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

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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.

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ABBREVIATIONS

arr  aminoglycoside response regulator
BCESM  *Burkholderia cepacia* 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 *Staphylococcus aureus*
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.
CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION AND OVERVIEW

Although cystic fibrosis and its complications have been progressively studied for decades in order to elucidate its pathogenesis and proper treatment, it is still one of the most severe genetic disorder diseases found in Caucasian populations (Boucher, 2004). About 30,000 children and adults in the United States manifest a variety of symptoms of this disease and at least 1,000 new cases are detected annually (http://www.cff.org viewed in April, 2006; http://www.who.org viewed in January, 2006). Chronic respiratory infection in cystic fibrosis primarily resulted from accumulation of desiccated mucus secretion, failure to eliminate pathogens from the cystic fibrosis lungs, and obstruction of respiratory ducts appears to be the major common cause of death in cystic fibrosis individuals (Tummler and Kiewitz, 1999; McAuley and Elborn, 2000; Lyczak et al., 2002; Chmiel and Davis, 2003). Only long-term antibiotic treatment for such chronic infection appears to be ineffective, conversely, induce the occurrence of antimicrobial resistance in several pathogens (Chow et al., 1989; Burns et al., 1989; Barclay et al. 1996; Cheng et al., 1996; Hancock, 1998; Ziha-Zarifi et al., 1999; Al-Aloul et al., 2004; Bagge et al., 2004a). Moreover, recurrent infection and virulence factors of such intractable antibiotic-resistant pathogens are predominantly responsible for the aetiology of disease, debilitation, and the increase of morbidity and mortality in patients (Burns et al., 1996a; Burns et al., 1996b; Carmeli et al., 1999; Lyczak et al., 2002; Chmiel and Davis, 2003). Combination of different antimicrobial agents, or of antimicrobials and non-antimicrobials, have been examined as alternative regimes for treatment, however, such chronic and recurrent infection appears to be incompletely eliminated (Govan and Deretic, 1996; Aaron et al., 2000; Walters III et al., 2003; Aaron et al., 2005). Furthermore, it remains uncertain about the optimal use of antibiotics to treat such intractable infection in terms of the optimal dose and also duration therapy (Ratjen,
2001a). Thus, it needs to be extended finding of the compatible combinations between antimicrobial and non-antimicrobial agents in order to effectively treat chronic respiratory infection in cystic fibrosis individuals with less toxicity and, possibly, not inducing the resistant mechanisms of pathogens from the overuse of antimicrobials.

### 1.2 CYSTIC FIBROSIS

Cystic fibrosis is an autosomal recessive disorder disease, which is mostly found in Caucasians, occurring at the rate of 1 in 2,500 (Campana et al., 2004; Rowe et al., 2005). The three regions of the highest incidence of cystic fibrosis are the USA, Europe, and Australia (http://www.who.org_viewed in January, 2006; Figure 1.2.1).

![Map showing incidence of cystic fibrosis around the world](image)

**Figure 1.2.1** Incidence of cystic fibrosis around the world. There are three main regions, as can be seen in black colour, in which have the highest incidences of cystic fibrosis: the USA, Europe, and Australia (http://www.who.org_viewed in January, 2006).

Although this figure shows the different incidence of cystic fibrosis in various populations around the world, the incidence between neighboring countries, for
instance, Norway, Sweden, Denmark, and Finland, seems to significantly be much different. This could possibly represent the effect of external factors between these countries, for example, geographical difference, that may influence such variations. Cystic fibrosis results from alterations in CFTR, a membrane-bound chloride channel, encoded by the cystic fibrosis transmembrane regulator (CFTR) gene (Fulmer et al., 1995; Vankeerberghen et al., 2002; Gibson et al., 2003a; Dorwart et al., 2004). Chloride ion transport is directly affected, which also induces the alterations of either sodium ion transport or water reabsorption (Middleton et al., 1994; Johnson et al., 1995; Mall et al., 1998; Drumm, 1999; Bachhuber et al., 2005; Jentsch et al., 2005). Dehydrated mucus then obstructs epithelial lining ducts, including sweat and airway, and also impedes mucociliary clearance (Pilewski and Frizzell, 1999; Andersson et al., 2002). Pathogens, for example, Pseudomonas aeruginosa and Burkholderia cepacia complex, can persistently colonise the mucosal surface (Gilligan, 1991; Govan and Deretic, 1996). Abnormal host immune response caused by recurrent infection or therapeutic side-effects can consequently lead to inflammation and host tissue destruction (Lammers, 2001; Meyer and Zimmerman, 2002; Conese et al., 2003), and then progressive destruction of the respiratory system (Govan and Deretic, 1996; Strausbaugh, 1996; Truninger et al., 2001; Taylor and Aswani, 2002). Moreover, other clinical sequelae, for instance, dysfunction of the immune system, diabetes mellitus, and osteoporosis, can also be manifested due to other functions of CFTR (Schoumacher et al., 1990; Tsongalis et al., 1994; Conway et al., 2000; Truninger et al., 2001; Brunzell and Schwarzenberg, 2002; Groman et al., 2002; Taylor and Aswani, 2002; Brenckmann et al., 2003; Conway, 2003; Pfeffer et al., 2003). In order to increase the clinical outcomes for cystic fibrosis individuals, improved care and the avoidance of microbial transmission in hospitals or in cystic fibrosis centres are necessary (Mahadeva et al., 1998; Jones et al., 2002; Jung et al., 2002; Jones et al., 2003a; Lee et al., 2003; Scott and Pitt, 2004). Additionally, newborn screening for cystic fibrosis has proved to be effective (Massie et al., 2005).
1.3 COMPLICATIONS OF CYSTIC FIBROSIS

As CFTR has numerous functions in addition to its ion channel activity, its dysfunction can result in abnormalities in a variety of human systems (Conway et al., 2000; Truninger et al., 2001; Taylor and Aswani, 2002; Conway, 2003; Maisonneuve et al., 2003; Grossman and Grossman, 2005). However, the most severe hallmark of cystic fibrosis is chronic pulmonary infectious disease (Gilligan, 1991; Pilewski and Frizzell, 1999; Wine, 1999; Gibson et al., 2003a; Boucher, 2004). Various altered conditions in cystic fibrosis, including excessive inflammatory response – massive influx of neutrophils and interleukin (IL)-8 overproduction – can increase the likelihood of infection, the survival of pathogens, and progression of the disease (May et al., 1991; Kammouni et al., 1997; Tabary et al., 1998; Hiemstra, 2001; Conese et al., 2003).

1.3.1 Chronic Bacterial Infection in Cystic Fibrosis

In almost all cystic fibrosis individuals, chronic bacterial infection in the lungs seems to be an inevitable consequence that raises the morbidity and mortality of patients, especially in childhood (Choi et al., 2002; Ullrich et al., 2002; Steinkamp et al., 2005). Prolonged colonisation of pathogens in cystic fibrosis patients may also increase inflammatory processes and poor prognosis for patients (Govan and Deretic, 1996). There are a variety of microorganisms responsible for long-term infection; but *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* complex are the four most virulent agents of chronic pulmonary disease (Gilligan, 1991; Lyczak et al., 2002; Saiman and Siegel, 2004). *Staphylococcus aureus* is almost always the first pathogen causing airway infection in cystic fibrosis individuals (Anderson, 1949 in Gilligan, 1991; Gibson et al., 2003a). *S. aureus* has a variety of virulence factors, such as capsular polysaccharide (Albus et al., 1988), which enhance its colonisation potential (Humphreys, 2002). Resistance to many types of antibiotics, especially β-lactam agents and methicillin, is typical for *S. aureus* (Nadesalingam et al., 2005). Methicillin-resistant *S. aureus* (MRSA) is strongly associated with treatment problems and poor prognosis in cystic fibrosis patients; and an important factor for emerging MRSA is a long-term use of antimicrobial therapy (Nadesalingam et al., 2005). Alteration of the efflux pump mechanisms in this pathogen
seems to also play another key role in resisting antibiotics (Grkovic et al., 2002; Li et al., 2003). Several lines of evidence have been established the collaboration between S. aureus and P. aeruginosa that primary colonisation by S. aureus assists secondary infection by P. aeruginosa, by providing an iron source for the latter (Mashburn et al., 2005). In addition, the prolonged use of anti-staphylococcal antimicrobial agents can synergistically be implicated in P. aeruginosa colonisation (Ratjen, 2001a).

Haemophilus influenzae is the second most common microbe colonising the cystic fibrosis airway epithelium (Moller et al., 1996; Regelink et al., 1999). Although this organism cannot persistently colonise the cystic fibrosis airway surface, it may enhance colonisation by other pathogens.

Pseudomonas aeruginosa and Burkholderia cepacia complex have been identified as the two most common pathogens colonising the airway surface of cystic fibrosis patients and, subsequently, correlate with high mortality and morbidity in these patients (Govan and Deretic, 1996; Quinn, 1998). P. aeruginosa is a Gram-negative, non-fermentative bacillus, which can act as a facultative pathogen in humans (Harris et al., 1999; da Silva Filho et al., 2001; Orrett, 2004). In nature, P. aeruginosa is found in planktonic form in several environments, such as water and soil. On the other hand, when it causes infection in humans, this pathogen can change from planktonic into mucoid form for surviving in different conditions (Govan and Deretic, 1996; Davies, 2002; Lau et al., 2005). Many researchers have emphasised the virulence factors that help P. aeruginosa survive and resist either host defence immune systems or antimicrobial agents (Flynn and Ohman, 1988; Kluftinger et al., 1989; Marcus et al., 1989; May et al., 1991; Goldberg and Pier, 1996; Pier, 2000; Choi et al., 2002; Wolfgang et al., 2003; Kipnis et al., 2006). Manno et al. (2005) recently demonstrated the trend of low susceptibility to aminoglycosides, imipenem, and ciprofloxacin of P. aeruginosa isolated from a cystic fibrosis centre. An increasing incidence of the antibiotic resistance of this microbe (Jung et al., 2002; Livermore, 2002) has been noted in the UK (Pitt et al., 2003; Scott and Pitt, 2004), Denmark (Jalal et al., 2000), and France (Hocquet et al., 2003). Additionally, colonies of P. aeruginosa from cystic fibrosis individuals appear to have demonstrated evidence of multidrug resistance and also cross-infection, owing to airborne transmission of the pathogen (Jones et al., 2001; Jones et al., 2003a). Before progressing into the chronic stage, there is also evidence that this pathogen might undergo an intermittent or latent period of pulmonary infection.
(Cantón et al., 2005; Nguyen and Singh, 2006). Hoiby et al. (2005) have stated that this latency could be the best opportunity for intervention and elimination of this pathogen from cystic fibrosis lungs.

1.3.2 Biofilm Formation and Quorum-sensing of *Pseudomonas aeruginosa*

Biofilm (alginate) formation is one of the adaptive mechanisms of *P. aeruginosa*, not only against the host immune system, but it also enables the organism to survive and persist in the natural environment (Costerton, 2001; Hentzer et al., 2001; Hoiby et al., 2001; Parsek, 2001; Rezaee et al., 2002; Sauer et al., 2002; Moskowitz et al., 2004; Hall-Stoodley and Stoodley, 2005; Lau et al., 2005). After this pathogen colonises the airways of cystic fibrosis individuals, some characteristics of the pathogen in planktonic forms, such as motility, can be adapted for survival in altered cystic fibrosis lung conditions (Mahenthiralingam et al., 1994; Ratjen, 2001a; Kipnis et al., 2006). This mucoid form of *P. aeruginosa* can be isolated from approximately 90% of cystic fibrosis individuals suffering chronic pulmonary infection (Lam et al., 1980; Hoyle et al., 1993; Ramsey and Whiteley, 2004). This alginated form of *P. aeruginosa* seems to be almost impossible to eradicate completely from the airway of patients with cystic fibrosis (Donlan and Costerton, 2002; Hall-Stoodley and Stoodley, 2005). Moreover, the occurrence of antibiotic resistance in the mucoid form of this pathogen has been demonstrated (Nichols et al., 1988; Giwerceanu et al., 1991; Aaron et al., 2002; Rezaee et al., 2002; Walters III et al., 2003). Long-term infection by mucoid *P. aeruginosa* is likely to have effects on the progression of airway infection and tissue damage in cystic fibrosis patients (Hoiby, 2002). Alginate or mucoid exopolysaccharide consists of acetylated D-mannuronic acid and L-guluronic acid (Deretic et al., 1995; Hoiby, 2002; Mah et al., 2003). A variety of factors have been considered as causes of such mucoidy in this microorganism (May et al., 1991; Martin et al., 1993; Deretic et al., 1994; Mathee et al., 1999; Bagge et al., 2004b; Häußler, 2004; von Götz et al., 2004; Bragonzi et al., 2005; Lau et al., 2005; Ramsy and Wozniak, 2005).

Recently, subinhibitory concentrations of aminoglycosides have been shown to induce biofilm formation in this pathogen (Hoffman et al., 2005). The relationship between the altered aminoglycoside response regulator (*arr*), a genetic determinant of biofilm-mediated antibiotic resistance in *P. aeruginosa*, and mucoid conversion of this bacteria
has also been discovered (Deretic et al., 1994; Høiby et al., 2001; Whiteley et al., 2001; Sriramulu et al., 2005). A cluster of genes on the P. aeruginosa chromosome designated algR, algS, and algT are responsible for alginate production (Deretic et al., 1989a, 1989b; Mathee et al., 1999). Quorum-sensing (QS) is a crucial mechanism that plays an essential role in cell-to-cell communication and controls other virulence factors in P. aeruginosa (Pearson et al., 2000; Smith and Iglewski, 2003; Schaber et al., 2004). Two QS systems have been found in P. aeruginosa, namely Las and Rhl, that regulate production of elastases, exotoxin A, pyocyanin, alkaline protease, and hydrogen cyanide (Arevalo-Ferro et al., 2003; Juhas et al., 2005; Kipnis et al., 2006). It is probable that the altered environment in cystic fibrosis can activate the expression of quorum-sensing regulated genes in this bacterium (Erickson et al., 2002; Hassett et al., 2002).

