treated with tigecycline at 0.1 - 1.6 mg/L + LPS all had similar IL-6 expression levels as the LPS control (60 pg/mL). This data was not significantly different.

Figure 3.11(D). Following 24 h treatment the media control expression levels had reduced by approximately 2-fold and the LPS control had increased by 25 pg/mL above the 4 h time point. IL-6 expression levels of cells stimulated with tigecycline at 0.1 mg/L, 0.4 mg/L and 1.6mg/mL were all lower than the LPS control, this reduction was not statistically significant. Cells stimulated with

tigecycline 0.1 - 1.6 mg/L + LPS all had significantly increased levels of IL-6 after 24 h when compared to the LPS control and 4h time point ($p < 0.05$).



Experiment were carried out in duplicate and represent the mean and standard deviation of three experiments (n=3).

3.2.6 Immunomodulatory effects of tigecycline upon IL-8 produced by pulmonary and oral epithelial cell lines.

These experiments were designed to evaluate the immunomodulatory activity of tigecycline against the synthesis of pro-inflammatory chemokine IL-8 by the pulmonary cell line (A, B) A549 and (C, D) oral cell line OKF6/TERT (Figure 3.12 – 3.13). Cells were stimulated with tigecycline at 0.1 mg/L, 0.4 mg/L and 1.6 mg/L \pm 50 mg/L of *E.coli* derived LPS for (A, C) 4 h and (B, D) 24 h. To ensure reliability all experiments were carried out in duplicate on three separate occasions, after 4 and 24 h cell supernatant were stored for analysis with ELISA and genomic material harvested for analysis by RT-qPCR.

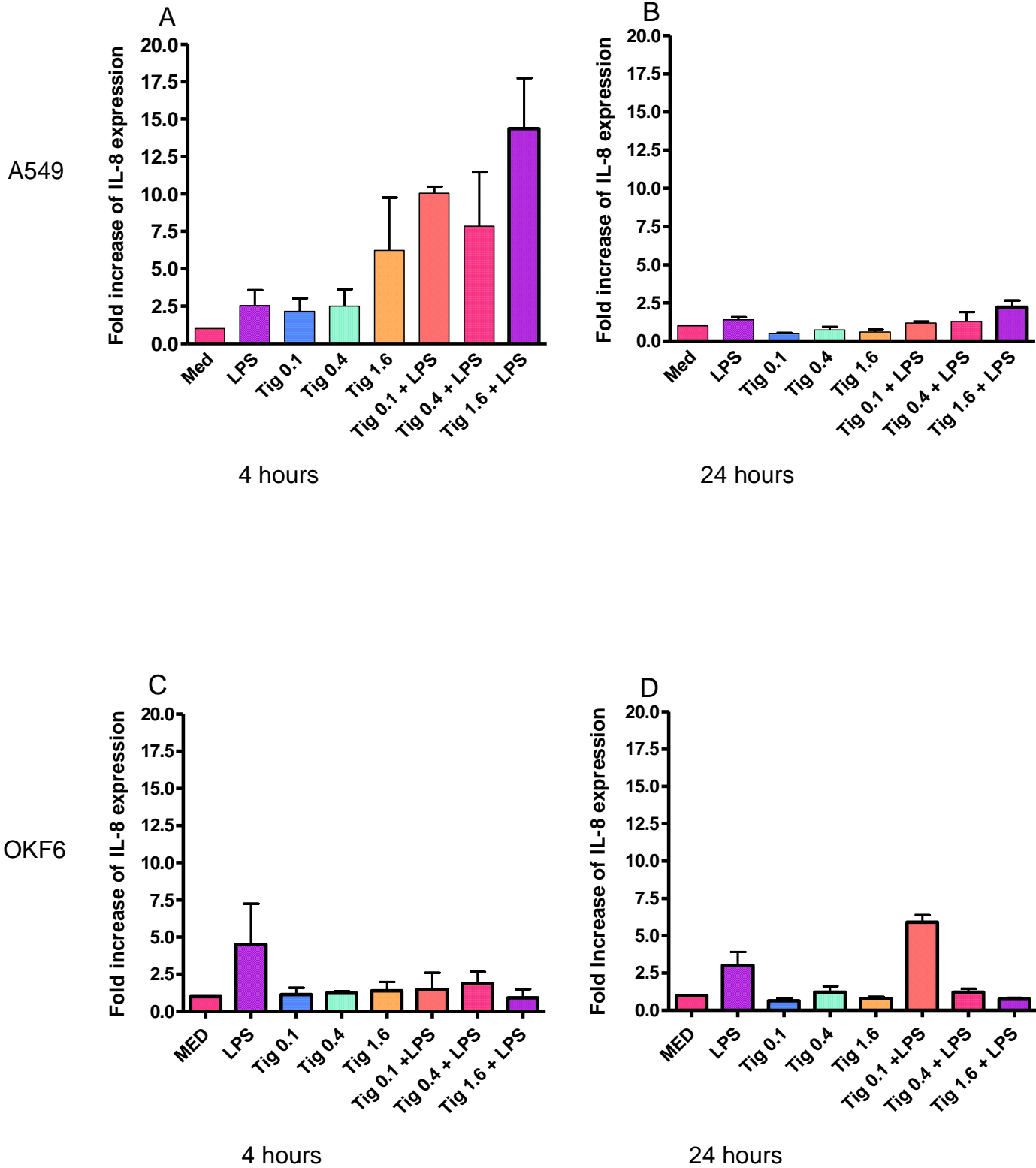
Figure 3.12 IL-8 mRNA expression after exposure to varying ranges of tigecycline ± LPS after 4 and 24 h.

