

In vitro ACTIVITY OF
TOBRAMYCIN, AMILORIDES
AND OTHER NON-ANTIBIOTICS
AGAINST *Pseudomonas aeruginosa*
AND *Burkholderia cenocepacia*

PUTHAYALAI TREERAT

2008

MASTER OF SCIENCE (RESEARCH)

Submitted in fulfilment of the requirements for the
degree of Master of Science (research) at the
University of Technology, Sydney

CERTIFICATE OF AUTHORSHIP/ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree, nor has it been submitted as part of the requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Signature of Student

Production Note:

Signature removed prior to publication.

ACKNOWLEDGEMENTS

First of all, I am very grateful my supervisor, Dr Tony George, for all his endeavours in assisting and guiding me throughout the time of my study. Secondly, I would like to thank my co-supervisors, Associate Professor Peter Middleton, Associate Professor Jon Iredell, and Dr Fred Widmer, from the Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clinical Pathology and Medical Research (ICPMR), Westmead Hospital, for kindly giving the clinical strain of *Burkholderia cenocepacia*, verapamil, essential information, including journals, and generous support in relation to thesis publication by the *Journal of Antimicrobial Chemotherapy*. I would also like to thank my co-supervisor Dr. Rachel Shepherd for kindly assisting me on doing my thesis. Moreover, I would really like to thank Pat Skinner for thoughtfully helping me with my writing and for proofreading my thesis. Finally, I would like to thank my family, friends, and all the staff in Building 4, especially Rochelle Seneviratne, for all their support and encouragement.

TABLE OF CONTENTS

CHAPTER ONE: LITERATURE REVIEW	1
1.1 INTRODUCTION AND OVERVIEW	1
1.2 CYSTIC FIBROSIS	2
1.3 COMPLICATIONS OF CYSTIC FIBROSIS	4
1.3.1 Chronic Bacterial Infection in Cystic Fibrosis	4
1.3.2 Biofilm Formation and Quorum-sensing of <i>Pseudomonas aeruginosa</i>	6
1.3.3 Efflux Pump Systems of <i>Pseudomonas aeruginosa</i>	7
1.3.4 Infection Caused by <i>Burkholderia cepacia</i> Complex	8
1.4 ANTIMICROBIAL AGENTS	10
1.4.1 β -lactams	10
1.4.2 Quinolones	10
1.4.3 Aminoglycosides	12
1.5 TREATMENT REGIMES FOR RESISTANT PATHOGENIC BACTERIA	14
1.5.1 The Amilorides and Analogues	15
1.5.2 Other Adjunctive Non-antibiotic Compounds	16
1.5.3 Combination (Adjunctive) Therapy	19
1.6 HYPOTHESIS AND RATIONALE	20
1.7 PROJECT AIMS	21

CHAPTER TWO: MATERIALS AND METHODS	22
2.1 MATERIALS	22
2.1.1 Chemicals, Media, and Microtitre Plates	22
2.1.2 Bacterial Strains	22
2.2 METHODS	23
2.2.1 Preparation of Stock and Working Solutions of Antibiotics, other Drugs, and Salts	23
2.2.2 Preparation of Bacterial Cell Suspensions	24
2.2.3 Minimum Inhibitory Concentration of Antibiotics against <i>P. aeruginosa</i> or <i>B. cenocepacia</i> by the Broth Microdilution Method	24
2.2.4 Minimum Inhibitory Concentrations of Antibiotics in Dimethyl Sulfoxide	26
CHAPTER THREE: RESULTS	29
3.1 MIC OF ANTIBIOTICS AND NON-ANTIBIOTICS	29
3.1.1 Tobramycin and Amikacin	30
3.1.2 Amiloride and Derivatives	32
3.1.3 Salbutamol	34
3.1.4 Verapamil and Amlodipine	35
3.2 COMBINATION (ADJUNCTIVE) THERAPY	38
3.2.1 Tobramycin with Amiloride, Benzamil, and Phenamil	39
3.2.2 Tobramycin, Amlodipine, Verapamil	44
3.2.3 Tobramycin and Salbutamol	47
3.2.4 Combinations of Three Drugs	48
3.3 SODIUM ANTAGONISM OF TOBRAMYCIN ACTIVITY	56
3.3.1 Effect of Sodium Chloride	56

3.3.2	Effect of Sodium Gluconate	58
3.3.3	Effect of Potassium Chloride	60
3.3.4	Effect of D-Mannitol	62
3.3.5	Effect of N-methyl-D-glucamine	64
3.4	SODIUM ANTAGONISM OF TOBRAMYCIN IS PARTIALLY REVERSED BY AMILORIDE	68
3.4.1	Tobramycin, Amiloride and Sodium Chloride	68
3.4.2	Tobramycin, Amiloride and Sodium Gluconate	70
3.4.3	Tobramycin, Amiloride and Potassium Chloride	72
3.4.4	Tobramycin, Amiloride and D-Mannitol	74
3.4.5	Tobramycin, Amiloride and N-methyl-D-glucamine	76
3.5	EFFECT OF ADJUNCTIVE COMPOUNDS ON AMIKACIN MICs	79
3.5.1	Two or Three Drug Combinations	79
3.6	ADJUNCTIVE AGENT COMBINATIONS AGAINST CYSTIC FIBROSIS ISOLATES OF <i>Pseudomonas aeruginosa</i> RESISTANT TO TOBRAMYCIN	82
3.6.1	Tobramycin MICs	82
3.6.2	Tobramycin-non-antibiotic combinations against PSM1, PSM2, PSS1 and PSS2 <i>P. aeruginosa</i> clinical cystic fibrosis isolates	82
	CHAPTER FOUR: DISCUSSION	89
4.1	HIGH POTENCY OF AMIKACIN COMPARED TO TOBRAMYCIN	90
4.2	SYNERGISTIC EFFECT OF TOBRAMYCIN WITH THE AMILORIDES	91