### 1.3.3 Efflux Pump Systems of Pseudomonas aeruginosa

In addition to the virulence factors, efflux pump systems are mechanisms that contribute to the intrinsic antimicrobial resistance of P. aeruginosa to many antibiotics (Hancock, 1998; Köhler and Pechere, 2001a; Schweizer, 2003; Webber and Piddock, 2003). Seven efflux pumps have been discovered and identified: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY-OprM, MexJK-OprM, MexHI-OpmD, and MexVW-OprM (Pumbwe and Piddock, 2000; Köhler et al., 2001b; Li et al., 2003; Schweizer, 2003). The differences between these efflux pumps relate directly to the different patterns of antimicrobial resistance (Nikaido, 1996; Nikaido, 1998; Fernandez-Recio et al., 2004; Middlemiss and Poole, 2004). Overproduction of MexAB-OprM directly contributes to β-lactam and fluoroquinolone resistances (Ziha-Zarifi et al., 1999; Poole, 2000; Hocquet et al., 2003; Nehme et al., 2004). Upregulation of MexXY-OprM is associated with resistance to aminoglycosides (Aires et al., 1999; Islam et al., 2004; Vogne et al., 2004). AmrAB-mediated efflux encoded by amrAB genes may also be implicated in aminoglycoside resistance in P. aeruginosa (Westbrock-Wadman et al., 1999). Resistance to antibiotics — for instance, faropenem, ritipenem, sulopenem, or even aminoglycosides — is a consequence of the interrelationship between active efflux systems, decreased permeability of the outer membrane barrier, and β-lactamase activity in this pathogen (Masuda et al., 1999; Okamoto et al., 2001; Grkovic et al., 2002; Llanes et al., 2004; Poole, 2005).
1.3.4 Infection Caused by *Burkholderia cepacia* Complex

The second most common microorganism found in cystic fibrosis individuals (Coenye *et al.*, 2001a; Coenye *et al.*, 2001b; Eberl and Tummler, 2004) and also certain diseases, such as chronic granulomatous diseases (Jones and Webb, 2003b), is *B. cepacia* complex (Table 1.3.1). Chronic respiratory infection in patients with cystic fibrosis caused by this pathogen appears to have a more considerable adverse effect on the survival of patients (Baird *et al.*, 1999; Lewenza *et al.*, 1999; De Kievit and Iglewski, 2000; Chaparro *et al.*, 2001; LiPuma, 2001; Soni *et al.*, 2002; Biddick *et al.*, 2003; Sokol *et al.*, 2003). Moreover, transient co-infection with more than one strain of this pathogenic complex can notably enhance the severity of infection (Huber *et al.*, 2002; Magalhães *et al.*, 2002; Yang *et al.*, 2006). *B. cepacia* complex, previously known as *P. cepacia*, was discovered and given the latin name “cepacia”, which means “of or like onion” by Walter H. Burkholder in 1949. *B. cepacia* complex was moved into the new genus, Burkholderia, in 1992, and was classified into “genomovars” by Vandamme and colleagues in 1997. Genomovar is used for *B. cepacia* strains that can be differentiated phylogenetically, but not phenotypically (Coenye and Vandamme, 2003). At present, nine genomovars have been given species names (Coenye *et al.*, 2001b; Coenye *et al.*, 2003). Although all genomovars of the complex can cause chronic infection in patients with cystic fibrosis (Coenye *et al.*, 2001a), *B. cenocepacia* (genomovar III) and *B. multivorans* (genomovar II) are the commonest strains isolated from cystic fibrosis patients (Speert *et al.*, 2002; McDowell *et al.*, 2004). In particular, a genomovar III infection is more likely to increase the progression and severity of chronic infection, as it appears to potentiate a more rapid decline in cystic fibrosis lung function (Frangolias *et al.*, 1999; Chaparro *et al.*, 2001; Courtney *et al.*, 2004; De Soyza *et al.*, 2001; Fauroux *et al.*, 2004), and also transmission of infection among patients (Smith *et al.*, 1993; Mahenthiralingam *et al.*, 2001; Biddick *et al.*, 2003). *B. cepacia* complex infections usually cause “cepacia syndrome”, which involves bacteraemia, the most severity clinical manifestation in cystic fibrosis and other patients (Coenye *et al.*, 2001b; Courtney *et al.*, 2004). In Australia, a novel cluster strain of *B. cepacia*, “the Hunter strain”, has been implicated in rapid deterioration of the respiratory system and nutrients status in cystic fibrosis patients (Fitzgerald *et al.*, 2001).
Table 1.3.1 Nomenclature of *Burkholderia cepacia* complex (modified from http://www.cftrust.org.uk viewed in January, 2006). *B. cepacia* complex have been classified into nine genomovars based on the variation of specific characteristics, including biochemical and whole-cell protein profiles, 16S rDNA, and *recA* gene sequences. Almost all genomovars of *B. cepacia* complex are commonly discovered in environment, except for genomovar II and III, which are primarily found in the airway of cystic fibrosis individuals.

<table>
<thead>
<tr>
<th>Genomovar</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td><em>Burkholderia cepacia</em></td>
</tr>
<tr>
<td>II</td>
<td><em>Burkholderia multivorans</em></td>
</tr>
<tr>
<td>III</td>
<td><em>Burkholderia cenocepacia</em></td>
</tr>
<tr>
<td>IV</td>
<td><em>Burkholderia stabilis</em></td>
</tr>
<tr>
<td>V</td>
<td><em>Burkholderia vietnamiensis</em></td>
</tr>
<tr>
<td>VI</td>
<td><em>Burkholderia dolosa</em></td>
</tr>
<tr>
<td>VII</td>
<td><em>Burkholderia ambifaria</em></td>
</tr>
<tr>
<td>VIII</td>
<td><em>Burkholderia anthinia</em></td>
</tr>
<tr>
<td>IX</td>
<td><em>Burkholderia pyrrocinia</em></td>
</tr>
</tbody>
</table>

Since *B. cepacia* complex is heterogeneous in terms of at least nine phenotypically similar but genotypically different characteristics relating to the severity of clinical manifestations in patients with cystic fibrosis, there are problems in the diagnosis of the infection (Coenye et al., 2001b; Coenye and Vandamme, 2003; Coenye et al., 2003; Steinkamp et al. 2005). Many researchers have been examined how to appropriately distinguish between these genomovars, especially genomovars II and III, the two most prevalent strains isolating from the lungs of cystic fibrosis patients (Coenye et al., 2001b; Mahenthiralingam et al., 2002; Mahenthiralingam et al., 2005). Several molecular identifications have been widely used as the preferred methods, for example, the restriction fragment length polymorphism (RFLP) analysis of 16S rDNA, *recA*-based PCR assays, and the epidemiological marker named *Burkholderia cepacia* epidemic strain marker (BCESM) (Henry et al., 2001; Kidd et al., 2003; Baldwin et al., 2004).
1.4 ANTIMICROBIAL AGENTS

As chronic progressive bronchopulmonary infection caused by bacteria appears to be the most severe complication in cystic fibrosis patients, a variety of antimicrobial agents have been examined in vitro, and used as treatment regimes for cystic fibrosis individuals (Table 1.4.1) (Cohn et al., 1995; Lyczak et al., 2002; Pitt et al., 2003). Types of antibiotics, administrative methods, drug concentration, therapeutic and side-effects, types of pathogens, progression of infection, are all factors that are taken into account during in vitro studies and in vivo treatments (Shawar et al., 1999; Aaron et al., 2000; Aaron et al., 2005; Blumberg et al., 2005; Cantón et al., 2005). Early detection of colonised pathogens in cystic fibrosis lungs is the chief way of beginning the process of alleviating chronic infection (Ratjen et al., 2001b).

1.4.1 β-lactams

β-lactam agents initially bind to bacterial penicillin-binding proteins (PBPs), located in the cell wall-cell membrane. Hydrolytic β-lactamases produced by some bacteria, such as S. aureus and H. influenzae, can inactivate β-lactam antibiotics (Greenwood and Ogilvie, 2002). Since P. aeruginosa normally contains chromosomal AmpC β-lactamase, which can be induced by exposure to β-lactam agents, resistance to such compounds is commonly found in this microbe (Giwereman et al., 1991; Senda et al., 1996; Campbell et al., 1997; Hancock and Speert, 2000; Bagge et al., 2004b; Aktas et al., 2005). In addition, an efflux pump MexAB-OprM of P. aeruginosa has been proved to act as another mechanism involved in resistance to these antibiotic compounds (Masuda et al., 1999; Livermore, 2002). In attempts to effectively treat such intractable infections, several β-lactamase inhibitors, for instance, clavulanic acid, sulbactam, and tazobactam, have been used as adjunctive combinations with β-lactam antibiotics (Aronoff et al., 1984; McGrath et al., 1993; Bonacorsi et al., 1999; Aktas et al., 2005).

1.4.2 Quinolones

Quinolones have been used widely as the broad-spectrum antibiotic agents in order to treat infection caused by Gram-positive and Gram-negative bacteria (Brooks et al.,
2001; Greenwood and Ogilvie, 2002). The target of quinolones is the α-subunit of bacterial DNA gyrase, an essential enzyme involved in Gram-negative bacterial DNA synthesis, or topoisomerase IV, an essential enzyme in Gram-positive bacteria (Nathwani et al., 1997).

Table 1.4.1  Antibiotics commonly used in the treatment of lung infection in cystic fibrosis patients (Gibson et al., 2003a). Antibiotic treatment regimes that can commonly be used to treat chronic respiratory infection caused by the most two common pathogens Pseudomonas aeruginosa and Burkholderia cepacia complex in cystic fibrosis appear to have smaller therapeutic options than Staphylococcus aureus and Haemophilus influenzae. In addition, the emergence of antibiotic resistance of such common cystic fibrosis pathogens appears to be much higher in incidence and severity. Therefore, combinations between different types of antibiotics, or antibiotics and the appropriate non-antibiotic agents are likely to be alternative therapeutic options in terms of long-term treatment regimes without inducing the occurring of antibiotic resistance in cystic fibrosis pathogens.

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Antibiotic</th>
<th>Pediatric Dose*</th>
<th>Adult Dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus</td>
<td>Choose one:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Doxycycline</td>
<td>6.25-12.5 mg/kg four times daily</td>
<td>250-500 mg four times daily</td>
</tr>
<tr>
<td></td>
<td>Ceftriaxone</td>
<td>12.5-25 mg/kg four times daily</td>
<td>500 mg four times daily</td>
</tr>
<tr>
<td></td>
<td>Amoxicillin</td>
<td>7.5-15 mg/kg four times daily</td>
<td>400-875 mg of amoxicillin component twice a day</td>
</tr>
<tr>
<td></td>
<td>Sulfamethoxazole</td>
<td>7.5-15 mg/kg four times daily</td>
<td>500 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Erythromycin (base)</td>
<td>7.5 mg/kg twice a day</td>
<td>500 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td>10 mg/kg initial dose followed by</td>
<td>500 mg initial dose followed by</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5 mg/kg every day</td>
<td>250 mg every day</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.5-7 mg/kg three times a day</td>
<td></td>
</tr>
<tr>
<td>Haemophilus influenzae</td>
<td>Choose one:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amoxicillin</td>
<td>7.5-15 mg/kg twice a day</td>
<td>500-875 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Amoxicillin/clavulanate</td>
<td>12.5-22.5 mg/kg of amoxicillin component twice a day</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Second/third generation cephalosporins:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cefuroxime axetil</td>
<td>15-20 mg/kg twice a day</td>
<td>250-500 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Cefuroxime</td>
<td>7.5-15 mg/kg twice a day</td>
<td>250-500 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Cefotaxime</td>
<td>10 mg/kg twice a day</td>
<td>200-400 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Cefpodoxime proxetil</td>
<td>5 mg/kg twice a day</td>
<td>100-200 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Loracarbef</td>
<td>7.5-15 mg/kg twice a day</td>
<td>400 mg twice a day</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>Choose one:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ciprofloxacin</td>
<td>10-15 mg/kg twice a day</td>
<td>500-750 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Tobramycin via inhalation</td>
<td>300 mg by nebulizer, twice a day</td>
<td>300 mg by nebulizer, twice a day</td>
</tr>
<tr>
<td></td>
<td>Colistin via inhalation</td>
<td>150 mg by nebulizer, twice a day</td>
<td>150 mg by nebulizer, twice a day</td>
</tr>
<tr>
<td>Burkholderia cepacia</td>
<td>Choose one:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trimethoprim/sulfamethoxazole</td>
<td>4-5 mg/kg of trimethoprim component twice a day</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Doxycycline</td>
<td>5 mg/kg initial dose followed by</td>
<td>200 mg initial dose followed by</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mg/kg twice a day</td>
<td>100 mg twice a day</td>
</tr>
<tr>
<td></td>
<td>Minocycline</td>
<td>4 mg/kg initial dose followed by</td>
<td>200 mg initial dose followed by</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2 mg/kg twice a day</td>
<td>100 mg twice a day</td>
</tr>
</tbody>
</table>

Ciprofloxacin is commonly used as drug of first choice in order to treat chronic P. aeruginosa infection in cystic fibrosis patients (Gibson et al., 2003a). It can be used as single antimicrobial agent and/or combined with other antibiotic compounds in order to delay the onset of resistance during long treatment periods (Fass et al., 1996; Lang et
al., 2000; Lomovskaya et al., 2001). However, clinical isolates from cystic fibrosis patients are often resistant to quinolones, owing to efflux pump mechanisms in this pathogen that are induced by exposure to the antibiotics (Hancock and Speert, 2000; Jalal et al., 2000; Poole, 2000).

1.4.3 Aminoglycosides

Aminoglycosides are potent bactericidal antibiotics used to treat infections by Gram-negative bacteria (Drasar et al., 1976; Smith, 2002; Vakulenko and Mobashery, 2003). Although the exact mechanisms of action is not completely described, in general, these antibiotics inhibit bacterial protein synthesis without interfering in or harming human cell functions by irreversible binding to the 30S bacterial ribosome, inhibiting translocation, and then misreading of mRNA (Davis, 1987; Brooks et al., 2001; Vakulenko and Mobashery, 2003). In addition to this target, the outer membrane of bacteria is another target of these bactericidal agents by creating gaps, and then the leakage of intracellular contents in the outer cell membrane. As a consequence, such leakage can increase the uptake of the antibiotics (Davis, 1987; Vakulenko and Mobashery, 2003). Tobramycin (Figure 1.4.1A) is used extensively for the treatment of chronic pulmonary infection in cystic fibrosis (Shawar et al., 1999; Beringer et al., 2000; Ramagopal and Lands, 2000; Master et al., 2001). Due to the use of oral tobramycin chemotherapy, accumulation of this antibiotic in serum, ototoxicity, and nephrotoxicity are the most irreversibly serious side effects in a dose-dependent manner (McCracken, 1986; Joly et al., 1991; Kennedy et al., 2005). Since the airway surface of cystic fibrosis individuals often contains a blanket of dehydrated mucus, poor penetration of tobramycin leading to a decrease of tobramycin concentration at the sites of infection occurs, and, as a consequence, can lead to the induction of resistance mechanisms in cystic fibrosis pathogens (Puchelle et al., 2002; Gibson et al., 2003a). In addition to desiccated mucus, production of bacterial biofilm can significantly inhibit tobramycin activity, principally by blocking the penetration of the antibiotic (Nichols et al., 1988; Hill et al., 2005). Antibiotic delivery by aerosol inhalation is the most appropriate means of delivery of the drug for chronic cystic fibrosis infections (Ramsey et al., 1999; Smith, 2002; Vakulenko and Mobashery, 2003). Aerosolisation of tobramycin has been used with moderate success as an alternative means of treating infections (Pai and Nahata, 2001; Bowman, 2002; Geller et al., 2002; Moss, 2002;
Gibson et al., 2003b; Vakulenko and Mobashery, 2003). Tobramycin has bactericidal effect on Gram-negative bacteria by inhibiting bacterial protein synthesis, enhancing bacterial cell membrane permeability, and then increasing the progressive disruption of the bacterial cell envelope. Amikacin has a mechanism of bactericidal activity similar to tobramycin, except for more potency and fewer undesirable effects on host cells.

![Chemical structures of tobramycin (A) and amikacin (B).](Image)

Figure 1.4.1 Chemical structures of tobramycin (A) and amikacin (B).

Recently, Pilcer and colleagues (2006) examined a novel formulation of tobramycin inhalation in the form of lipid-coated particles in order to deliver the highest concentration of this agent at the site of infection without exposure to other areas. Nevertheless, tobramycin resistance, especially in *P. aeruginosa* and *B. cepacia* complex, remains the most prevalent problem during the prolonged use of aerosolized drug (Prober et al., 2000; Smith, 2002; Manno et al., 2005). Combination of different antimicrobial combinations, for instance, tobramycin and ceftazidime, has been an alternative regime effectively treating chronic cystic fibrosis infection even though only tobramycin can be enough as a drug of the front line (Weiss and Lapointe, 1995; Cappelletty and Rybak, 1996; Bonacorsi et al., 1999; Aaron et al., 2000; Aaron et al., 2005). Furthermore, combination between aminoglycosides and non-antibiotic agents can be an alternative approach for long treatment of cystic fibrosis infections, since it can delay the occurrence of multidrug resistance in cystic fibrosis pathogens (Cohn and Aronoff, 1989; Cohn et al., 1995; Rajyaguru and Muszynski, 1997; Middleton et al., 2005). One explanation for such synergistic effects is that some chemical moieties of non-antibiotics could assist aminoglycoside activity in terms of its penetration and binding to the target site (Rajyaguru and Muszynski, 1998). Amikacin (Figure 1.4.1B)
(Garraffo et al., 1990; Vakulenko and Mobashery, 2003) is less toxic than tobramycin, as determined in animal models (Hottendorf and Gordon, 1980). Although this aminoglycoside is generally used as a single drug against tobramycin-resistant pathogens, amikacin has been widely used in combination with other types of antibiotics, for instance, imipenem, in order to delay antibiotic resistance in cystic fibrosis pathogens (McGrath et al., 1993). Moreover, it can also be used in combination with non-antibiotic agents (Rajyaguru and Muszynski, 1998). Several non-antibiotic compounds, especially those containing amine group (-N-CH₃), appear to have synergistic effects on aminoglycoside activity. Rajyaguru and Muszynski (1998) demonstrated the synergistic capability of sub-inhibitory concentration of certain amine compounds with amikacin, against B. cepacia complex. In their study, theobromine, theophylline, and famotidine, xanthine derivatives that are commonly used as antihypertensive and diuretic drugs, decreased amikacin MICs. One explanation for this synergistic effect is that the amine group (-N-CH₃) can assist the penetration of aminoglycoside into the bacteria and/or help increase the uptake of the antibiotic into bacterial cells.