Figure 3.12(A). IL-8 mRNA expression of the A549 cell line after 4 h stimulation with tigecycline at 0.1 and 0.4 mg/L had similar expression levels as the LPS control, in contrast cells treated with tigecycline at 1.6 mg/L were 3-fold higher than the LPS control. Cells treated with tigecycline at 0.1 - 1.6 mg/L + LPS all had higher abundance of IL-8 transcripts when compared to media and LPS controls. When compared to the LPS control cells treated with tigecycline at 0.1, 0.4 and 1.6 mg/L + LPS had a 4-fold, 3-fold and a 5-fold higher expression, respectively, however this data was not significant.

Figure 3.12(B). After 24 h cells stimulated with tigecycline at 0.1, 0.4, and 1.6 mg/L IL-8 mRNA expression levels had reduced by 3-fold, 3-fold, and 6-fold respectively when compared to the 4 h time point. Cells treated with tigecycline at 0.1, 1.4 and 1.6 mg/L had lower levels of expression compared to media and LPS. Cells treated with tigecycline 0.1, 0.4 and 1.6 mg/L + LPS IL-8 gene expression had reduced by 7-fold, 5-fold and 6-fold respectively when compared to the 4 h time point, this data was not significantly different.

Figure 3.12(C). OKF6 cells treated with tigecycline 0.1 - 1.6 mg/L had similar expression levels of IL-8 mRNA compared to the media control after 4 h. Treatment of cells with tigecycline at 0.1 - 0.4 mg/L + LPS have slightly higher levels of expression when compared to the media control and those samples without treatment of LPS, however, all samples have approximately 3-fold reduction of IL-8 expression when compared to the LPS control, this data was not significantly different.

Figure 3.12(D). After 24 h the LPS control had reduced by 2-fold compared to 4 h. Cells treated with tigecycline at 0.1 and 1.6 mg/L expressed lower levels of IL-8 mRNA compared to media control. Levels of expression for cells treated with tigecycline 0.4 and 1.6 mg/L + LPS were approximately the same as the media control, tigecycline 0.1 mg/L + LPS had increased by 4-fold from 4 h expressing a 2-fold increase when compared to the LPS control at 24 h, this data was not significantly different.



Experiment were carried out in duplicate and represent the mean and standard deviation of three experiments (n=3).

Figure 3.13 IL-8 protein expression after exposure to varying ranges of tigecycline ± LPS after 4 and 24 h.