4.3	SYNERGISTIC EFFECTS OF OTHER ADJUNCTIVE NON-ANTIBIOTICS WITH TOBRAMYCIN	93
4.4	ANTAGONISTIC EFFECT OF SODIUM ON ACTIVITY OF TOBRAMYCIN	96
4.5	SYNERGISTIC EFFECTS OF TOBRAMYCIN AND NON-ANTIBIOTICS AGAINST CLINICAL ISOLATES FROM CYSTIC FIBROSIS PATIENTS	99
4.6	CONCLUSION	101
	BIBLIOGRAPHY	102

FIGURE LEGENDS

Figure 1.2.1	Incidence of cystic fibrosis around the world.	2
Figure 1.4.1	Chemical structures of tobramycin and amikacin.	13
Figure 1.5.1	Chemical structures of unprotonated and protonated forms of amiloride.	16
Figure 1.5.2	Chemical structures of benzamil and phenamil.	16
Figure 1.5.3	Chemical structure of salbutamol.	17
Figure 1.5.4	Chemical structure of verapamil, a calcium channel blocker.	18
Figure 1.5.5	Structure of amlodipine.	18
Figure 2.1	Pattern of filling wells of a microtiter plate for the MIC assays.	26
Figure 2.2	Evaluation of the effect of 0.1% of dimethyl sulfoxide (DMSO) in MHB without two aminoglycoside antibiotics against <i>Pseudomonas aeruginosa</i> NCTC 10662 and <i>Burkholderia cenocepacia</i> .	27
Figure 2.3	Evaluation of tobramycin or amikacin dihydrate activity under 0.1% of dimethyl sulfoxide (DMSO) in MHB against <i>Pseudomonas aeruginosa</i> NCTC 10662 and <i>Burkholderia cenocepacia</i> .	28
Figure 3.1.1	Comparison between tobramycin and amikacin MICs against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	31

Figure 3.1.2	Comparison of benzamil and phenamil MICs against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	33
Figure 3.1.3	MICs of salbutamol against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	35
Figure 3.1.4	Verapamil MICs against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	37
Figure 3.1.5	Amlodipine MICs against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	38
Figure 3.2.1	Comparison of tobramycin combining with 0.1 mM or 1 mM of amiloride against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	41
Figure 3.2.2	Tobramycin with different concentrations of benzamil.	42
Figure 3.2.3	Tobramycin with different concentrations of phenamil against both microorganisms.	43
Figure 3.2.4	Combination between tobramycin and 0.03 or 0.06 mM of amlodipine against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	45
Figure 3.2.5	Tobramycin MICs for <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> in combination with 2 mM verapamil.	46
Figure 3.2.6	Combination between tobramycin and salbutamol against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	48

- Figure 3.2.7** Combinations of tobramycin, 2 mM salbutamol, and 2 mM of verapamil; and of tobramycin, 2 mM salbutamol, and 1 mM amiloride against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 50
- Figure 3.2.8** Combinations of tobramycin, 2 mM salbutamol, and 0.125 or 0.25 mM benzamil against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 51
- Figure 3.2.9** Tobramycin, 2 mM salbutamol, and 0.03 mM amlodipine; and tobramycin, 2 mM salbutamol, and 0.06 mM amlodipine against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 52
- Figure 3.3.1** Effect of different sodium chloride concentrations on tobramycin MICs for *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 57
- Figure 3.3.2** Effect of different concentrations of sodium gluconate on increasing tobramycin MICs for *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 59
- Figure 3.3.3** Effect of different concentrations of potassium chloride on increasing the tobramycin MICs for *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 61
- Figure 3.3.4** Effect of different concentrations of D-mannitol on tobramycin MICs for *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 63
- Figure 3.3.5** Comparison between adjusted pH at 7.2 and unadjusted pH of N-methyl-D-glucamine (NMDG) concentrations by measuring the growth of *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 65