1.5 TREATMENT REGIMES FOR RESISTANT PATHOGENIC BACTERIA

Of all of the potential therapeutic interventions, antibiotic control of infections by the primary pathogens P. aeruginosa and B. cepacia is still the subject of most attention, primarily because the consequences for the quality of life of patients, even life expectancy, are most closely allied to the aetiology of chronic infections (Chaparro et al., 2001; Lyczak et al., 2002; Chmiel and Davis, 2003). In recent decades, the overuse of antimicrobial agents has led to serious public health problems of bacterial resistance and treatment in patients with cystic fibrosis and other human diseases (Hurley et al., 1995; Carmeli et al., 1999; Levy, 2002). Single antibiotic treatments for chronic bacterial infection in cystic fibrosis patients are now largely ineffective. Additionally, discovery and development of new antibiotics is both time-consuming and almost prohibitively expensive, given the cost to effect potential benefits. Thus, combinations of antimicrobial agents, or of antibiotic with non-antibiotic compounds, have been
employed as alternative treatment regimes (Cohn et al., 1995; Weiss and Lapointe, 1995; Fass et al., 1996; Rajyaguru and Muszynski 1998; Chiu et al., 2001; Brennan and Geddes, 2004; Aaron et al., 2005; Blumer et al., 2005; Lekkas et al., 2006).

1.5.1 The Amilorides and Analogues

Since the treatment of chronic respiratory infection in cystic fibrosis individuals is by and large inefficient due to various factors, alternative approaches have been studied both in vitro and in vivo (Rodgers and Knox, 2001; Hirsh, 2002). The most important pathogens in cystic fibrosis lung infection are P. aeruginosa and B. cepacia complex, especially multi-drug resistant strains isolated from patients, and most approaches have focused on eliminating these pathogens from the airway surface (Robinson, 2001; Smith, 2002). In addition to the problem of antibiotic penetration of the mucous layers on the airway surface liquid in the cystic fibrosis lung epithelia, penetration of antibiotics into bacterial cells is counteracted to a large degree by the activity of bacterial sodium and other cation pumps (Lamb et al., 1972; Cohn et al., 1988). Sodium ion channel activity by bacteria has been proved to be overcome by the inhibitors, including amiloride and its derivatives (Horisberger, 1998; Rodgers and Knox, 2001; Hirsh, 2002). As a consequence, the mucociliary clearance activities and viscosity of cystic fibrosis mucus can then be improved mainly due to a decrease in water reabsorption (Cohn et al., 1992; Jones et al., 1997). Moreover, desiccated blanket mucus entrapping bacterial pathogens can also be removed from the airway surface more easily (Noone et al., 1997; Yu et al., 1998; Knowles and Boucher, 2002). Nevertheless, the binding affinity of amiloride for bacterial sodium pumps is comparatively low, and it also has a short half-life due to rapid absorption by the airway epithelium (Noone et al., 1997; Rodgers and Knox, 1999). For these reasons, thus, amiloride-substituted analogues, such as benzamil and phenamil, have been considered, and, in some cases, tested as alternatives (Hirsh, 2002; Hirsh et al., 2004). Benzamil and phenamil are much more lipophilic than amiloride. This could assist their pharmacological activity on human airway surfaces to enhance duration and potency (Blank et al., 1997; Hofmann et al., 1998; Rodgers and Knox, 1999).
Figure 1.5.1 Chemical structures of unprotonated (A) and protonated (B) forms of amiloride (Hirsh, 2002). Amiloride is an $N$-amidino-3,5-diamino-6-chloropyrazine carboxamide. Under normal physiological conditions, it is protonated in order to become an active form, and then function at the target site.

Figure 1.5.2 Chemical structures of benzamil (A) and phennil (B). Benzamil, a benzyl substitute, and phennil, a phenyl substitute, on the terminal nitrogen atom of the guanidino moiety of amiloride, appear to be more potent sodium channel inhibitors than amiloride.

1.5.2 Other Adjunctive Non-antibiotic Compounds

Since the diffusive movement of an aerosolised antibiotic, for instance, tobramycin, is somewhat restricted by its effect in constricting bronchiolar passages, this problem can be overcome largely by including agents, such as salbutamol in the antibiotic aerosol formulation (Nikolaizik et al., 1996; Brand, 2000; Ramagopal and Lands, 2000). Salbutamol (Figure 1.5.3) is a $\beta_2$ adrenergic agonist, which appears to have a variety of
therapeutic effects on cystic fibrosis, for instance, improving airway clearance by bronchodilation \citep{ziebach2001, groshaus2004, broadley2006}. In order to effectively clear the airway passage, aerosolisation could be a proper route administration of salbutamol for treating cystic fibrosis individuals, as it can be absorbed across bronchial cell layers \citep{vaisman1987a, vaisman1987b, ziebach2001, dodd2005, ehrhardt2005}. Therefore, using such an agonist to clear airway passages of cystic fibrosis before, or concomitantly with, antibiotics, could be an effective regime treatment for chronic respiratory infections.

Figure 1.5.3 Chemical structure of salbutamol.

Salbutamol is used clinically to alleviate the symptoms of bronchospasm, as a short-acting $\beta_2$-adrenergic receptor agonist. After binding of this agent to the receptors in the lungs, the bronchial smooth muscles in the lungs can be relaxed, and then resulting in the dilation of the airway. Desensitisation of salbutamol can occur after regular use or long-term therapy \citep{groshaus2004, haney2005a, haney2005b}. Another disadvantage is that it can affect the resting energy expenditure, which is usually increased in cystic fibrosis individuals \citep{vaisman1987a, vaisman1987b}. Combinations of salbutamol and tobramycin have been examined previously \citep{nikolaizik1996, ramagopal2000}, but have focused on salbutamol only in terms of bronchodilation activity, and not for antimicrobial activity with tobramycin. In addition to salbutamol, another non-antibiotic agent, which is generally used in clinics and has also been previously examined as the adjunctive compound with tobramycin, is verapamil \citep{cohn1995}. 
Verapamil (Figure 1.5.4) is used to treat cardiac diseases, such as hypertension. In brief, this compound blocks the internal flux of calcium ions, resulting in the prevention of calcium ion releasing from the sarcoplasmic reticulum, a store of calcium ions in striated muscle that triggers muscle contractions when releasing calcium (Spurlock et al., 1991). Another calcium ion channel inhibitor is amlodipine (Asok Kumar et al., 2003; Asok Kumar et al., 2004). Amlodipine (Figure 1.5.5) is an antihypertensive agent for treating patients with cardiovascular diseases (Nissen et al., 2004), but it also appears to have effective bactericidal activity against a diverse spectrum of bacterial species (Asok Kumar et al., 2003).
1.5.3 Combination (Adjunctive) Therapy

Synergistic combinations of antibiotics and other chemical compounds have been tried as alternative methods for treating intractable bacterial infections (Kelly et al., 1976; Cohn et al., 1988; Cappelletty and Rybak, 1996; Rodgers and Knox, 2001). A combination of β-lactam and a β-lactamase inhibitor has been used for the treatment of *P. aeruginosa* infections, since the inhibitor can protect β-lactams from inactivating enzymes (Frank et al., 2003). However, this method has not been very successful in eliminating bacterial infections from the airways of cystic fibrosis patients, as altered conditions in cystic fibrosis, for example, mucous blanket and high salt concentration, impede the activities of antimicrobial and non-antimicrobial agents (Boucher, 2002), and organisms, such as *P. aeruginosa* and *B. cepacia* complex, have intrinsic resistance to β-lactams (Hancock and Speert, 2000; Aktas et al., 2005). Moreover, under such conditions, the efficacy of antibiotics including tobramycin is reduced (Montgomery et al., 1999; Bowman, 2002). One possible explanation for this is that the thickened mucous blanket, which substantially obstructs human airways, delays the penetration and efficacy of antibiotics (Cohn and Aronoff, 1989; Leviton et al., 1995; Boucher, 2004). Combinations of different antibiotic agents, for example, β-lactams and aminoglycosides, have been proposed as a therapeutic option to eliminate pathogens and reduce acquired resistance in long-term treatment of chronic infections (Aronoff and Klinger, 1984; Lewin et al., 1993; Bonacorsi et al., 1999; Aaron et al., 2000; Lang et al., 2000; Schidlow, 2000; Karakoç and Gerçeker, 2001; Smith, 2002; Aaron et al., 2005). However, long-term treatment with single antibiotics or antibiotic combinations increases the risk of either acquired resistance in bacteria or toxicity from the antibiotics themselves (Brown, 1997; Levy, 2002; Gibson et al., 2003a). The use of non-antibiotic agents with antibiotics has been proposed as alternative treatment options. Several pilot studies have examined the synergistic effect of combined amiloride-tobramycin against *P. aeruginosa* or *B. cepacia* in vitro (Cohn et al., 1988; Cohn and Aronoff, 1989) or in cystic fibrosis patients (Middleton et al., 2005). Three of four patients were successfully treated without any reverse effect, including bronchoconstriction and alteration of serum electrolytes. Since the genomovars of *B. cepacia* in these four patients were not identified, it is possible that amiloride-tobramycin was particularly effective on specific genomovar(s) only. Moreover, only four patients were examined and there was no
control group in this pilot study in order to compare the sputum cultures before and after treatment. The original in vitro study of Cohn and his colleagues (1988) showed that amiloride-tobramycin eliminated only 50% of B. cepacia isolates, but as this study pre-dated the genomovar classification of B. cepacia complex, it remains uncertain whether the susceptibility was related to differences within or between genomovars. Moreover, this combination inhibited only 11% (3/27) of P. aeruginosa isolates. The use of combined tobramycin and amiloride needs to be examined further in vitro and in vivo to assess fully the effectiveness of this adjunctive therapy. Amiloride derivatives that have longer duration and more potency than amiloride, for instance, benzamil and phenamil, should be examined as alternative and/or more appropriate combinations with tobramycin against these pathogens.

1.6 HYPOTHESIS AND RATIONALE

According to the original study of Cohn’s groups (1988, 1989, and 1992) and the previous preliminary study of Middleton and colleagues (2005), amiloride and its derivatives, benzamil and phenamil, which are generally more potent in terms of therapeutic effect and longer therapeutic duration than amiloride, could demonstrate synergistic effect on tobramycin activity against P. aeruginosa and B. cenocepacia in this in vitro study. In addition to this hypothesis, amiloride inhibition of the sodium channels in the human lung or infectious microorganisms, or both, could also overcome the sodium antagonism of tobramycin uptake by both pathogens. One possible explanation for this is that sodium ion might interfere with the initial ionic binding of tobramycin to negatively charged lipopolysaccharides within the cell envelope (Cohn and Aronoff, 1989). According the previous studies (Cohn et al., 1988; Cohn and Aronoff, 1989; Blank et al., 1997), sodium is the chief agonist of aminoglycoside activity against Gram-negatives. This study could confirm and extend the findings of the potential effectiveness of tobramycin in combination with amiloride and other non-antibiotics against P. aeruginosa and B. cenocepacia; and check which of sodium, potassium or chloride ions, are the major inhibitor(s) of tobramycin efficacy.
Several studies (Vaisman, et al., 1987a, 1987b; Cohn et al., 1995; Ziebach et al., 2001; Asok Kumar et al., 2003; Asok Kumar et al., 2004) have suggested that some non-antibiotic compounds, including the bronchodilator salbutamol and calcium channel inhibitors, verapamil and amlodipine, could produce synergistic activity with tobramycin against *Pseudomonas* and *Burkholderia* spp. Therefore, this study could point the way to the use of such agonists to clear airway passages in cystic fibrosis patients before, or concomitantly with, antibiotics, as an effective regime treatment for chronic respiratory infections.

### 1.7 PROJECT AIMS

This project aims to examine *in vitro* susceptibility to tobramycin and amikacin dihydrate against selected clinical strains of *P. aeruginosa* and *B. cenocepacia* from cystic fibrosis patients, using the standard broth microdilution technique. In attempts to demonstrate the improved efficacy of tobramycin and/or amikacin, different compound combinations between tobramycin or amikacin with selected non-antibiotics will also be examined.

In order to broaden the scope of the study, tobramycin-resistant strains of *P. aeruginosa* will be included in the drug combination *in vitro* tests.

In addition, this project will examine problems associated with sodium and other cations in terms of reducing bactericidal activity of individual antibiotics and/or the combinations against the test microorganisms.

This study is a prelude to establishing the potential for clinical trials to treat infections in cystic fibrosis lungs.
CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals, Media, and Microtitre Plates

Mueller-Hinton broth (MHB) in powder form was obtained from Oxoid (Hampshire, England). Tobramycin, amiloride hydrochloride hydrate (98%), benzamil hydrochloride, phenamil, D-mannitol (98%), sodium gluconate, salbutamol, and N-methyl-D-glucamine (NMDG) were purchased in powder form from Sigma-Aldrich Inc. (St. Louis, Mo.). Amikacin dihydrate was purchased from Fluka Biochemika (Switzerland). Amlodipine was purchased from Sequoia Research Products (United Kingdom). Verapamil hydrochloride (Tocris Biosciences, United Kingdom) was a gift from the Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clinical Pathology and Medical Research [ICPMR], Westmead Hospital. Microtitre plates (96 wells) were purchased from Greiner Bio-one (Germany). All other chemicals and reagents were of analytical or molecular biological grade.

2.1.2 Bacterial Strains

Bacterial strains using in this study were: Pseudomonas aeruginosa NCTC 10662, four additional Pseudomonas aeruginosa strains (two tobramycin-susceptible and two tobramycin-resistant strains isolated from cystic fibrosis patients at the Centre for Infectious Diseases and Microbiology Laboratory Services, Institute of Clinical Pathology and Medical Research, Westmead Hospital, Australia), an isolate of Burkholderia cenocepacia (genomovar III) from the cystic fibrosis sputum of a 36-year-old male out-patient at Westmead Hospital. Two of such four additional tobramycin-resistant strains of P. aeruginosa (PSM1 and PSM2) produced mucoid colonies on agar plates.
2.2 METHODS

2.2.1 Preparation of Stock and Working Solutions of Antibiotics, other Drugs, and Salts

Stock solutions (40x) of antibiotics tobramycin or amikacin dihydrate, powder forms of tobramycin or amikacin dihydrate were dissolved in sterile distilled water and kept at -20°C. The stock solutions were serially diluted to prepare the working solutions on the day of experiment, using Mueller-Hinton Broth (MHB) as diluent. When testing the Minimum Inhibitory Concentration (MIC) of drugs against *P. aeruginosa* or *B. cenocepacia*, 50 μl of tobramycin or amikacin dihydrate serial dilutions were added into the wells of microtitre plates. After adding MHB diluent (50 μl for antibiotic assays only) or non-antibiotics (50 μl for combination assays), 100 μl of a bacterial cell suspension was added into the wells, for a total of 200 μl.