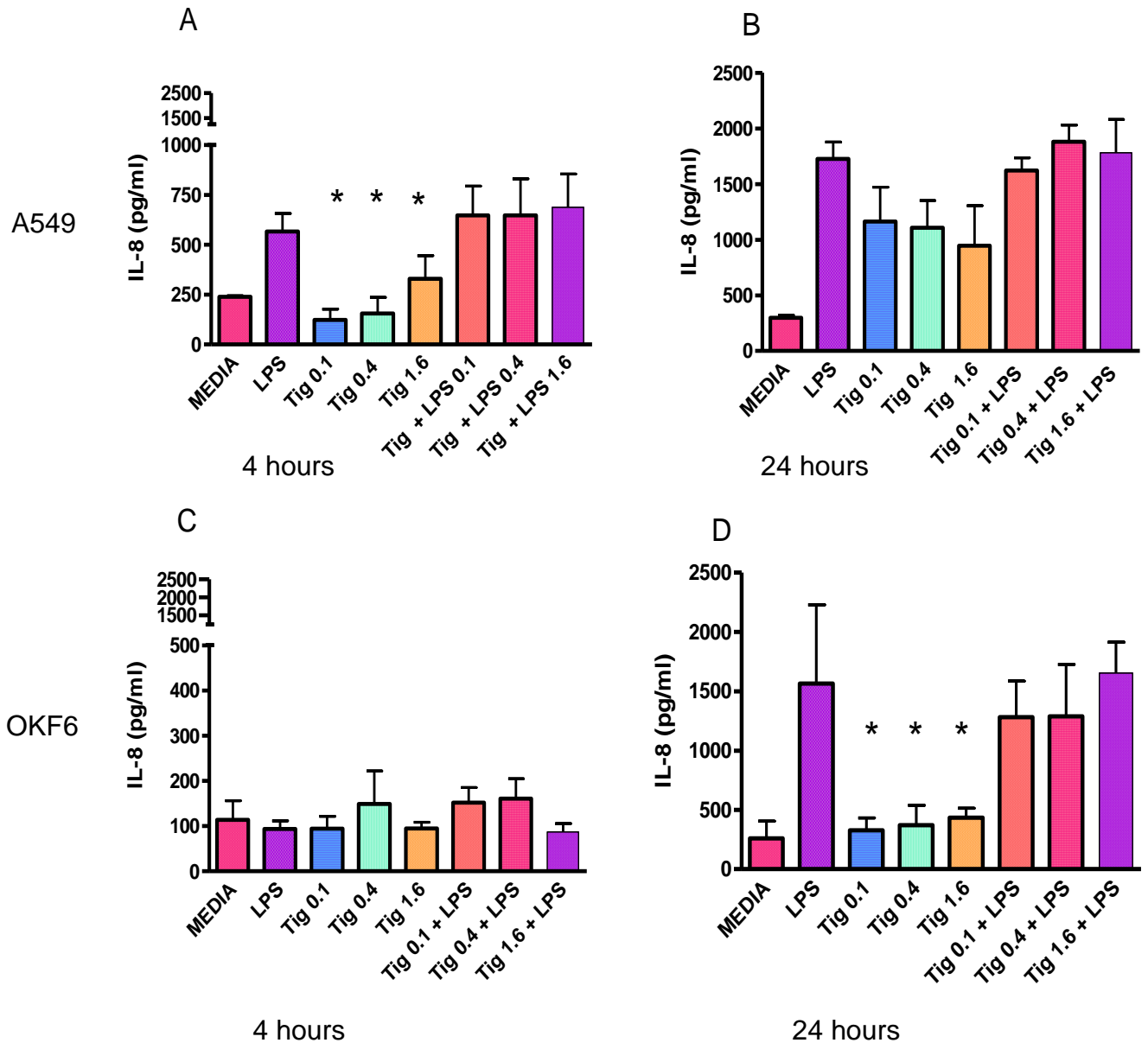
Figure 3.13(A). Lower levels of IL-8 protein were expressed by the A549 cell line after treatment with tigecycline 0.1 to 1.6 mg/L after 4 h when compared to the LPS control ($p < 0.05$). Tigecycline 0.1, 0.4 and 1.6 mg/L reduced IL-8 levels 4-fold, 4-fold and 2-fold, respectively. Cells stimulated with tigecycline 0.1 to 1.6 + LPS all expressed moderate increases in IL-8 protein when compared to the LPS control and those cells treated with tigecycline 0.1 - 1.6 mg/L.

Figure 3.13(B). After 24 h the LPS control had increased IL-8 levels by 3-fold compared to levels detected at 4 h, Cell treated with tigecycline 0.1, 0.4 and 1.6 mg/L exponentially increased in IL-8 expression by 4-fold, 3-fold and 2-fold respectively compared to levels at 4 h, all levels remained lower than the LPS control. Treated cells with tigecycline 1.6 mg/L + LPS had similar levels of protein expression compared to the LPS control. The cells treated with tigecycline 0.1 and 0.4 mg/L + LPS had marginally lower and higher IL-8 levels than the LPS control, respectively, this data was not significantly different.

Figure 3.13(C). After 4 h line media control and LPS control of the OKF6 cell had similar levels of expression. Cells treated with tigecycline 0.1 and 1.6 mg/L had the same levels of expression as the LPS control. Expression levels of cells treated with tigecycline 0.4 mg/L were elevated when compared to the LPS control. Stimulation of cells with tigecycline 0.1 and 0.4 mg/L + LPS had the highest levels of IL-8 expression and tigecycline 1.6 mg/L + LPS had the lowest levels of expression; marginally lower than the LPS control. This data was not significantly different.

Figure 3.13(D). At 24 h cells treated with tigecycline 0.1 - 1.6 mg/L all demonstrate significantly lower expression levels of IL-8 when compared to LPS control, and had slightly higher levels when compared to measurements at 4 h ($p < 0.05$). Cells treated with tigecycline 0.1 and 0.4 mg/L + LPS had both increased by 10-fold compared to 4h time point, those stimulated with

tigecycline at 1.6 mg/L + LPS had increased IL-8 levels 15-fold compared with expression at 4 h and showed a marginal increase in IL-8 expression when compared to the LPS control, this data was not significantly different.