- Figure 3.3.6** Effect of different N-methyl-D-glucamine concentrations from 0.125 mM to 1 mM on tobramycin MICs for *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*, and from 3.125 mM to 50 mM on tobramycin MICs for *P. aeruginosa* and *B. cenocepacia*. 66-67
- Figure 3.4.1** Combination of tobramycin and 1 mM amiloride in different concentrations of sodium chloride against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 69
- Figure 3.4.2** Combination of tobramycin and 1 mM amiloride in different concentrations of sodium gluconate against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 71
- Figure 3.4.3** Combination of tobramycin and 1 mM amiloride in different concentrations of potassium chloride against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 73
- Figure 3.4.4** Combination of tobramycin and 1 mM amiloride in different concentrations of D-mannitol against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 75
- Figure 3.4.5** Combination of tobramycin and 1 mM amiloride in different N-methyl-D-glucamine (NMDG) concentrations from 0.125 mM to 1 mM and from 3.125 mM to 50 mM against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 77-78
- Figure 3.5.1** Combination of amikacin with 1 mM amiloride against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 80
- Figure 3.5.2** Comparison between three drug combinations: amikacin serial dilutions, 2 mM salbutamol and 1 mM amiloride; and amikacin, 2 mM salbutamol and 2 mM verapamil against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. 81

Figure 3.6.1	MICs of tobramycin alone or in the combinations with non-antibiotic compounds against <i>P. aeruginosa</i> PSM1.	84
Figure 3.6.2	MICs of tobramycin alone or in the combinations with non-antibiotic compounds against <i>P. aeruginosa</i> PSM2.	85
Figure 3.6.3	MICs of tobramycin alone or in the combinations with non-antibiotic compounds against <i>P. aeruginosa</i> PSS1.	85
Figure 3.6.4	MICs of tobramycin alone or in the combinations with non-antibiotic compounds against <i>P. aeruginosa</i> PSS2.	86

TABLES

Table 1.3.1	Nomenclature of <i>Burkholderia cepacia</i> complex.	9
Table 1.4.1	Antibiotics commonly used in the treatment of lung infection in cystic fibrosis patients.	11
Table 3.1	Minimum inhibitory concentration (MIC) for tobramycin, amikacin, and different non-antibiotic compounds used in this study against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	29
Table 3.2	Comparison of tobramycin MICs ($\mu\text{g/ml}$ and μM) in various combinations with non-antibiotic compounds, amiloride, benzamil, phenamil, salbutamol, verapamil, or amlodipine, to tobramycin itself against <i>Pseudomonas aeruginosa</i> and <i>Burkholderia cenocepacia</i> .	53-55
Table 3.3	Minimum inhibitory concentrations for tobramycin and non-antibiotic compounds against four cystic fibrosis strains of <i>P. aeruginosa</i> (PSM1, PSM2, PSS1, PSS2).	87
Table 3.4	Comparison of tobramycin MICs in various combinations with non-antibiotic compounds, amiloride, benzamil, salbutamol, or amlodipine, four cystic fibrosis strains of <i>P. aeruginosa</i> (PSM1, PSM2, PSS1, PSS2).	88

ABBREVIATIONS

<i>arr</i>	aminoglycoside response regulator
BCESM	<i>Burkholderia cepacia</i> epidemic strain marker
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane regulator
cfu	colony forming unit
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
IL	interleukin
MHB	Mueller-Hinton broth
MIC	minimum inhibitory concentration
mRNA	messenger RNA
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
NMDG	N-methyl-D-glucamine
PBPs	penicillin-binding proteins
QS	quorum-sensing
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SD	standard deviation
SEM	standard error of the mean

ABSTRACT

Chronic respiratory infection, mainly caused by *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex, is the major cause of complications and eventually of death in patients with cystic fibrosis. These problems are exacerbated by drug resistance mechanisms induced in the infectious microorganisms, and by persistence of the microorganisms by sequestration in viscous mucus or biofilms. The sequestration prevents effective antibiotic access to the bacteria. Such problems have led to the search for alternative treatments and therapies, but none of these alternative techniques have yet been tested rigorously or successfully in clinical patients. In this project, we used a standard strain of *P. aeruginosa* (NCTC 10662) and a *B. cenocepacia* isolate from cystic fibrosis sputum to appraise tobramycin/amikacin efficacy in combination with clinically relevant concentrations of the adjunctive agents amiloride, benzamil hydrochloride, phenamil, salbutamol, verapamil, and amlodipine. Altered conditions in the cystic fibrosis lung were simulated by using different concentrations of sodium chloride, potassium chloride, sodium gluconate, D-mannitol, and N-Methyl-D-glucamine. Benzamil hydrochloride was the most potent additive compound against the organisms tested; enhancing the antibacterial effect of tobramycin. A sub-inhibitory concentration of amlodipine was only marginally useful, even though its minimum inhibitory concentration (MIC) against both microbes was the lowest of all the non-antibiotic compounds tested. Conversely, salbutamol, verapamil, and amlodipine were antagonistic in some combinations with tobramycin. Amikacin was generally more potent than tobramycin. Sodium and potassium chlorides and sodium gluconate increased the tobramycin MIC up to 8-fold at salt concentrations from 50–400 mM. This antagonistic effect of cations appeared to be partially reversed by adding amiloride, verapamil, or salbutamol. This study needs to be extended by further assays with more clinical isolates, but it has shown that non-antibiotic adjunctive agents can be used with antibiotics to produce effective results *in vitro*; and potentially *in vivo* as an alternative regime for the treatment of chronic airway infections in cystic fibrosis patients.