For the stock solutions of non-antibiotic compounds, powdered amiloride hydrochloride hydrate, benzamil hydrochloride, phenamil, salbutamol, verapamil hydrochloride, and amlodipine were dissolved in 10% dimethyl sulphoxide (DMSO) and kept at -20°C. These stock solutions (40x) were diluted to prepare working solutions (4x), or were serially diluted, on the day of experiment, using MHB as diluent. Fifty microlitres of compound solutions was added into the wells of microtitre plates as above, either alone, when estimating MICs of these compounds, or with 50 μl of tobramycin or amikacin dihydrate, when testing combination effects on antibiotic MICs. MHB diluent and bacterial cell suspensions were added into the wells as above. The concentrations of non-antibiotic solutions were diluted to the required concentration ranges of: 200 to 3,200 μg/ml (amiloride hydrochloride hydrate), 2.78 (7.81 x 10⁻³ mM) to 1,424.8 (4 mM) μg/ml (benzamil hydrochloride), 0.298 (9.77 x 10⁻⁴ mM) to 152.5 (0.5 mM) μg/ml (phenamil), 79.8 (0.5 mM) to 7,657.6 (32 mM) μg/ml (salbutamol), 245.54 (0.5 mM) to 15,714.24 (32 mM) μg/ml (verapamil hydrochloride), or 3.125 to 1,600 μg/ml (amlodipine).
Sodium chloride, potassium chloride, sodium gluconate, D-mannitol, or NMDG were prepared as stock and working solutions as above, again using MHB as diluent. The ranges of concentrations of these compounds tested were:

- Sodium chloride (50, 100, 150, 200, 300, or 400 mM)
- Potassium chloride (50, 100, 150, 200, 300, or 400 mM)
- Sodium gluconate (50, 100, 150, 200, 300, or 400 mM)
- D-mannitol (100, 200, 300, 400, 600, or 800 mM)
- NMDG (0.125, 0.25, 0.5, 1, 3.125, 6.25, 12.5, 25, or 50 mM)

2.2.2 Preparation of Bacterial Cell Suspensions

Single colonies of *P. aeruginosa* NCTC 10662, two antibiotic-sensitive and two antibiotic-resistant clinical isolates of *P. aeruginosa*, or *B. cenocepacia* (genovar III) were cultured overnight in MHB at 37°C, with shaking at 200 rpm. Each bacterial cell culture was also kept as a stock culture in 8% DMSO at -80°C. After overnight culture, cell turbidity was measured in a spectrophotometer at a wavelength (λ) of 625 nm, using MHB as the blank solution. Cultures were then adjusted to an absorbency of 1.0, equivalent to a McFarland Standard of 1.0 (equivalent to cell density ~10^8 cfu/ml, determined earlier by plate counts of serial dilutions). Cell suspensions were diluted 1:100 (equivalent to ~10^6 cfu/ml) and then 1:10 (~10^5 cfu/ml) in MHB before adding 100 μl aliquots into the wells of microtiter plates (Isenberg, 1998).

2.2.3 Minimum Inhibitory Concentration of Antibiotics against *P. aeruginosa* or *B. cenocepacia* by the Broth Microdilution Method

In this *in vitro* study, MHB was prepared without supplementing the medium with calcium and magnesium ions. The reason for this is that the project was intended to mimic the conditions in the cystic fibrosis lung as nearly as possible, and this involved checking and controlling parameters such as the concentrations of exogenous ions, such as Na^2+, K^+, and Cl^−. In many of the assays, the direct effects of these ions on the MIC assays – added at defined concentrations – would be a crucial part of the investigation. Even for assays not involving these ions, other cations, such as Mg^2+ and Ca^2+ that are sometimes added routinely to MHB, the effects on growth and subsequent MIC
measurements for the test bacteria would be affected by the presence of these cations, as would the comparisons of data for assays with and without other ions examined in this project. This approach is not without precedent as many previous studies, including one that is directly relevant to this project, have used MHB without Mg$^{2+}$ and Ca$^{2+}$ supplementation (Medeiros et al., 1971; Ramirez-Ronda et al., 1975; Fass and Barnishan, 1979; Casillas et al., 1981; Zuravleff et al., 1982; Barry et al., 1987).

In the MIC assays, 50 µl of antibiotic, other drug or compound, or salt was added at a single concentration or as serial dilutions into designated wells, followed by 50 µl of MHB when required, and finally 100 µl of bacterial cell suspension diluted from overnight cultures as described in Section 2.2.2. Every assay was routinely monitored using positive controls (100 µl of MHB and 100 µl of bacterial cell suspension) and negative controls (200 µl of MHB only), in the last two rows or columns of microtiter plates, as indicated in Figure 2.1. In addition, the internal control, which is 50 µl of antibiotics, 50 µl of MHB, and 100 µl of bacterial cell suspension, was performed when doing the assays of the combinations in order to check that the MICs for the antibiotic only were consistent in each assay. All microtitre plates were incubated overnight at 35°C, with agitation at 100 rpm. Plates were then read and analysed in a microtitre plate reader (Bio-Rad model 3550-UV) at a wavelength of 450 nm, with agitation for 20 seconds before reading. The results were entered into tables and plotted. The MIC was defined as the lowest concentration of antibiotic that inhibited the growth of bacteria. In this study, the MIC values of every compound were calculated by using the difference between the highest and lowest values of bacterial cell count (cfu x 10$^8$/ml) on the Y-axis in the graphs, and then divided by two, before reading the antibiotic concentration on the X-axis, taken as the MIC. All assays were performed at least in triplicate on the same or different days.

The standard error of the mean (SEM) was plotted along with the mean values for the MICs in all graphs. SEM is basically used to give an estimate of how the mean of the sample is related to the mean of the underlying population. The SEM is an accurate estimate of the error in low sample numbers as it gets smaller with increasing sample size. On the other hand, the standard deviation (SD) shows variation between individual readings and is used mainly if the interest is in the actual degree of variation itself.
Since the variations in readings in these MIC assays were only slight, the SEM, rather than the SD, is a more appropriate estimate of the scatter of values about the mean for replicates (Nagele, 2003; Altman and Bland, 2005).

**Figure 2.1** Pattern of filling wells of a microtiter plate for the MIC assays. Antibiotic, other drugs, compounds, or salts were added left to right into the wells of the plate, except for the last two rows and columns for the positive and negative controls, respectively. The positive and negative controls were routinely added in every assay to monitor the assay conditions.

### 2.2.4 Minimum Inhibitory Concentrations of Antibiotics in Dimethyl Sulfoxide

Since the stock solutions of amiloride hydrochloride hydrate, benzamil hydrochloride, phenamil, salbutamol, verapamil, and amlodipine were made up in 10% dimethyl sulfoxide (DMSO), any solvent effect on either the growth of *P. aeruginosa* and *B. cenocepacia*, or the MICs of antibiotics and drugs, needed to be tested. The final concentration of DMSO in any MIC assay was never higher than 0.05%, but DMSO at double this concentration (0.1%) was evaluated in the assays. One hundred microlitres of MHB containing DMSO (0.1% v/v) and 100 µl of bacterial cell suspension were added into the wells of microtiter plates, respectively, for the effect on cell growth. The effect of 0.1% DMSO on the activity of antibiotics was also examined against representative microorganisms. All plates were incubated overnight and analysed as in the standard assay described in Section 2.2.3. Results were plotted as depicted below, in
Figures 2.2 and 2.3. These results showed that there was no inhibition of growth of either organism in the presence of 0.1% DMSO; nor any change in the MICs for tobramycin and amikacin dihydrate against both organisms in the presence (figures below) or absence (see relevant graphs in Chapter 3) of 0.1% DMSO.

Figure 2.2 Evaluation of the effect of 0.1% of dimethyl sulfoxide (DMSO) in MHB without two aminoglycoside antibiotics against *Pseudomonas aeruginosa* NCTC 10662 (A) and *Burkholderia cenocepacia* (B). Serial dilutions started from the highest concentration of DMSO (0.1%) in well number 9 back to one. The point marked zero in each plot was the control without DMSO. After incubating the plates overnight at 37°C, absorbances were read at λ 450 nm, and then converted by calculation into bacterial cell counts [cfu x (10^8)]/ml. Data were corrected by (±) SEM, where the number of replicates was four. Some of the SEM bars are concealed by the symbols.
Figure 2.3  Evaluation of tobramycin or amikacin dihydrate activity under 0.1% of dimethyl sulfoxide (DMSO) in MHB against *Pseudomonas aeruginosa* NCTC 10662 (A) and *Burkholderia cenocepacia* (B). Serial dilutions of both antibiotics with the highest concentration of DMSO (0.1%) were examined against the microorganisms. Assays were performed as for Fig. 2.2. Some SEM bars were concealed by the symbols. TOB = tobramycin, AMIK = amikacin.
CHAPTER THREE

RESULTS

3.1 MIC OF ANTIBIOTICS AND NON-ANTIBIOTICS

Minimum inhibitory concentration (MIC) results of *P. aeruginosa* versus *B. cenocepacia* against two aminoglycoside antibiotics: tobramycin and amikacin, and non-antibiotic compounds used in this study: verapamil, salbutamol, amlodipine, amiloride, benzamil, and phenamil, were calculated from kill curves and summarised in Table 3.1.

**Table 3.1** Minimum inhibitory concentration (MIC) for tobramycin, amikacin, and different non-antibiotic compounds used in this study against *Pseudomonas aeruginosa* and *Burkholderia cenocepacia*. These MIC values were the average of between 4 to 20 estimations that did not vary by more than two values for all of the compounds.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Minimum Inhibitory Concentration (MIC)</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>P. aeruginosa</em></td>
<td><em>B. cenocepacia</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>µg/ml</td>
<td>µM</td>
<td>µg/ml</td>
<td>µM</td>
</tr>
<tr>
<td>Tobramycin</td>
<td></td>
<td>0.090</td>
<td>0.193</td>
<td>0.040</td>
<td>0.086</td>
</tr>
<tr>
<td>Amikacin</td>
<td></td>
<td>0.017</td>
<td>0.027</td>
<td>0.008</td>
<td>0.013</td>
</tr>
<tr>
<td>Verapamil</td>
<td></td>
<td>12,768</td>
<td>26,000</td>
<td>638</td>
<td>1,300</td>
</tr>
<tr>
<td>Salbutamol</td>
<td></td>
<td>3,111</td>
<td>13,000</td>
<td>3,470</td>
<td>14,500</td>
</tr>
<tr>
<td>Amlodipine</td>
<td></td>
<td>88</td>
<td>215</td>
<td>39</td>
<td>96</td>
</tr>
<tr>
<td>Amiloride</td>
<td></td>
<td>4,790</td>
<td>18,000</td>
<td>4,790</td>
<td>18,000</td>
</tr>
<tr>
<td>Benzamil</td>
<td></td>
<td>712</td>
<td>2,000</td>
<td>134</td>
<td>375</td>
</tr>
<tr>
<td>Phenamil</td>
<td></td>
<td>563</td>
<td>1,400</td>
<td>542</td>
<td>1,350</td>
</tr>
</tbody>
</table>
3.1.1 Tobramycin and Amikacin

In this study, two aminoglycosides tobramycin and amikacin were chosen in order to assess the MICs against *P. aeruginosa* and *B. cenocepacia* since both antibiotics have been widely used as front line drugs for treating infections caused by Gram-negative bacteria (Shawar *et al.*, 1999; Beringer *et al.*, 2000; Master *et al.*, 2001; Vakulenko and Mobashery, 2003). Furthermore, amikacin seems to have more therapeutic effect and is less toxic than tobramycin (Hottendorf and Gordon, 1980; Vakulenko andMobashery, 2003). Thus, it is possible that amikacin combined with some of the non-antibiotic compounds used in this study could exhibit more potent therapeutic effects than the same combinations with tobramycin. From Table 3.1, the MICs of tobramycin against *P. aeruginosa* and *B. cenocepacia* were 0.193 μM (0.090 μg/ml) and 0.086 μM (0.040 μg/ml), respectively. However, the MICs of amikacin were 0.027 μM (0.017 μg/ml) and 0.013 μM (0.008 μg/ml). From a comparison of these two aminoglycosides, it can be seen that amikacin was about seven times more potent than tobramycin against both *P. aeruginosa* (Figure 3.1.1A) and *B. cenocepacia* (Figure 3.1.1B).
Figure 3.1.1 Comparison between tobramycin and amikacin MICs against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Serial dilutions of antibiotics were examined against both test organisms using the standard broth microdilution method. After incubating bacterial cell suspensions with each antibiotic at 37 °C overnight, absorbance was evaluated by at a wavelength of 450 nm, and then converted by calculation from growth curves into bacterial cell count [cfu x (10^8)]/ml. Data were corrected by the average MICs ± SEMs. The replicates for each assay (n) were three. SEMs of both assays ranged from 0.002 to 0.137. Some SEM bars were concealed by the symbols as they were smaller than the symbols. TOB = tobramycin, AMIK = amikacin, cfu = colony forming unit.
3.1.2 Amiloride and Derivatives

Apart from the sodium channel inhibitor activity of amiloride, it has been reported in combination with tobramycin to treat chronic cystic fibrosis lung infections (Cohn et al., 1988; Middleton et al., 2005). In this project, benzamil and phenamil, substituted analogues of amiloride, were generally more potent antibacterials than amiloride (Table 3.1); and therefore might be more effective adjuncts with tobramycin. All three amilorides were compared and assessed for synergistic activity with tobramycin against *P. aeruginosa* and *B. cenocepacia*. The MICs of these three sodium channel inhibitors against both microorganisms were displayed in Table 3.1; and the MIC traced curves for benzamil and phenamil were depicted in Figure 3.1.2. For *B. cenocepacia*, benzamil was the most potent non-antibiotic, with its MIC being 48 times lower than that of amiloride. For *P. aeruginosa*, the phenamil MIC was 13 times lower than that of amiloride. These data suggest that benzamil and phenamil could be used at concentrations closer to or lower than their individual MICs against both microorganisms, producing potentially more effective results than amiloride *in vivo*, especially in combination with aminoglycosides or other antibiotics used to treat pulmonary infections. The earlier success of a pilot clinical trial of amiloride-tobramycin therapy on four cystic fibrosis patients (Middleton et al., 2005) argues strongly for benzamil or phenamil to be assessed in future trials.
Figure 3.1.2 Comparison of benzamil and phenamil MICs against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Serial dilutions of benzamil and phenamil were evaluated against both microorganisms in order to obtain the MICs. Data were corrected by the average MICs ± SEMs. The replicates of each assay (n) were three. SEMs of both assays ranged between 0.000 to 0.017. Some SEM bars were concealed by the symbols since they were smaller than the symbols. PHEN = phenamil, BEN = benzamil, cfu = colony forming unit.
3.1.3 Salbutamol

In this study, the bronchodilator salbutamol was chosen for combination MICs with tobramycin since it is generally used to alleviate the symptoms of bronchospasm in asthma and chronic obstructive pulmonary disease (Ziebach et al., 2001; Broadley, 2006). In addition, two studies have demonstrated moderate synergistic effects of this bronchodilator with aminoglycosides (Nikolaizik et al., 1996; Ramagopal and Lands, 2000). In this study, the MICs for salbutamol (Table 3.1) were 13 mM against *P. aeruginosa* (Figure 3.1.3A) and 14.5 mM against *B. cenocepacia* (Figure 3.1.3B).
Figure 3.1.3 MICs of salbutamol against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). The assays and methodology is otherwise as described in the legend to Fig. 3.1.2. SAL = salbutamol. For this figure and all subsequent figures, unless specified otherwise, n was equal to four replicates.