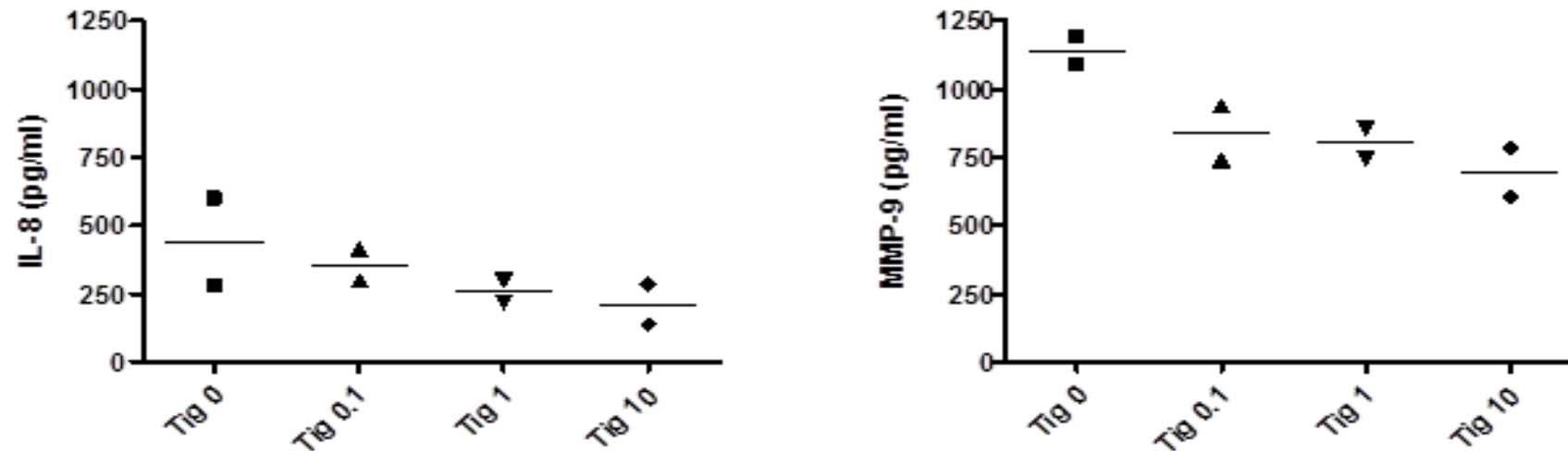


Experiment were carried out in duplicate and represent the mean and standard deviation of three experiments (n=3).

3.2.7 Immunomodulatory effects of tigecycline upon IL-8 and MMP-9 production by human neutrophils.

These experiments aimed to investigate the immunomodulatory effects tigecycline had upon the synthesis of pro-inflammatory mediators IL-8 (Figure 3.14A) and MMP-9 (Figure 3.14B) produced by human neutrophils during an inflammatory response as described in section 2.2.9. An increase of tigecycline reduced expression of IL-8 and MMP-9 synthesis when neutrophils were costimulated with 1 mg/L phorbol 12-myristate acetate (PMA)

Figure 3.14 Immunomodulatory effects of tigecycline to IL-8 and MMP-9 produced by human neutrophils in the presence of 1 mg/L PMA. Human neutrophil cells were stimulated with 0 mg/L, 0.1 mg/L, 1 mg/L and 10 mg/L of tigecycline + PMA (1 mg/L) for 24 hr in microaerophilic conditions (5% CO₂) at 37°C. The experiment was carried out in triplicate (n=2). The supernatant was harvested and the concentration of (A) IL-8 and (B) MMP-9 determined for each sample quantified using antibody specific ELISA. A 10-fold increase of tigecycline concentration inhibited expression of IL-8 and MMP-9 in a dose dependent manner when co-stimulated with 1 mg/L PMA compared to the negative control.



Chapter 4
Discussion

4 Discussion

4.1 Introduction

CF is a disease characterised by bacterial biofilm infection and chronic inflammation of the airways, management of which is both complex and multifactorial. Therefore, establishing new ways of treating this chronic condition with fewer medications is attractive both for the patient, clinician and from an economic standpoint. This study has evaluated a compound, tigecycline, which potentially has the capacity as an antibacterial agent and anecdotally as an immunomodulatory agent. The data from this study has shown that tigecycline has limited activity against planktonic *B. cepacia*, which is contributed to the activity of efflux pump mediated resistance. This is further compounded when *B. cepacia* is growing in a biofilm, which was associated with high-level resistance. Conversely, analysis of stimulated epithelial cells and human neutrophils demonstrated down-regulation of inflammatory mediators, indicating a potential use in CF. Moreover, given the exquisite sensitivity of pathogens associated with periodontal disease to tigecycline, another chronic inflammatory disease, then there is a potential for the compound to be used in the management of periodontitis. These findings will now be discussed.

P. aeruginosa and *B. cepacia* aggregate in mixed biofilms in the lungs of CF patients, both are problematic due to their biofilm forming capacity (Riedel *et al*, 2002; TomLin *et al* 2005). It is well documented that *P. aeruginosa* exhibits resistance to tigecycline. Cheng *et al* (2005) identified that 96% of *P. aeruginosa* isolates tested exhibited degrees of resistance to tigecycline MIC₉₀ 16 mg/L. However, the same study also reported that 67% of *B. cenocepacia* isolates which is a member of the *Burkholderia cepacia complex* (Bcc) as is *B. cepacia* were sensitive to tigecycline (Cheng *et al*, 2005). This is inconsistent with the data reported herein, which indicated that both planktonic and sessile *B. cepacia* and *B. gladioli* of different genovars all exhibited some degree of resistance to tigecycline (n=20). This finding is interesting and clinically relevant as multidrug resistant *B. cepacia* have emerged as a major infection control challenge for CF carers (Govan *et al*, 1996). This was therefore investigated to elucidate a potential mechanism of resistance.