### 3.1.4 Verapamil and Amlodipine

According to several studies (Cohn *et al.*, 1995; Asok Kumar *et al.*, 2003; Asok Kumar *et al.*, 2004), verapamil and amlodipine, calcium ion channel inhibitors, have been tested as adjunctive compounds with tobramycin against *P. aeruginosa* and *B. cepacia* complex, even though both are generally used for treating cardiovascular diseases. The MICs for verapamil and amlodipine against *P. aeruginosa* were 26 mM (12,768 μg/ml) and 0.215 mM (88 μg/ml), and against *B. cenocepacia* 1.3 mM (638 μg/ml) and 0.096
mM (39 μg/ml), respectively (Figure 3.1.4 and Table 3.1). The exceptional susceptibility of *B. cenocepacia* to verapamil versus that of *P. aeruginosa* cannot be easily explained, except to cite a specific strain difference, in which case verapamil MIC testing of more strains of both organisms is warranted. However, an earlier study (Cohn *et al.*, 1995) of verapamil-tobramycin synergy in these two organisms found that the verapamil MICs for four strains of each were about the same, at >8 mM, suggesting that a species-specific effect is probable in this instance. When MIC values of amlodipine to any other additive compounds in this study were compared, it was obvious that amlodipine was the most potent non-antibiotic agent for *in vitro* testing against *P. aeruginosa* (Figure 3.1.5A; Table 3.1) and *B. cenocepacia* (Figure 3.1.5B; Table 3.1).
Figure 3.1.4 Verapamil MICs against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). VER = verapamil.
Figure 3.1.5 Amlodipine MICs against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). AMLO = amlodipine.

### 3.2 COMBINATION (ADJUNCTIVE) THERAPY

Additive and/or synergistic effects of tobramycin with non-antibiotic agents were assessed by combining tobramycin with sub-inhibitory clinical relevant concentrations of these adjunctive agents against both microorganisms. For the *in vitro* MIC assays,
amiloride was tested at 1 mM; verapamil and salbutamol at 2 mM; benzamil at 0.125 (for *B. cenocepacia* only), 0.25, 0.5 and 1 mM; phenamil at 0.25, 0.5 and 1 mM; and amlodipine at 0.03 and 0.06 mM. The results for antibiotics/non-antibiotics combination MIC assays are summarised in Table 3.2. Synergistic effects were either marginal (tobramycin MICs up to 2-fold lower), moderately enhanced (up to 4-fold) or significantly enhanced (up to 32-fold).

### 3.2.1 Tobramycin with Amiloride, Benzamil, and Phenamil

Since amiloride apparently appears to moderately assist tobramycin against *P. aeruginosa* and *B. cepacia* complex, its lipophilic derivatives, benzamil and phenamil, with lower MICs, could have more powerful therapeutic effects with tobramycin. According to Table 3.2, Figure 3.2.1 (A and B), amiloride at 1 mM appeared to slightly enhance tobramycin activity against *P. aeruginosa* (0.063 µg/ml or 0.134 µM) and *B. cenocepacia* (0.031 µg/ml or 0.067 µM). Amiloride at 0.1 mM was essentially ineffective with tobramycin activity against *P. aeruginosa* (0.187 µg/ml or 0.401 µM) and *B. cenocepacia* (0.063 µg/ml or 0.134 µM). Lipophilic compounds are more permeable across membranes, and therefore the antibacterial activities of phenamil and benzamil in combination with tobramycin should be stronger than amiloride, as both are more lipophilic. Indeed, this was confirmed in this study since the MICs for phenamil and benzamil were approximately 10 to 50 times lower than that of amiloride, respectively (Table 3.1). Although phenamil is about 20 times more lipophilic than amiloride and twice more than benzamil in terms of sodium inhibitor action (Hirsh *et al.*, 2004), its various subinhibitory concentrations (0.25, 0.5, and 1 mM) exhibited the same slight inhibition of tobramycin activity, which were 0.125 µg/ml (0.267 µM) (Figure 3.2.3A; Table 3.2) against *P. aeruginosa* and 0.047 µg/ml (0.100 µM) against *B. cenocepacia* (Figure 3.2.3B; Table 3.2). The structures of phenamil and benzamil differ only by the latter having a methylene bridge between the terminal guanidine amino moiety and a phenyl ligand. Amilorides are weak bases that are most potent as inhibitors in the protonated form (Hirsh *et al.*, 2004). The pKas of phenamil, benzamil and amiloride are 7.8, 8.1 and 8.7, respectively, with phenamil being slightly less basic than the other two, yet the antibacterial potencies of the three compounds do not appear to correlate with the pKa values. On the other hand, the lipophilicities of phenamil and...
benzamil suggest that their MICs should be similar and far lower than for the more hydrophilic amiloride (Table 3.1). Benzamil at a concentration above 0.5 mM exhibited a dramatically synergistic effect on tobramycin against *P. aeruginosa*, that is, about 24-fold (at 0.5 mM) and 32-fold (at 1 mM) lower concentrations than tobramycin itself (Table 3.2). However, benzamil hydrochloride at 0.25 mM showed only slight synergy. This seemingly higher potency of tobramycin-benzamil against *P. aeruginosa* does not correlate with the actual potency of the drug against both organisms, as the benzamil concentration [1 mM] was not much below its MIC for *P. aeruginosa*, but several-fold higher than the benzamil MIC for *B. cepacia* and the benzamil concentration used in the tobramycin- benzamil assay.
Figure 3.2.1 Comparison of tobramycin combining with 0.1 mM or 1 mM of amiloride against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, AMIL = amiloride.
Figure 3.2.2  Tobramycin with different concentrations of benzamil. All results were corrected by the average MICs ± SEMs. TOB = tobramycin, BEN = benzamil.
Figure 3.2.3 Tobramycin with different concentrations of phenamil against both microorganisms. Phenamil at 0.25 mM, 0.5 mM, or 1 mM was assessed with tobramycin against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, PHEN = phenamil.
3.2.2 Tobramycin, Amlodipine, Verapamil

According to Cohn et al. (1995), a combination of tobramycin with the calcium channel inhibitor verapamil was found to be effective against *B. cepacia* complex but not *P. aeruginosa*. Another calcium channel blocker, amlodipine, was found to have bactericidal activity against various bacterial pathogens (Asok Kumar et al., 2003; Asok Kumar et al., 2004). In this study, verapamil and amlodipine were assessed as adjunctive agents with tobramycin against *P. aeruginosa* and *B. cenocepacia*. Although MIC values for amlodipine alone against both microorganisms were the lowest of all the non-antibiotic compounds tested (Table 3.1), sub-inhibitory concentrations of this calcium channel inhibitor (0.03 and 0.06 mM) with tobramycin were not significantly synergistic against *P. aeruginosa* (Figure 3.2.4A; Table 3.2), and were antagonistic against *B. cenocepacia* (Figure 3.2.4B; Table 3.2). This result appeared to be generally consistent with a previous study (Asok Kumar et al., 2004). On the other hand, verapamil demonstrated an additive effect with tobramycin against *P. aeruginosa*. Conversely, verapamil at 2 mM, chosen from the original study (Cohn et al., 1995), killed *B. cenocepacia* as this concentration was slightly higher than verapamil MIC (Figure 3.2.5B). Therefore, as a follow up, two sub-inhibitory concentrations of verapamil, 0.325 and 0.65 mM, were then assessed with tobramycin.
Figure 3.2.4 Combination between tobramycin and 0.03 or 0.06 mM of amlodipine against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, AMLO = amlodipine.
Figure 3.2.5 Tobramycin MICs for *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B) in combination with 2 mM verapamil. TOB = tobramycin, VER = verapamil.
3.2.3 Tobramycin and Salbutamol

As salbutamol is known to help clear the airway passage, its use as a potential adjunctive agent with tobramycin was considered. In this study, a sub-inhibitory and clinical relevant concentration of salbutamol [2 mM] was used for performing the combination assays with tobramycin. The MICs of tobramycin with salbutamol were identical against both microorganisms, which was 0.031 μg/ml (0.067 μM) (Table 3.2). Salbutamol with tobramycin, was marginally more potent against *P. aeruginosa* (Figure 3.2.6A) than *B. cepacia* (Figure 3.2.6B), decreasing the tobramycin MICs by factors of 2.9-fold and 1.3-fold against *P. aeruginosa* and *B. cepacia*, respectively.
Figure 3.2.6 Combination between tobramycin and salbutamol against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, SAL = salbutamol.

3.2.4 Combinations of Three Drugs

An interesting outcome of the MIC assays was the almost identical MICs for amiloride and salbutamol for both organisms. Combination between the clinical relevant concentrations of salbutamol (2 mM) and amiloride (1 mM) had the most potent synergistic effect on tobramycin by decreasing the original tobramycin MICs, from 0.090 μg/ml (0.193 μM) to 0.023 μg/ml (0.050 μM) against *P. aeruginosa*; and from...
0.040 μg/ml (0.086 μM) to 0.016 μg/ml (0.033 μM) against *B. cenocepacia*. Interestingly, before discovering the low MIC for verapamil alone against *B. cenocepacia*, the tobramycin-verapamil-salbutamol combined MIC (Figure 3.2.7B) was almost the same as the tobramycin-salbutamol MIC, even though 2 mM verapamil with tobramycin was ordinarily enough to kill the bacteria (Figure 3.2.5; Table 3.2). It was presumed that the activity or availability of verapamil in these *in vitro* assays was antagonised by salbutamol. This unusual result was not observed for the corresponding assays for *P. aeruginosa* (Figure 3.2.7A), as the concentration of verapamil [2 mM] was well below the 26 mM MIC for this organism (Table 3.1). The second most potent combinations against *B. cenocepacia* were either tobramycin-salbutamol-verapamil (Figure 3.2.7B) or tobramycin-salbutamol-benzamil (Figure 3.2.8B). In contrast, both combinations had antagonistic effects against *P. aeruginosa*. Benzamil at 0.125 mM was antagonistic with tobramycin-salbutamol against *P. aeruginosa* (Figure 3.2.8A), but synergistic against *B. cenocepacia* (Figure 3.2.8B). This can only be explained by strain differences and/or differential effects of the triple drug combination. For *P. aeruginosa*, the second most potent triple combination was tobramycin-salbutamol-amlopidine (Figure 3.2.9A), which decreased the tobramycin MIC to 0.044 μg/ml (0.094 μM) at either 0.03 or 0.06 mM amlopidine concentrations. However, the same combinations were ineffective for *B. cenocepacia* (Figure 3.2.9B), with the tobramycin MIC increasing to 0.125 μg/ml (0.267 μM) at both amlopidine concentrations.
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* (A) and *Burkholderia cenocepacia* (B). n was 3 to 6. TOB = tobramycin, AMIL = amiloride, SAL = salbutamol, VER = verapamil.
Figure 3.2.8 Combinations of tobramycin, 2 mM salbutamol, and 0.125 or 0.25 mM benzamil against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, SAL = salbutamol, BEN = benzamil.
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* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, SAL = salbutamol, AMLO = amlodipine.
Table 3.2  Comparison of tobramycin MICs (µg/ml and µM) in various combinations with non-antibiotic compounds, amiloride, benzamil, phenamil, salbutamol, verapamil, or amlodipine, to tobramycin itself against *Pseudomonas aeruginosa* and *Burkholderia cepacia*. "ND = not determined.

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3.3 SODIUM ANTAGONISM OF TOBRAMYCIN ACTIVITY

The mechanisms related to the antagonistic effect of sodium chloride on the activity of aminoglycoside remain unclear, but there is evidence that sodium it interferes with the binding or uptake of certain antimicrobials, including aminoglycosides (Cohn and Aronoff, 1989). In this in vitro study, different concentrations of test compounds sodium chloride, sodium gluconate, potassium chloride, D-mannitol, and N-methyl-D-glucamine (NMDG) were examined in order to determine if sodium itself was the interfering ion, or if other ions, such as chloride, potassium, or positive amines, or osmolarity had any contributing effects. It was noted that MHB is formulated with sodium chloride at about 90 mM in liquid medium. Since this basal concentration would be present in all assays and tobramycin MICs against the test organisms were consistent, this basal level of sodium chloride in MHB could essentially be ignored.

3.3.1 Effect of Sodium Chloride

In order to mimic high salt in the cystic fibrosis lung, serial dilutions of sodium chloride ranged from 50 to 400 mM. For *P. aeruginosa* (Figure 3.3.1A), tobramycin MICs with 50 or 100 mM sodium was 0.188 μg/ml (0.402 μM); and for 150 or 200 mM sodium was 0.333 μg/ml (0.712 μM). The tobramycin MICs for 300 and 400 mM was 0.469 μg/ml (1.003 μM) and 0.667 μg/ml (1.427 μM), respectively. For *B. cepacia* (Figure 3.3.1B), the corresponding tobramycin MICs for 50, 100, 150, 200, 300, and 400 mM sodium were 0.188 μg/ml (0.402 μM), 0.208 μg/ml (0.445 μM), 0.375 μg/ml (0.802 μM), 0.406 μg/ml (0.868 μM), 0.688 μg/ml (1.472 μM), and 1.500 μg/ml (3.209 μM), respectively. These data clearly showed increasing tobramycin MICs against both organisms in the presence of increasing sodium concentrations, and generally support previous observations (Cohn and Aronoff, 1989).
Figure 3.3.1 Effect of different sodium chloride concentrations on tobramycin MICs for *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Different concentrations of sodium chloride ranging from 50 to 400 mM were examined with the serial dilutions of tobramycin. Fifty microlitres of sodium chloride solutions were added into the wells of microtitre plates followed by 50 μl of tobramycin serial dilutions and then 100 μl of bacterial cell suspension. n was 6 for *P. aeruginosa* and 3 for *B. cenocepacia*. The data were corrected by the average MICs ± SEMs. SEMs of these assays ranged from 0.001 to 0.037. Some SEM bars were covered by the symbols. TOB = tobramycin, NaCl = sodium chloride.
3.3.2 Effect of Sodium Gluconate

In order to discount any effect of chloride and to also confirm the sodium effect, different concentrations of sodium gluconate ranging from 50 to 400 mM were assessed with tobramycin. For *P. aeruginosa*, tobramycin MICs under sodium gluconate concentrations ranging from 50 to 400 mM were from 0.102 μg/ml (0.218 μM) to 0.458 μg/ml (0.980 μM), and, for *B. cenocepacia*, the MICs were from 0.156 μg/ml (0.334 μM) to 1.500 μg/ml (3.209 μM), respectively (Figure 3.3.2 A and B). The MIC values of tobramycin in both organisms under different sodium gluconate concentrations were almost equivalent to those for sodium chloride at the same concentrations. In addition, tobramycin MIC of *B. cenocepacia* when increasing sodium gluconate concentration displayed about two times higher than that of *P. aeruginosa*. Thus, it can then presumptively conclude that sodium, and not chloride, antagonistically affects tobramycin activity *in vitro*, by increasing the tobramycin MICs for *P. aeruginosa* and *B. cenocepacia*. 
Figure 3.3.2 Effect of different concentrations of sodium gluconate on increasing tobramycin MICs for *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Different concentrations of sodium gluconate (50, 100, 150, 200, 300, and 400 mM) were individually examined with tobramycin serial dilutions. n was 3. The data were corrected by the average MICs ± SEMs. SEMs of these assays ranged from 0.000 to 0.025. SEM bars were covered by the symbols. TOB = tobramycin, NaGlu = sodium gluconate.
3.3.3 Effect of Potassium Chloride

In order to be sure that only sodium monovalent cations were responsible for the antagonistic effect on the tobramycin MIC in these organisms, we decided to test potassium chloride. In these assays, there was no need to account for the chloride ion of this potassium salt, as the earlier identical data for sodium chloride and sodium gluconate had already demonstrated that sodium, not chloride, was the probable cause of the increased tobramycin MICs. For *P. aeruginosa* (Figure 3.3.3A), tobramycin MICs ranged from 0.188 μg/ml (0.402 μM) to 1.337 μM; and for *B. cenocepacia* (Figure 3.3.3B), MICs ranged from 0.188 μg/ml (0.402 μM) to 1.500 μg/ml (3.209 μM). According to these potassium chloride results, the tobramycin MICs increased by almost the same factor as for sodium chloride and sodium gluconate in both *P. aeruginosa* and *B. cenocepacia*. As the effect of potassium on aminoglycoside MICs against Gram-negatives had never been studied before, this *in vitro* result is novel in affirming that not only monocation sodium but also potassium – and, by inference, possibly other cations can antagonise the activity of aminoglycosides against Gram-negative bacteria.
Figure 3.3.3  Effect of different concentrations of potassium chloride on increasing the tobramycin MICs for *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Different concentrations of potassium chloride (50, 100, 150, 200, 300, and 400 mM) were independently appraised with serial dilutions of tobramycin. TOB = tobramycin, KCl = potassium chloride.
3.3.4 Effect of D-mannitol

Since osmotic strength could be another parameter affecting the MICs of antibiotic compounds, D-mannitol at the concentrations of 100, 200, 300, 400, 600, and 800 mM were assayed with tobramycin activity against P. aeruginosa and B. cenocepacia. Interestingly, D-mannitol appears to assist tobramycin activity against only P. aeruginosa by decreasing the MIC of tobramycin, except for the concentrations from 200 mM to 400 mM, which exhibited an identical tobramycin MIC (0.083 μg/ml or 0.178 μM) (Figure 3.3.4A). Moreover, the MIC for this microorganism at 800 mM of D-mannitol, the highest testing concentration, was decreasing to 0.039 μg/ml (0.083 μM), which seems to be the lowest tobramycin MIC for P. aeruginosa among the combinations of tobramycin with the other compounds. However, D-mannitol appears to have inhibitory effect on tobramycin against B. cenocepacia by moderately increasing the MIC values (Figure 3.3.4B). From 100 mM to 400 mM D-mannitol, tobramycin MICs were identical, which were 0.104 μg/ml (0.222 μM), while, at 600 mM and 800 mM, the MICs were 0.109 μg/ml (0.233 μM) and 0.125 μg/ml (0.267 μM), respectively.
Figure 3.3.4 Effect of different concentrations of D-mannitol on tobramycin MICs for *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Different D-mannitol concentrations ranging from 100 to 800 mM were examined with the serial dilutions of tobramycin. n was 6 for *P. aeruginosa* and 3 for *B. cenocepacia*. TOB = tobramycin, Mann = D-mannitol.
3.3.5 Effect of N-methyl-D-glucamine

N-methyl-D-glucamine (NMDG) was chosen to examine the effect of a slightly basic but non-ionisable amine group on tobramycin activity. In order to ascertain whether different concentrations of NMDG were associated with a changing pH of media culture or not, NMDG solutions adjusted to a pH of 7.2, or not adjusted at all, were tested by monitoring the growth of \textit{P. aeruginosa} and \textit{B. cenocepacia}. Fifty microlitres of serial dilutions of pH adjusted or unadjusted NMDG were added into the wells of the plates followed by 50 µl of MHB as a diluent, and then 100 µl of bacterial cell suspension. The plates were shaking incubated and analysed as described in Chapter 2. It was noted (Figure 3.3.5) that the pH of the medium directly affected the growth of both test bacteria. Thus, only adjusted pH NMDG solutions were used in the assays with tobramycin. The ranges of NMDG concentrations in this study were separated into two major groups, low (from 0.125 mM to 1 mM) and high (from 3.125 to 50 mM) NMDG concentrations. For \textit{P. aeruginosa} (Figure 3.3.6 A and B), the lowest NMDG concentration (0.125 mM) exhibited the highest tobramycin MIC (0.106 µg/ml or 0.227 µM), whereas, from 1 mM to 50 mM NMDG, the MICs were identical (0.094 µg/ml or 0.201 µM). In contrast, tobramycin MIC at 25 mM of NMDG for \textit{B. cenocepacia} (Figure 3.3.6 C and D) was the highest (0.063 µg/ml or 0.135 µM), while from 0.125 mM to 1 mM or 3.125 mM to 12.5 mM were 0.044 µg/ml (0.094 µM) or 0.051 µg/ml (0.109 µM), respectively. Thus, the effect of NMDG on the tobramycin MICs was essentially negligible, except at the highest concentrations.
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* (A) and *Burkholderia cenocepacia* (B). Data were corrected by the average MICs ± SEMs. SEMs of the assays ranged from 0.000 to 0.083. ——: growth of *P. aeruginosa* in adjusted pH 7.2 NMDG, ——: growth of *B. cenocepacia* in adjusted pH 7.2 NMDG, ——: growth of *P. aeruginosa* in unadjusted pH NMDG, ——: growth of *B. cenocepacia* in unadjusted pH NMDG. NMDG = N-methyl-D-glucamine.
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* (A) and *Burkholderia cenocepacia* (C), and from 3.125 mM to 50 mM on tobramycin MICs for *P. aeruginosa* (B) and *B. cenocepacia* (D). TOB = tobramycin, NMDG = N-methyl-D-glucamine.
3.4 SODIUM ANTAGONISM OF TOBRAMYCIN IS PARTIALLY REVERSED BY AMILORIDE

3.4.1 Tobramycin, Amiloride and Sodium Chloride

The inhibitory effect of high [sodium] on tobramycin may be partially reversed by the sodium channel blocker amiloride (Cohn et al., 1992; Hirsh, 2002). To test this in our system, we combined 1 mM amiloride, the clinically relevant concentration, with tobramycin and sodium chloride at 50 to 400 mM. For *P. aeruginosa* (Figure 3.4.1A), the inhibitory effect of sodium (up to 100 mM) on the tobramycin MIC, was reversed by amiloride. The tobramycin MIC of 0.086 μg/ml (0.184 μM) in sodium plus amiloride was almost identical to the MIC of tobramycin-amiloride without sodium chloride. However, the tobramycin MIC above 200 mM sodium chloride increased to 0.250 μg/ml (0.535 μM), and then up to 0.310 μg/ml (0.663 μM) at 400 mM. In contrast to the *P. aeruginosa* result, the tobramycin MICs for *B. cepacia* (Figure 3.4.1B) were much more affected by sodium chloride, as the 1 mM amiloride only maintained the tobramycin MIC of 0.052 μg/ml (0.111 μM) at the lowest concentration of sodium chloride [50 mM]. Beyond that, sodium chloride at 150 mM to 800 mM and 1 mM amiloride gave tobramycin MICs about two-fold lower than the MICs without amiloride (see Section 3.3.1). These results appear to show that the antagonistic effects of sodium chloride against tobramycin were only partially reversed in the presence of amiloride.
Figure 3.4.1 Combination of tobramycin and 1 mM amiloride in different concentrations of sodium chloride against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, AMIL = amiloride, NaCl = sodium chloride.
3.4.2 Tobramycin, Amiloride and Sodium Gluconate

Tobramycin, 1 mM amiloride, and sodium gluconate at 50 to 400 mM exhibited similar tobramycin MIC outcomes against *P. aeruginosa* (Figure 3.4.2A) and *B. cenocepacia* (Figure 3.4.2B) as was seen earlier for sodium chloride, confirming the antagonistic effect of only sodium and its partial reversal by amiloride.
Figure 3.4.2 Combination of tobramycin and 1 mM amiloride in different concentrations of sodium gluconate against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). TOB = tobramycin, AMIL = amiloride, NaGlu = sodium gluconate.
3.4.3 Tobramycin, Amiloride and Potassium Chloride

Since this study indicated that monovalent potassium antagonised tobramycin activity by almost the same as sodium chloride and sodium gluconate (Section 3.3.3), an attempt was made to see if amiloride could overcome the antagonistic effect on tobramycin MICs as seen earlier for sodium. For *P. aeruginosa* (Figure 3.4.3A), 1 mM amiloride and potassium chloride from 50 to 400 mM gave tobramycin MICs from 0.089 μg/ml (0.190 μM) to 0.583 μg/ml (1.247 μM). For *B. cenocepacia* (Figure 3.4.3B), tobramycin MICs ranged from 0.188 μg/ml (0.402 μM) to 1.500 μg/ml (3.209 μM) for KCl at 50 to 400 mM in 1 mM amiloride. These data were similar to those for sodium chloride seen above. That is, amiloride appeared to reverse the potassium chloride effect at the lowest concentration, but was only partially effective at higher KCl concentrations.
Figure 3.4.3 Combination of tobramycin and 1 mM amiloride in different concentrations of potassium chloride against Pseudomonas aeruginosa (A) and Burkholderia cenocepacia (B). Potassium chloride solutions from 50 mM to 400 mM were combined with 1 mM amiloride and serial dilutions of tobramycin, and bacterial cell suspension. TOB = tobramycin, AMIL = amiloride, KCl = potassium chloride.
3.4.4 Tobramycin, Amiloride and D-mannitol

D-mannitol should have an osmotic effect on the growth of microorganisms, mimicking conditions – partially at least – in the lungs of cystic fibrosis patients. We attempted to assess the potential of amiloride to reverse the effect of mannitol on tobramycin activity, using concentrations of D-mannitol ranging from 100 mM to 800 mM. These were combined individually with 1 mM amiloride and tobramycin serial dilutions, against the test microorganisms. It appears that D-mannitol slightly assisted the tobramycin-amiloride combination against *P. aeruginosa* (Figure 3.4.4A) since the MIC of tobramycin even at 800 mM of D-mannitol was still only 0.089 μg/ml (0.190 μM), which was about the original MIC in the absence of mannitol. However, the tobramycin MIC against *B. cenocepacia* (Figure 3.4.4B) was 0.088 μg/ml (0.188 μM), which was twice the original MIC. It was surmised that amiloride was slightly synergistic with tobramycin in the presence of increasing concentrations of D-mannitol, against both microorganisms. The effect was slighter greater with *B. cenocepacia*, as higher concentrations of D-mannitol were partially inhibitory of the growth of *P. aeruginosa* – not an undesirable outcome in itself, since inhibition of growth is the purpose of these experiments.
Figure 3.4.4 Combination of tobramycin and 1 mM amiloride in different concentrations of D-mannitol against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Each concentration of D-mannitol (100, 200, 300, 400, 600, 800 mM) was combined with 1 mM of amiloride followed by tobramycin serial dilutions and then bacterial cell suspension. All data were corrected by using the average MICs ± SEMs. n was 3 to 6 for *P. aeruginosa* and *B. cenocepacia*, respectively. SEMs of the tests ranged from 0.001 to 0.156. SEM bars were covered by the symbols. TOB = tobramycin, AMIL = amiloride, Mann = D-mannitol.
3.4.5 Tobramycin, Amiloride and N-methyl-D-glucamine

Although the result from Section 3.3.5 demonstrated an insignificant effect of NMDG, except at the highest concentration (50 mM), on tobramycin MICs, it was still decided to check any possible synergistic effects of combined amiloride and NMDG on tobramycin MICs against \textit{P. aeruginosa} and \textit{B. cenocepacia}. As can be seen in Figure 3.4.5 (A, B) for \textit{P. aeruginosa}, 1 mM amiloride and NMDG from 0.125 mM to 50 mM decreased the tobramycin MIC about two-fold to 0.044 µg/ml (0.094 µM). For \textit{B. cenocepacia} (Figure 3.4.5 C, D), tobramycin MIC was only marginally reduced under the same amiloride and NMDG concentrations as were used for the \textit{P. aeruginosa} assays.
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* (A and B) and *Burkholderia cenocepacia* (C and D). TOB = tobramycin, AMIL = amiloride, NMDG = N-methyl-D-glucamine.
3.5 EFFECT OF ADJUNCTIVE COMPOUNDS ON AMIKACIN MICs

3.5.1 Two or Three Drug Combinations

It has been proved that the aminoglycoside amikacin has a greater therapeutic effect and is less toxic than tobramycin (Garraffio et al., 1990; Vakulenko and Mobashery, 2003). According to Table 3.1 in this in vitro project, it shows that amikacin was indeed more potent than tobramycin against the test organisms. Thus, amikacin combined with the adjunctive compounds previously tested with tobramycin, might exhibit even better chemotherapy outcomes against P. aeruginosa and B. cenocepacia. Amikacin MICs with 1 mM amiloride alone (Figure 3.5.1 A and B) were moderately to slightly lower for P. aeruginosa and B. cenocepacia, respectively. However, when the amikacin MIC assays were performed with amiloride (1 mM) and verapamil (2 mM) or salbutamol (2 mM) or all three (Figure 3.5.2 A and B), the combined amiloride-salbutamol effect was pronounced with much lower amikacin MICs for both organisms. In contrast, the triple combination of amiloride, salbutamol and verapamil demonstrated antagonistic effects, albeit with still lower amikacin MICs than for amiloride alone.
Figure 3.5.1  Combination of amikacin with 1 mM amiloride against *Pseudomonas aeruginosa* (A) and *Burkholderia cenocepacia* (B). Serial dilutions of amikacin combined with 1 mM amiloride were appraised against both microorganisms. The results were corrected by the average of MICs ± SEMs, which ranged from 0.001 to 0.014. AMIK = amikacin, AMIL = amiloride.
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* (A) and *Burkholderia cenocepacia* (B). AMIK = amikacin, SAL = salbutamol, AMIL = amiloride, VER = verapamil.
3.6 ADJUNCTIVE AGENT COMBINATIONS AGAINST CYSTIC FIBROSIS ISOLATES OF *Pseudomonas aeruginosa* RESISTANT TO TOBRAMYCIN

3.6.1 Tobramycin MICs

The earlier results for successful combinations of non-antibiotics in reducing tobramycin MICs, especially for the benzamil-tobramycin combination, induced us to explore the usefulness of combination chemotherapy on several tobramycin-resistant cystic fibrosis isolates of *P. aeruginosa*. Tobramycin MICs for four such isolates, PSM1, PSM2, PSS1, and PSS2 are given in Table 3.3. Direct comparison of the tobramycin MICs of these strains with the standard NCTC *P. aeruginosa* tobramycin-susceptible test strain indicates that the clinical isolates were – in the order given above – 28, 8.7, 8.9 and 2.4 times more resistant to tobramycin, respectively, than the test strain. Of the non-antibiotic compounds tested, the MICs for amiloride, benzamil and salbutamol were about the same as seen earlier for the NCTC *P. aeruginosa* strain, but the amlodipine MICs were unexpectedly about 5 times lower for the four clinical strains, PSM1, PSM2, PSS1, and PSS2, at 45 μM (18.5 μg/ml), 44 μM (18 μg/ml), 37 μM (15 μg/ml), and 38 μM (15.5 μg/ml), respectively (Tables 3.1 and 3.3). Two of these strains are mucoid, but this feature is more typically found in agar and biofilm colonies. This might explain why, in these broth assays, there was little difference in the MICs for non-antibiotics for all four CF isolates and the NCTC test *P. aeruginosa* strain.

3.6.2 Tobramycin-non-antibiotic combinations against PSM1, PSM2, PSS1 and PSS2 *P. aeruginosa* clinical cystic fibrosis isolates

The chief purpose of these combination assays was to determine if the moderate to exceptional synergistic effects of amiloride, verapamil, salbutamol, amlodipine, and benzamil with tobramycin, seen in the susceptible type strain of *P. aeruginosa*, could be reproduced in four tobramycin-resistant *P. aeruginosa* cystic fibrosis clinical isolates. If so, then the synergistic effects of the non-antibiotics might reduce the tobramycin MICs
in these resistant strains. The data for the combination MIC assays are summarized in Table 3.4 and Figures 3.6.1 to 3.6.4.