Resistance to antibiotic therapy is generally mediated by the RND type transporters present in many bacterial species (Hasdemir, 2007, Chan *et al*, 2003). Given that tigecycline efflux pump activity had been reported to be involved in *P. aeruginosa* PA01 resistance previously, then it was a logical starting point for *B. cenocepacia* (Dean *et al*, 2003; Gugliera *et al*, 2006). Moreover, Peleg *et al* (2007) identified bloodstream isolates of *Acinetobacter baumannii* with reduced susceptibilities to tigecycline. Peleg *et al* identified that tigecycline susceptible and non-susceptible isolates had a gene coding for the transmembrane component of the AdeABC efflux pump, *adeB*, and the two-component regulatory system comprising *adeS* and *adeR*. They also

identified point mutations in the regulatory system in tigecycline non-susceptible isolates, indicating that an efflux-based mechanism plays a role in reduced tigecycline susceptibility of *A. baumannii* (Peleg *et al*, 2007). *Salmonella enterica* strains have also exhibited decreased susceptibility to tigecycline; the involvement of multidrug efflux pumps and AcrAB regulators are responsible for resistance to tigecycline (Horiyama *et al*, 2010).

Horiyama *et al* investigate the mechanisms by which efflux pumps inhibited those systems, using a broad spectrum efflux pump inhibitor MC-207,110. These experiments are based on work conducted by Lomovskaya *et al* (2001) in which they identified and characterised inhibitors of multidrug resistance efflux pumps in *P. aeruginosa*. In their investigation a broad-spectrum EPI was used that was active against all three known Mex efflux pumps from *P. aeruginosa* MC-207,110. Previous studies have demonstrated MC-207,110 as a potential inhibitor of efflux pumps in Gram-negative bacteria that potentiated the activities of levofloxacin and chloramphenicol (Lomovskaya, 2001). However, according to Chan *et al* (2004), MC-207,110 did not display any advantageous effect on efflux of erythromycin or streptomycin by the BpeAB-OprM pump in *Burkholderia pseudomallei*. In the present study it was shown that MC-207,110 increased sensitivity to tigecycline in members of the *Burkholderia cepacia* complex by over 250 times, demonstrating that these microorganisms use efflux pumps as a resistance mechanism. The potency ($MIC_{90} = 32 \text{ mg/L}$) of tigecycline in the absence of MC-207,110 was similar to the results published by Milatovic and coworkers (2003), but in the presence of MC-207,110 the MICs decreased significantly ($MIC_{90} = 1 \text{ mg/L}$). Direct assessment of the efflux activity, using the sensitive fluorescent ala-nap assay

system demonstrated a correlation between efflux activity and high MIC values, and conversely the lower MICs of *B. gladioli* were correlated with low efflux pump activity. Furthermore, combination therapy with MC-207,110 and tigecycline showed that they acted synergistically to reduce the viability of *B. cepacia* biofilms. Results from these experiments were similar to results demonstrated by Kvist *et al* (2008). Combination therapy of tetracycline and the EPI 1-(1-naphthylmethyl)piperazine significantly reduced susceptibility of *E. coli* to tetracycline and also significantly reduced biofilm formation (Kvist *et al*, 2008). Therefore, these results indicate a dual role of MC-207,100 inhibiting efflux activity resulting in an increased sensitivity to drug treatment and reducing established biofilm biomass of this important CF pathogen.

Overall, the data supports the hypothesis that members of the *B. cepacia* complex utilise efflux pumps which have evolved to become integral to their natural physiological function, and which also confer inherent resistance to tigecycline. However, these pumps can be inactivated using inhibitors to restore the activity of tigecycline. Whether EPIs can be used for combination therapy with tigecycline in CF patients remains to be seen. Using EPIs in combination with tigecycline in CF patients may provide the opportunity for an antibiotic with unrestricted broad-spectrum antimicrobial activity. Of interest, EPIs are undergoing phase I trials in CF patients utilising an aerosol agent for administration and have been well tolerated in humans (Kvist *et al*, 2008). Moreover, another potential benefit of EPIs in combination with an antibiotic is to delay the development of resistance, as the antibacterial effects are achieved at lower concentrations. Nevertheless, tigecycline is disappointing in the context of treating Gram-negative infections of the CF lung, yet given the

potential immunomodulatory properties of the molecule then it may still have a role in CF management.