Generally, the tobramycin non-antibiotic combinations were very promising, but the results varied according to the individual *P. aeruginosa* cystic fibrosis strains, with one exception. Firstly, highlighting the best combinations for each of the strains, the tobramycin MICs for PSM1 were most markedly reduced with benzamil, amlodipine and verapamil. For PSM2, all of the non-antibiotic synergists were effective. For PSS1 and PSS2, benzamil and amiloride were the most effective synergists. Overall, the best results were achieved with benzamil at either 0.5 or 1.0 mM for all four strains, in parallel to the very promising benzamil-tobramycin data seen earlier for the NCTC *P. aeruginosa* and *B. cenocepacia* strains. Most importantly, it can be concluded that, insofar as this was only a small sample group of clinical strains, benzamil universally, and some of the other non-antibiotics more specifically, was an effective adjunctive that reduced the tobramycin MICs in all four tobramycin-resistant cystic fibrosis clinical strains. For either 0.5 or 1.0 mM benzamil (Table 3.4), the tobramycin MIC was reduced by a factor of 13.7, 6.2, 61.5, or 4, for PSM1, PSM2, PSS1, and PSS2, respectively. These reductions made the new tobramycin susceptibilities of the four strains near to or even below the MIC for the tobramycin-susceptible NCTC *P. aeruginosa* test strain (Table 3.1), which is a very promising result that augers well for extending this study to a broader range of clinical cystic fibrosis isolates of *P. aeruginosa* and *B. cenocepacia*. 
Figure 3.6.1 MICs of tobramycin alone or in the combinations with non-antibiotic compounds against *P. aeruginosa* PSM1. TOB = tobramycin, AMIL = amiloride, BEN = benzamil, SAL = salbutamol, VER = verapamil, AMLO = amlodipine.
**Figure 3.6.2** MICs of tobramycin alone or in the combinations with non-antibiotic compounds against *P. aeruginosa* PSM2. TOB = tobramycin, AMIL = amiloride, BEN = benzamil, SAL = salbutamol, VER = verapamil, AMLO = amlodipine.

**Figure 3.6.3** MICs of tobramycin alone or in the combinations with non-antibiotic compounds against *P. aeruginosa* PSS1. TOB = tobramycin, AMIL = amiloride, BEN = benzamil, SAL = salbutamol, VER = verapamil, AMLO = amlodipine.
**Figure 3.6.4** MICs of tobramycin alone or in the combination with non-antibiotic compounds against *P. aeruginosa* PSS2. TOB = tobramycin, AMIL = amiloride, BEN = benzamil, SAL = salbutamol, VER = verapamil, AMLO = amlodipine.
Table 3.3 Minimum inhibitory concentrations for tobramycin and non-antibiotic compounds against four cystic fibrosis strains of *P. aeruginosa* (PSM1, PSM2, PSS1, PSS2). These MIC values are the average of 4 estimations that did not vary by more than two values for all of the compounds.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Minimum Inhibitory Concentration (MIC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>P. aeruginosa</em></td>
</tr>
<tr>
<td></td>
<td>PSM1</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>2.58</td>
</tr>
<tr>
<td></td>
<td>5.52</td>
</tr>
<tr>
<td>Amiloride</td>
<td>4,930</td>
</tr>
<tr>
<td></td>
<td>18,527</td>
</tr>
<tr>
<td>Benzamil</td>
<td>890</td>
</tr>
<tr>
<td></td>
<td>2,499</td>
</tr>
<tr>
<td>Salbutamol</td>
<td>3,820</td>
</tr>
<tr>
<td></td>
<td>15,963</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>45</td>
</tr>
</tbody>
</table>
Table 3.4  Comparison of tobramycin MICs in various combinations with non-antibiotic compounds, amiloride, benzamil, salbutamol, or amlodipine, four cystic fibrosis strains of *P. aeruginosa* (PSM1, PSM2, PSS1, PSS2). MIC values are the average of 4 estimations that did not vary by more than two values for all of the compounds. "ND = not determined.

<table>
<thead>
<tr>
<th>Amloride (mM)</th>
<th>Benzamil (mM)</th>
<th>Salbutamol (mM)</th>
<th>Verapamil (mM)</th>
<th>Amlodipine (mM)</th>
<th>Tobramycin MIC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>PSM1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>µg/ml</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2.58</td>
</tr>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.780</td>
</tr>
<tr>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.188</td>
</tr>
<tr>
<td>-</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>&quot;ND&quot;</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>2.330</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>2</td>
<td>-</td>
<td>0.620</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.06</td>
<td>1.100</td>
</tr>
</tbody>
</table>
CHAPTER FOUR

DISCUSSION

In this chapter, sections 4.1 to 4.4 discuss the results for the NCTC *P. aeruginosa* and *B. cenocepacia* test strains. The final section, namely 4.5, has been added in the revised version of this thesis and it discusses new data for four *P. aeruginosa* cystic fibrosis clinical isolates.

Opportunistic pathogens, such as *P. aeruginosa* and *B. cepacia* complex, are the most common causative agents of chronic pulmonary infections in cystic fibrosis patients, and they are among the most difficult to treat and eradicate (Hoiby *et al.*, 2005). This difficulty is exacerbated by the ability of these organisms to form biofilm communities and to elicit multidrug resistance mechanisms (Hoffman *et al.*, 2005; Mashburn *et al.*, 2005). These virulence characteristics can be controlled and also triggered by the quorum-sensing signals of bacteria and the persistent use of antibiotics (Pearson *et al.*, 2000; Smith and Iglewski, 2003; Schaber *et al.*, 2004). Aminoglycosides actually enhance the ability of infectious organisms to form biofilms (Hoffman *et al.*, 2005).

Both *P. aeruginosa* and *B. cepacia* complex are intrinsically resistant to many antibiotics, and have inducible resistance and multidrug resistance mechanisms that are more prolific in hospitals than in the wider community (Hancock and Speert, 2000; Schweizer, 2003; Poole, 2005), rendering antibiotic therapies generally suppressive rather than curative. Recurrent infection or intractable antibiotic-resistant pathogens are chiefly responsible for the aetiology of disease, debilitation, and often the death of cystic fibrosis patients (Lyczak *et al.*, 2002; Chmiel and Davis, 2003). These problems can only be solved by developing new antibiotics and/or alternative therapies. Unfortunately, the occurrence of bacterial resistance appears to progress more quickly than the discovery of antibiotics (Brennan and Geddes, 2004). Moreover, the development of new antibiotics has been largely abandoned because this is time-consuming and costly, with only linezolid and daptomycin being introduced in the past four decades (Wenzel *et al.*, 2005). Therefore, the use of different drug combinations
and therapeutic regimes are the best options for long-term treatment of chronic infections.

4.1 HIGH POTENCY OF AMIKACIN COMPARED TO TOBRAMYCIN

Evidence from several experiments has demonstrated amikacin as an aminoglycoside which is more effective than tobramycin (Garraffo et al., 1990; Vakulenko and Mobashery, 2003). In this project, amikacin dihydrate was approximately seven times more potent than tobramycin against *P. aeruginosa* and *B. cepacia* (Table 3.1). Bearing in mind that tobramycin has been widely used as antibiotic of choice for treating infections in cystic fibrosis, resistance mechanisms and bacterial biofilms are of major concern (Shawar et al., 1999; Beringer et al., 2000; Master et al., 2001), and although the same problems would be expected to apply to amikacin dihydrate, its lower MIC suggests that it could be more effective than tobramycin if delivered at lower dosage levels. Another potential advantage of amikacin over tobramycin is its lower toxicity (Hottendorf and Gordon, 1980). Although amikacin is more potent than tobramycin, strain-specifics might affect some results and therefore many more clinical isolates need to be tested for a complete assessment. To this end, the occurrence of pathogen resistance should be monitored (Barclay et al. 1996; Ziha-zarifi et al., 1999; Al-Aloul et al., 2004). For example, Manno and colleagues (2005) demonstrated the decreasing aminoglycoside susceptibility of *P. aeruginosa* in cystic fibrosis centres. Ceftazidime—a third-generation cephalosporin that is strongly active against *P. aeruginosa*—was the most potent agent, killing 86% of isolates, whereas tobramycin and amikacin eradicated 76% and 69%, respectively.
4.2 SYNERGISTIC EFFECT OF TOBRAMYCIN WITH THE AMILORIDES

Due to an increasing incidence of resistant pathogens caused by single antibiotic usage, chemotherapeutic effects of non-antibiotics in combination with antibiotics might be more effective against pathogens (McGrath et al., 1993; Schidlow, 2000; Chiu et al., 2001; Brennan and Geddes, 2004). Amiloride hydrochloride hydrate and its derivatives function by blocking sodium pumps. Sodium is known to antagonise tobramycin uptake by bacteria (Leviton et al., 1995), and this antagonism can be partially reversed by the sodium ion channel blocker, amiloride (Cohn et al., 1988). In this project, amiloride hydrochloride hydrate was used at a clinically relevant concentration of 1 mM, which is 18 times lower than its MIC against the test organisms, giving more powerful in vitro synergistic effects with tobramycin against P. aeruginosa and B. cenocepacia than only tobramycin by itself (Table 3.1). This approach has been shown to be effective in vivo in a pilot trial of aerosolised amiloride-tobramycin therapy (Middleton et al., 2005), in which three out of four patients were cleared of infection after six months. Since amiloride hydrochloride hydrate has the lowest potency and also the shortest duration, benzamil hydrochloride and phenamil could potentially be even more efficacious with tobramycin. Benzamil hydrochloride and phenamil are more lipophilic than amiloride hydrochloride hydrate and might be expected to cross the cell envelope more easily. However, while benzamil hydrochloride, at a sub-inhibitory concentration, exhibited excellent efficacy against both microbes with tobramycin, phenamil was less effective. This could mean that other factors might be implicated in the uptake mechanisms of these bacteria. Since benzamil hydrochloride and phenamil have never been tested in combination with tobramycin against these organisms, this result could be a pointer for the treatment of cystic fibrosis infections. Giunta et al. (1984) suggested that amiloride could function as an antimicrobial agent, especially against Gram-positive bacteria; and that the inhibitory effect of amiloride on the sodium ion channel could be an important factor in the control of bacterial cell proliferation. Moreover, as benzamil has been reported to have a therapeutic duration at least twice longer than amiloride (Hofmann et al., 1998; Rodgers and Knox, 1999), it could be more useful for long-term cystic fibrosis treatment. Previous amiloride-tobramycin in vitro studies from the Cohn group (1988; 1992; 1994) showed only moderately successful synergistic effects – up to four-
fold lower MICs for tobramycin – in fewer than 50% of clinical strains of *Pseudomonas* spp and *B. cepacia* tested. One of these studies reported only a two-fold lower tobramycin MIC for benzamil, compared to amiloride.

Adding a lipophilic moiety to amiloride can increase its ability to block sodium ion transport, which can be seen in different MIC values (Cohn *et al.*, 1992). Since the permeability of these agents across cell membranes depends on the lipophilicity, the activities of phenamil and benzamil should be stronger (Hirsh *et al.*, 2004). Phenamil is 20 times more lipophilic than amiloride and twice more than benzamil (Kleyman and Cragoe, 1988), but the structures of phenamil and benzamil differ only by the latter having a methylene bridge between the terminal guanidine amino moiety and a phenyl ligand. Amilorides are weak bases that are most potent as inhibitors in the protonated form (Kleyman and Cragoe, 1988). The pKas of phenamil, benzamil and amiloride are 7.8, 8.1 and 8.7, respectively, with phenamil being slightly less basic than the other two, yet the antibacterial potencies of the three compounds do not appear to correlate with the pKa values. On the other hand, the lipophilicities of phenamil and benzamil suggest that their MICs should be lower than for the more hydrophilic amiloride, and this was the case (Table 3.1).

Recently, a new antibiotic, tigecycline, was approved by the U.S. Food and Drug administration for treating multidrug-resistant bacteria (http://www.fda.gov_viewed in September, 2006). Although it is a derivative of the tetracycline analogue minocycline, tigecycline is not affected by classical tetracycline resistance mechanisms; and tigecycline has broad spectrum activity against Gram-positive and Gram-negative bacteria (Smith *et al.*, 2005; Stein and Craig, 2006); and although this tetracycline analogue was shown to inhibit some *Burkholderia* species (Thamlikitkul and Trakulsomboon, 2006), many *P. aeruginosa* and *B. cepacia* strains appear to be intrinsically resistant to this analogue, owing to their chromosomal efflux pumps (Livermore, 2005; Stein and Craig, 2006). *P. aeruginosa* is also intrinsically resistant to β-lactam antibiotics, aminoglycosides and quinolones, but the carbapenem derivative, doripenem, has been reported to have more antibacterial efficacy than that of imipenem and meropenem (Mushtaq and Livermore, 2004; Brown and Traczewski, 2005; Rice, 2006). Tigecycline and doripenem might be excellent candidates for synergistic
assessing synergy with the non-antibiotic amilorides against *P. aeruginosa* and *B. cepacia* complex.

4.3 SYNERGISTIC EFFECTS OF OTHER ADJUNCTIVE NON-ANTIBIOTICS WITH TOBRAMYCIN

An interesting outcome of the MIC assays was the almost identical MICs for amiloride hydrochloride hydrate and salbutamol for both test organisms; but a surprising difference in verapamil’s MICs, with that for *B. cenocepacia* being 20-fold lower than for *P. aeruginosa* (Table 3.1). The exceptional verapamil susceptibility of *B. cenocepacia* versus *P. aeruginosa* cannot be easily explained, except by citing a specific strain difference, in which case verapamil MIC testing on more strains of both organisms could confirm this notion. An earlier study of verapamil-tobramycin synergy in these two organisms found that the verapamil MICs for four strains of each were about the same at >8 mM (Cohn et al., 1995), suggesting that a species-specific effect might explain the single result in this project. Segal et al. (2002) examined verapamil in A6 cell lines which are generally used for the study of transepithelial sodium ion transport, finding that verapamil can possibly inhibit the transport of sodium ion by acting on the epithelial sodium ion channel. This suggests that verapamil could be a promising adjunctive drug with tobramycin, as already noted for the amilorides.

Salbutamol-tobramycin-amiloride hydrochloride hydrate produced the lowest tobramycin MIC against *B. cenocepacia*, whereas all other salbutamol combinations were less effective (Table 3.2). Furthermore, with the exception of benzamil hydrochloride-tobramycin, the salbutamol and amiloride hydrochloride hydrate combination was also the most effective against *P. aeruginosa*. Certainly, these *in vitro* results might not reflect their effectiveness in the cystic fibrosis lung, which would require a thorough *in vivo* assessment trial. Nevertheless, their generally synergistic effect in delivering lower tobramycin MICs against *P. aeruginosa* and *B. cenocepacia* argues in favour of their potential therapeutic use, especially given the success of a pilot clinical trial of aerosolised tobramycin-amiloride therapy (Middleton et al., 2005). Salbutamol in human airway epithelial cells is acts as a bronchodilator (Groshaus et al.,
2004; Broadley, 2006), a role that is clearly different from the synergistic antimicrobial effects seen in the *in vitro* MIC assays. Despite this promising *in vitro* data, it will be essential to test and monitor aerosolized chemotherapeutic formulations in order to avoid the antagonistic effects of some compound combinations with tobramycin.

Of the non-antibiotic compounds tested in this study, amlodipine, a calcium ion channel inhibitor, had the lowest MIC against the test organisms; and this was about one third lower than the next best drug, benzamil hydrochloride (Table 3.1). It also exhibited a slightly synergistic effect with salbutamol and tobramycin against *P. aeruginosa*. However, amlodipine antagonized tobramycin activity against *B. cenocepacia*, even though amlodipine alone had a very low MIC result that is consistent with its recognised potency against other Gram-negative species (Asok Kumar et al., 2004). Amlodipine-streptomycin was moderately synergistic against Gram-negatives *in vitro* and also in a mouse model (Asok Kumar et al., 2004), suggesting some traction for this approach, but against that is the risk of using a cardiovascular drug in cystic fibrosis patients. Also, amlodipine does not reverse the sodium antagonism of tobramycin antibacterial potency. Despite these caveats, amlodipine was the most potent of the compounds tested other than antibiotics, and although its potential in cystic fibrosis chemotherapy should not be dismissed out of hand, it cannot be used with tobramycin, as it is a strong agonist.