Tigecycline is derived from tetracycline, which has been shown to have the capacity to modulate inflammatory processes, therefore, has the potential for suppressing inflammation of the CF lung. The rationale for the tigecycline concentrations used in this study was adapted from Golub *et al* (2001), in which 20 mg/L oral doses of doxycycline were used, which was reported to be the equivalent to a therapeutic dose of 2.6 mg/L. Initially, human derived A549 pulmonary epithelial cells and OKF6/TERT2 oral epithelial cells were used, and latterly human neutrophils were used in this study. Tigecycline was shown to have no toxicity in these cell types and was also shown to have no impurities. Low doses of tigecycline did not result in any significant reduction of metabolic activity associated with either cell line, which was critically important as damaged cells may have produced spurious data when in the progression of an inflammatory response initiated by chronic bacterial infections (Laberge *et al*, 2004). Tigecycline's possible immunomodulatory properties were investigated by co-stimulating human derived A549 pulmonary epithelial cells and OKF6/TERT2 oral epithelial cells with 50 mg/L *E. coli* LPS and subinhibitory doses of tigecycline. The rationale of the concentration of LPS used was adapted from Krauker *et al* (2002), which used 100 mg/L to stimulate peripheral blood mononuclear cells and pulmonary A549 epithelial cells during the investigation of pro-inflammatory cytokines and chemokines (Krauker *et al*, 2002). A549 epithelial cells stimulated with 0–200 mg/L of LPS demonstrated a dose dependent increase in IL-8. Based on this 50 mg/L of LPS was used throughout subsequent

investigations. The data obtained from the immunological work produced for both cells lines can be considered to be spurious in some cases. Cells treated with tigecycline \pm LPS displayed contradictory results producing both pro and anti-inflammatory effects. A further point to add is the expression of mRNA after 4 hours represented higher expression levels of transcripts when compared to the 24 h time point (Figure 3.12 A/B). This possibly results from a combination of one or more of the following: 1) the optimal time point for carrying out measurements of mRNA were not identified nor used, 2) the transient effect of the tigecycline and LPS on transcription, 3) the labile nature of mRNA molecules, i.e. they are rapidly degraded in cells 4) the compounds brought about opposite effects on transcription and mRNA stability. Any of these would ensure that changes in mRNA levels did not match protein expression levels. These findings are not in line with evidence reported in literature describing the anti-inflammatory effects of the tetracycline based molecule (Amin *et al*, 1996, Salvatore *et al*, 2009). Gene and protein expression of IL-8 from the A549 cell line was drastically lower than that seen in the literature when stimulated with LPS alone, Krauker (2002) displayed IL-8 expression levels in excess of 5000 pg/ml after 24 hours when stimulated with *E.coli* LPS compared to the 2000 pg/ml expressed in these investigations from the A549 cell line (Standiford *et al*, 1990; Krauker *et al*, 2002). However, Ultra pure LPS do not always elicit an effect in epithelial cells (Hedlund *et al*, 2001). Older preparations contain contaminants that assist LPS in stimulating TLRs. In some instances treatments with tigecycline alone did result in a significant reduction of IL-8 from both cell lines (figure 3.13A/D ($p < 0.05$)). However, these results may not prove fruitful as they represent tigecycline

treatments independently of co-stimulation with LPS, which doesn't represent a true model of infection. Therefore these results prove to be of little benefit and do not display immunomodulatory properties associated with inflammatory disease. Expression of IL-6 mRNA and protein serum levels were also low when expressed from treated A549 cells. These findings are in line with an investigation conducted by Yang (2003) which reported low levels of IL-6 mRNA and supernatant protein that were not significantly altered following infection. Figure 3.10B is the only experiment to display a significant pro-inflammatory effect of IL-6 mRNA transcripts following from co-stimulation with tigecycline and LPS at all three tigecycline concentrations compared to the LPS control. However as with the IL-8 result these data was contradictory to changes in the levels of protein expression, which indicted marginal pro-inflammatory effects. Taking these results into consideration it may have proven advantageous to use an LPS derived from the common CF pathogens or the bacteria themselves, which would have truly reflected an accurate cytokine and chemokine response during these investigations. *B. cepacia* LPS and *P. aeruginosa* LPS are available for use *in vitro*. *B. cepacia* has been shown to be 9 times more potent than *P. aeruginosa* LPS, inducing A549 cells to significantly secrete IL-8 in a more potent manner compared to *P. aeruginosa* (Hendry *et al*, 1999, Reddi *et al*, 2003).

Cytokine and chemokine production by oral epithelial cells plays a major role in the recruitment and activation of professional phagocytes in the diseased periodontium. Inflammatory cytokines such as IL-6, IL-1b and IL-8 have been reported to be higher in patients with periodontal disease than in healthy subjects (Cardoso *et al*, 2009). In the present investigation the response of

OKF6 cell line to treatment with tigecycline \pm LPS appeared to generate spurious data. As with the A549 cells, the OKF6 cell line produced pro- and anti-inflammatory responses to the same stimuli. Figure 3.10A depicts IL-6 mRNA production at 4 h, showing what may be considered to be a pro-inflammatory effect of low dose tigecycline, however when this was compared to the 24 h time point the high levels of IL-6 had reduced to the same level of expression as the LPS control. These contrasting results might be attributed to similar mechanisms as described above for the A549 cell lines. Levels of IL-8 production recorded in figure 3.13D are higher than those reported by Dongari-Bagtzoglo (2003). The work conducted by Dongari-Bagtzoglo (2003) directly infected OKF6 cell line with whole cell *Candida albicans* for 24 h after which approximately 500 pg/ml of IL-8 was produced. In contrast results, obtained within this report by OKF6 cell treated with *E.coli* LPS was recorded at 1500 pg/ml. This may be that greater numbers of cells were added to culture dishes in this study and or subtle differences in the culture conditions which had a stimulatory effect on cytokine production.

Cells treated with tigecycline and *E.coli* LPS recorded similar expression levels of IL-8, therefore, results suggested that low doses of tigecycline did not exert immunomodulation. However treatment with low doses of tigecycline alone appeared to have an anti-inflammatory effect by reducing the abundance of mRNA transcripts (figure 3.12D), and these are in-line with the reductions in the level of IL-8 recorded in figure 3.13D. The contradictory results in expression post-stimulation may be due to the choice of LPS. It seems reasonable to speculate that treatment with a whole cell stimulus such

as with a bacterium commonly associated periodontal such a *P. gingivalis* (Domisch *et al*, 2010) may have been a better choice. The immunology investigations in this study were somewhat disappointing, levels of IL-6 and IL-8 recorded in the results contained combination therapy of tigecycline and LPS which did not significantly reduce these pro-inflammatory mediators, displaying low levels of immunomodulation for both cell lines investigated. However, the cell types were epithelial and not a professional immune cell, so work then progressed to investigate human neutrophils.

Neutrophils are the origin of destructive enzymes in inflamed tissue, which are rapidly recruited to sites of inflammation, and are a key cell responsible for clearance of bacterial infections (Koller *et al*, 2009). It was of interest to investigate if tigecycline may be able to reduce pro-inflammatory mediators produced by these professional immune cells. Tigecycline displays rapid intracellular penetration into neutrophils with concentrations reaching 20 – 30 times higher when compared to extracellular drug concentrations (Ong *et al*, 2005). This may indicate why these low antimicrobial concentrations display immunomodulatory properties for neutrophil production of IL-8 and MMP-9, which in this study were reduced in a dose dependant manner. Indeed, an initial decrease was evident at 0.1 mg/L, below the 2.6 mg/L therapeutic dose used in Periostat®. The exquisite nature of low dose concentrations tigecycline to modulate neutrophil derived IL-8 and MMP-9 is very intriguing, however this data can not be evaluated statistically due to the low number of replicates in this study. Neutrophil derived MMPs degrade all protein components of the extracellular matrix, studies have indicated that MMP-9 is

up-regulated in the bronchial compartment of the CF lung after tissue inflammation, the increased expression of tissue remodeling enzymes at sites of inflammation are linked to impaired lung function (Roderfeld *et al*, 2009). Moreover, Gaggar and coworkers (2008) reported that MMP-8 and MMP-9 are both involved in the production of peptide proline-glycine-proline, which acts as a strong ECM derived neutrophil chemoattractant and could contribute to neutrophil influx and airway damage in CF patients (Gaggar *et al*, 2008). As MMPs are viewed as key extracellular processing enzymes that regulate cell responses and signaling it is interesting that low doses of tigecycline may provided a beneficial treatment for disease symptoms caused by MMPs (Kassim *et al*, 2007). All MMPs are expressed at low levels in several types of tissue, even in the absence of clinical inflammation, an increase in expression levels may account for destruction of soft tissue in the clinical symptoms of CF (Dursun *et al*, 2001; Gaggar *et al*, 2008; Roderfeld *et al*, 2009; Wong *et al*, 2009). Acute pulmonary infections with *P. aeruginosa* induce MMP-7 and MMP-10, suggesting discrete roles for MMPs in airway epithelial cell regulation of immune responses, cell proliferation, cell death, and other process key to initial host defences against bacterial infections (Kassim *et al*, 2007). Roderfeld *et al* (2009) showed that serum derived from adult CF patients increased MMP-8 and MMP-9 expression, after antibiotic treatment active MMP-9 protein levels in serum were significantly reduced compared to a placebo control group (Roderfeld *et al*, 2009). These findings support the *in vitro* observations reported in this present study, and may lend support for the use of tigecycline in CF and other chronic inflammatory diseases.

Periodontal disease displays similar inflammatory processes to CF given the complex microbial ecology, clinical symptoms of an exacerbated immune response, inflamed tissues linked to neutrophil activity and the presence of MMPs often found in oral serum samples of patients suffering from oral inflammatory disease (Manicone and McGuire, 2008). In the present study it was shown that tigecycline has activity against several key periodontal pathogens growing as biofilms, supporting its potential dual role in periodontal therapy, similar to Periostat®. MMPs are involved in the physiological turnover of periodontal tissue and appear to be involved in tissue destruction. Neutrophil derived MMP-8 and MMP-9 are the main proteinases related to tissue destruction and remodelling events in periodontal tissue (Choi *et al*, 2004). Work by Garlet and coworkers (2006) showed that increased levels of IFN- γ were directly associated with an increase in the expression of MMP-2 and MMP-9 from 24 h until 15 days of infection with *A. actinomycetemcomitans* (Garlet *et al*, 2006). If tigecycline is able to reduce these mediators at low doses, it might be able to provide some relief to patients who suffer the side effects of IL-8 induced neutrophil migration and excessive neutrophil activity (Krakauer 2002). Doxycycline is a similar tetracycline based molecule and has been used extensively in periodontal therapy (Alpagot *et al*, 2001/2006; Choi *et al*, 2004; Emingal *et al*, 2004a/b, Golub *et al*, 2001; Gu *et al*, 1996; Preshaw *et al*, 2004b). A sub-antimicrobial dose of Periostat® (doxycycline) at 20 mg has been reported to be a safe and effective adjunct when taken twice daily for at least 3 months and up to 24 months in a randomized placebo controlled clinical trial (Caton and Ryan 2011). Periostat® is currently the only FDA approved inhibitor of MMP's

implicated in plaque-induced pathologic degradation of connective tissue collagen of the supporting structures of the periodontium (Caton and Ryan 2011). Current studies indicate the potential benefits of the immunomodulatory properties of SDD (Caton *et al*, 2001; Choi *et al*, 2004; Novak *et al*, 2002, Raza *et al*, 2006). Emingil *et al* (2011) demonstrated that SDD can stabilise the inflammatory response by significantly reducing IL-6, TNF- α and MCP-1 in GCF samples from patient with chronic periodontitis (Emingil *et al*, 2011). Therefore, it was of interest to evaluate if any immunomodulatory properties were associated with tigecycline. As well as the reported immunomodulatory properties, SDD has also been shown to be a positive inhibitor of MMPs associated with chronic inflammatory disease. Currently this is the only pharmacological MMP-inhibitor approved by the FDA, and has shown to reduce the severity of chronic periodontitis whilst avoiding the long term side effects associated with tetracycline therapy (Lee *et al*, 2004). Thomas and coworkers (2000) demonstrated that long-term use of SDD does not lead to changes in antimicrobial susceptibility, demonstrating the potential positive role for this antibiotic (Thomas *et al*, 2000). Choi and coworkers (2004) investigated the use of SDD and reported that after 120 days of scaling and root planning combined with SDD that this resulted in a reduction in both MMP8 and MMP9. The group concluded that SDD therapy did not have a quantifiable antimicrobial affect and caused no apparent resistance, indicating that this type of therapy can be used safely and effectively (Choi *et al*, 2004). Similarly, Salvatore and coworkers (2009) reported that administering tigecycline at 10 mg/kg in a *Mycoplasma pneumoniae* pneumonia murine model significantly reduced key pro-

inflammatory cytokines and chemokines (IL-1 β , IL-12, IFN- γ and TNF- α) over a 6 day period (Salvatore *et al*, 2009). As well as SDD, oral macrolide antibiotics possess immunomodulatory activity and have shown to down regulate prolonged inflammation. Indeed, randomised low dose azithromycin improved the clinical symptoms of CF lung disease prior to colonisation with *P. aeruginosa* (Fayon, 2006).

Conclusion

In this study these experiments have demonstrated:

- The poor antimicrobial effects of tigecycline against planktonic and sessile bacterial cells associated with inflammatory disease of the lung and oral cavity.
- An increased sensitivity to these planktonic and sessile pathogens to tigecycline when incorporated with a competitive EPI.
- The exquisite effects of EPI on disrupting biofilm biomass.
- At subinhibitory concentrations tigecycline did not demonstrate any significant immunomodulation of pro-inflammatory cytokines and chemokines expressed by two clinical relevant cell lines.
- However, subinhibitory doses of tigecycline did reduce mediators involved with inflammation expressed by neutrophils.

In particular this study had demonstrated the effects of a single antibiotic compound, tigecycline, in combination with an EPI was investigated on single spp. biofilms, which was found to be advantageous in reducing antibiotic susceptibility. Chronic inflammatory diseases are characterised by a persistent and dysregulated inflammatory response, which are associated with the presence of established bacterial biofilms and an exaggerated host immune response. An EPI in combination with tigecycline has demonstrated positive effects on disrupting biofilm biomass and increasing sensitivity of bacterial spp. to tigecycline, thus indicating that EPIs could constitute to a new class of antibiotics. To expand upon this microbiological work it would be of interesting to evaluate the effects of multiple EPI on multiple species biofilm.

This could encompass investigating the effects these compounds may have on biofilm formation, relative biomass and sensitivity to dual and triple combination therapy with different antibiotics. Furthermore, subinhibitory concentrations of antibiotic compounds could be utilised to determine if low doses have any effect of QS molecules involved in biofilm formation. Microarray assays could be employed to determine which genes are down or up regulated in the genome of these organisms and what part these may play in biofilm mediated disease. In addition to these microbiological aspects, tigecycline at subinhibitory concentrations has been demonstrated to reduce pro-inflammatory mediators associated with neutrophils (IL-8 and MMP-9). Therefore, tigecycline could potentially be classed as an immune modulator, and may provide a beneficial treatment to reduce key MMPs associated with other inflammatory diseases.

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