The bismuth-thiols perhaps represent more promise as, in addition to demonstrated *in vitro* synergy with tobramycin against *B. cepacia* complex, they possess the added advantages of antibiofilm activity and MICs against *B. cepacia* complex (Veloira et al., 2003). However, bismuth-thiols are toxic to eukaryotic cells at low concentrations (5–15 µM) and any proposed clinical use would need to be at sub-inhibitory concentrations. This leaves amiloride and its analogues as probably the most promising candidates for adjunctive therapy in treating chronic infection in cystic fibrosis patients. Benzamil hydrochloride and phenamil, in particular, are relatively non-toxic at levels that not only enhance the antibacterial effects of aminoglycosides, but may serve as antibacterials in their own right and at concentrations below that used currently for the parent compound amiloride. Apart from sodium ion channel blockers, chloride ion channel-openers, for instance, forskolin and chlorzoxazone, could be tried as adjunctive
compounds in antimicrobial combinations (Cuthbert, 2001; Rodgers and Knox, 2001). Phenanthrolines, benzoquinolines, and chelating compounds for metal ions have also been revealed to have activities affecting CFTR and also potassium ion channels (Duszyk et al., 2001). These channel openers should be further studied in combinations with antimicrobials, bronchodilators, or even channel-blockers, as they could lead to successful adjunctive options for cystic fibrosis therapy.

Calcium and sodium channel blockers, bronchodilators, antihistamines, anaesthetics, psychotropics and others exhibit in vitro and in vivo antibacterial action against most Gram-negatives (see references in Asok Kumar et al. 2004). Results from the present and previous studies on the antibacterial activity of tobramycin against P. aeruginosa and B. cepacia complex indicate that moderate synergy can be demonstrated by adjunctive treatment with some of these clinical drugs. Their potential as adjunctive agents with antibiotics – particularly as aerosols used to treat intractable pulmonary infections in patients with cystic fibrosis – argues strongly in favour of more clinical trials. However, some problems remain to be elucidated, the chief of which are knowledge of the precise mechanism of aminoglycoside uptake in Gram-negatives and the antagonistic effects of small cations and anions, and of some drugs on these mechanisms. Certainly, some preliminary effects of these ions and compounds are known, but more evidence is required; if only to be more certain that antibiotics and adjunctive compounds will be effective in patient treatments. Sodium, potassium, and chloride ions are ubiquitous in nature – so much so that elaborate pumping mechanisms for maintaining homeostasis are present in all organisms. The use of drugs may disturb or antagonise this homeostasis, or one or more of the ions in constant flux may antagonise a therapeutic drug.

Another aminoglycoside, amikacin, has been used with other antibiotics (Aronoff and Klinger, 1984; Aaron et al., 2000; Aaron et al., 2005), but pathogens, especially P. aeruginosa and B. cepacia complex, eventually become resistant through induction or mutation of host resistance mechanisms (Hurley et al., 1995; Carmeli et al., 1999; Levy, 2002). The use of non-antibiotics with antibiotics may be more appropriate, especially for long-term treatment (Cohn et al., 1995; Chiu et al., 2001; Brennan and Geddes, 2004). Rajyaguru and Muszynski (1998) found that theobromine and theophylline, typically used as diuretics, vasodilators, or myocardial stimulants, can considerably
4.4 ANTAGONISTIC EFFECT OF SODIUM ON ACTIVITY OF TOBRAMYCIN

The mechanisms of uptake of aminoglycosides by bacterial cells have been studied by several groups (Thompson et al., 1985; Taber et al., 1987; Leviton et al., 1995). Tobramycin uptake in *E. coli* is regulated by the electrical potential component of the proton motive force; and is probably mediated by a voltage-gated channel in the cytoplasmic membrane that is inhibited by compounds that depolarise the membrane potential. The binding of aminoglycosides by cations probably occurs in the bacterial outer membrane (Ramirez-Ronda et al., 1975; Hancock et al., 1981; Loh et al., 1984).

This form of inhibition is competitive and is probably due to divalent cations or polycations antagonising the initial binding of aminoglycosides to lipopolysaccharides in the outer membrane (Zemelis and Jackson, 1973; Fass and Barnishan, 1979; Cohn and Aronoff, 1989). Although a sodium antagonising effect on tobramycin MICs was suggested from earlier data (Medeiros et al., 1971; Cohn and Aronoff, 1989), the present study interprets more entirely the moderating effect of amiloride hydrochloride hydrate on sodium antagonism of tobramycin MICs, and extends this to consider the effects, if any, from potassium and chloride ions (see Chapter 3). The absence or dysfunction of the CFTR chloride ion channel caused by *CFTR* mutation induces alterations of sodium transport and water reabsorption. As a consequence, desiccated mucus can then obstruct the ducts of the respiratory epithelium (Johnson et al., 1995; Bachhuber et al., 2005). It is well known that sodium chloride affects the antimicrobial activity of aminoglycosides against Gram-negative bacteria (Medeiros et al., 1971; Lamb et al., 1972; Cohn and Aronoff, 1989). The concentrations of sodium and chloride ions in patients with cystic fibrosis are present in concentrations that vary according to the severity of the cystic fibrosis syndrome (Giunta and Groppa, 1984). Unfortunately,
these studies did not provide conclusive proof that sodium was the chief agonist of the aminoglycoside activity against Gram-negatives.

As already mentioned, the importance of both sodium and chloride in the cystic fibrosis lung warrants confirmation that it is sodium, not chloride, that is responsible for the observed effect, primarily because it would be much more difficult to change or inhibit chloride flux in the absence of CFTR-chloride ion channel activity. When assays were performed with potassium chloride at the same concentrations used for sodium chloride and sodium gluconate, the tobramycin MICs against both organisms increased by the same factor of two- to four-fold for 50 to 300 mM potassium chloride. Since no previous study had tested for the effect of potassium on the activity of aminoglycosides against Gram negatives, this is a novel finding. There could be three possible reasons for the observed potassium effect on tobramycin MICs. The first is that potassium inhibition of tobramycin binding to cell envelope lipopolysaccharides is weaker than sodium inhibition, but both are reversed by amiloride hydrochloride hydrate, possibly by a relative mechanism of ion/proton exchange. The second possibility relates to the ion specificity of amiloride-sensitive sodium ion channels. Amiloride is known to inhibit specific sodium ion channels in eukaryotes and, by inference, also in prokaryotes. Therefore, if there is any amiloride effect on the potassium antagonism of tobramycin activity that is based on ion channel selectivity, it would need to involve a specific bacterial potassium ion channel. There is also evidence for such an interaction in eukaryotes, in which an amiloride analogue showed 30–40% inhibition of a rectifier potassium ion channel (Bielefeld et al., 1986); but no evidence of this type of interaction has appeared yet for bacterial potassium ion channels. A third explanation for the amiloride hydrochloride hydrate-potassium data is that there is not a potassium effect at all, and that the partial decrease in tobramycin MICs in the potassium chloride assays is due to amiloride hydrochloride hydrate overcoming only the sodium-induced inhibition of tobramycin uptake, where the sodium source is the endogenous sodium chloride in the MHB growth medium. If this third explanation is correct, then the use of potassium in the assays is an inadvertent but useful control for unmasking background sodium effects in the medium; and there is some evidence to support this notion. Nutrient broth has almost the same content of potassium concentration as MHB, but a nine-fold lower content of sodium concentration (Medeiros et al., 1971).
The use of compounds which can increase the permeability of the membrane might be an option for treating infections (Loh et al., 1984; Vaara, 1992). Polyamines at millimolar concentrations have been shown to increase the MICs of aminoglycosides, β-lactams, and other antibiotics (Kwon and Lu, 2006a; Kwon and Lu, 2006b). This polyamine-induced effect was unrelated to the disorganisation of the outer membrane, typically caused by agents, such as EDTA and polymyxin B, or to transcriptional control of β-lactamase expression. The polycationic property of polyamines suggests some affinity with increased tobramycin MICs caused by other exogenous cations, such as sodium and potassium ions. However, the effects of these different cations are unrelated to the positive charge, since it has been shown that polyamines and magnesium block cytoplasmic pores, probably by binding to conserved glutamate residues in the lumen of these channels, therefore antagonising inward potassium ion movement (Booth et al., 2003). The significance of this information for the present study becomes apparent by noting that most cells maintain internal concentrations of potassium and sodium at 300 and 1 mM, respectively. In contrast, the extracellular concentration of sodium is notably much higher than that of potassium, and effects of the former on aminoglycoside antagonism are therefore likely to be much greater. Inward movement of sodium ions is controlled by Na⁺/H⁺ antiporters and is only required in response to alkalinisation of the cytoplasm. The usual physiological situation notices inward movement of potassium and outward movement of sodium ions, which makes sodium ions much more relevant as an antagonist of binding and uptake of aminoglycosides on the extracellular side of bacterial membranes.

Finally, in order to determine if the ionic or osmotic strength of a solution can alter aminoglycoside MIC values, tobramycin MICs were measured in the presence of increasing concentrations of D-mannitol (Fig. 3.3.4). No changes or adverse effects were seen by D-mannitol on the tobramycin MICs against the test organisms. In human airway epithelial cells, osmotic compounds, including D-mannitol, have been discovered to enhance mucus clearance by the stimulation of mucus secretion (Salathe et al., 1996; Daviskas et al., 2001). Moreover, D-mannitol may be able to increase the amount of water in the airway surface, resulting in decreasing mucoviscosity, and then increasing the transportability of mucus (Salathe et al., 1996; Daviskas et al., 2001). Thus, D-mannitol might a useful agent in formulations that might be used against cystic fibrosis pathogens (Daviskas et al., 1999; Robinson et al., 1999; Daviskas et al., 2005).
Xylitol, another osmotic agent, has been found to decrease high salt concentrations in human airway surface liquid, resulting in increasing potency of antimicrobial peptides (Zabner et al., 2000), and therefore, by inference, the potency of antimicrobials.

4.5 SYNERGISTIC EFFECTS OF TOBRAMYCIN AND NON-ANTIBIOTICS AGAINST CLINICAL ISOLATES FROM CYSTIC FIBROSIS PATIENTS

In attempts to further study the synergistic efficacy of tobramycin combined with non-antibiotics, four strains of *P. aeruginosa*, isolated from cystic fibrosis patients at Westmead Hospital, two of them producing mucoid colonies on agar plates, were examined. All four strains were chosen deliberately for their clinical resistance to tobramycin. In this way, the real efficacy of tobramycin and non-antibiotic adjunctive synergy, seen earlier for two tobramycin susceptible strains, could be assessed. When comparing tobramycin MIC values alone for these four clinical strains (Table 3.3), PSS2 was the most susceptible to tobramycin, and the mucoid isolate PSM1 was the most tobramycin-resistant. Whether this variation in tobramycin resistance would translate to differences in efficacy of tobramycin-non-antibiotic combinations would be a true test of the significance of this project for clinical chemotherapeutic potential.

From Table 3.3 it is clear that, except for tobramycin, the four clinical strains of *P. aeruginosa* demonstrated similar drug susceptibilities to each of the non-antibiotic agents, with the possible exception of amlodipine. These MIC results meant that if there were any real differences between these four strains and the *P. aeruginosa* NCTC and *B. cenocepacia* strains, apart from tobramycin resistance or susceptibility, these differences could only be determined in the drug combination synergy assays (Figures 3.6.1 to 3.6.4; Table 3.4). Synergistic combinations were observed for all four tobramycin-resistant clinical strains, which is a promising conclusion that backs up the earlier data for the two tobramycin-susceptible strains. On an individual basis, the best results - summarized in Table 3.4 - were as follows:
For PSM1, the combination tobramycin-benzamil was 13.7 times more potent than tobramycin alone and, more importantly, decreased the tobramycin MIC to just two-fold higher than that seen in the susceptible *P. aeruginosa* assays (Table 3.1 and Table 3.2). Tobramycin-verapamil was the next best combination against PSM1 at a 4-fold lower tobramycin MIC than with tobramycin alone. For PSM2, all combinations generated effective synergy, with the reductions in tobramycin MICs ranging from just over 6-fold (with benzamil) to just over 5-fold (amiloride) to 3-fold (salbutamol, amlodipine, verapamil). For PSS1, benzamil was best (from 7-fold to 64-fold lower MICs for 0.5 and 1.0 mM benzamil, respectively), followed by amiloride at over 3-fold and salbutamol and verapamil at about 1.5-fold. Amlodipine was ineffective for this strain. Finally, for PSS2, which had the lowest tobramycin resistance of the four strains at only about 2.5-fold above tobramycin susceptibility, benzamil was again the best in combination with tobramycin with the MIC reduced four-fold. Amiloride was the only other effective drug with a two-fold MIC reduction.

Points to note for these data are that although the four clinical strains of *P. aeruginosa* were resistant to tobramycin, three of them (PSM2, PSS1, PSS2) showed more susceptibility to tobramycin-amiloride than the two organisms chosen initially for their tobramycin susceptibility. In addition, the clear conclusion from the entire project is that the combination of tobramycin with benzamil is particularly potent, at least *in vitro*, and beckons for testing in the clinic, as has been done in the pilot study with amiloride (Middleton *et al.*, 2005) that was the genesis of this project.

This preliminary project confirms the effectiveness of using combined drug chemotherapy, particularly with benzamil, to obtain synergy with tobramycin against tobramycin susceptible and resistant strains and against mucoid and non-mucoid strains of *P. aeruginosa*. Some success with similar *in vitro* studies, though less so than this project, has been reported, especially against mucoid strains (Rajyaguru and Muszynski, 1997; Rajyaguru and Muszynski, 1998; Høiby, 2002; Abdi-Ali *et al.*, 2006; Bye and Elkins, 2007; Henke and Ratjen, 2007). Garo and colleagues (2007) demonstrated the use of the biofilm inhibitors asiatic and corosolic acids in combination with either tobramycin or ciprofloxacin against *P. aeruginosa* biofilms. Since bacterial biofilms are commonly encountered in the CF lung, this should be the next phase of focus of this research.
4.6 CONCLUSION

The most critical problems in treating chronic infections caused by *P. aeruginosa* and *B. cepacia* complex is that these opportunistic pathogenic organisms thrive in “antibiotic-rich” clinical environments by expressing an array of intrinsic antibacterial mechanisms, such as permeability barriers, efflux pumps, and biofilms. The infecting microorganisms are refractory to most traditional chemotherapies, and prolonged antibiotic treatments seem to mostly be suppressive rather than curative. This *in vitro* project has confirmed the greater potency of amikacin dihydrate, compared to tobramycin, either as a single antibiotic, or with non-antibiotic adjunctive compounds, against *P. aeruginosa* and *B. cenocepacia*. Moderate to excellent antibacterial efficacies were also demonstrated by non-antimicrobial agents, such as the amilorides, verapamil, amlodipine, and salbutamol. Factors, such as lipophilicity, pH, and osmotic strength, could impact on the activity of the non-antibiotic adjuncts in clinical situations. High sodium concentration, a common condition in cystic fibrosis patients, appears to antagonize activity of many antibiotics. The antagonistic effects of sodium and potassium demonstrated a reverse correlation with aminoglycosides that could be partially overcome – at least for sodium – by using amilorides or other non-antibiotics with tobramycin against the test organisms. Even certain triple drug synergistic combinations (tobramycin-amiloride-salbutamol and amikacin-amiloride-salbutamol) were quite effective as inhibitors of the test organisms *in vitro*. This preliminary project confirmed the effectiveness of using combined drug chemotherapy, particularly with benzamil, to obtain synergy with tobramycin against tobramycin susceptible and resistant strains and against mucoid and non-mucoid strains of *P. aeruginosa*. The next phase of investigation should be to assess a large number of *P. aeruginosa* and *B. cenocepacia* isolates from cystic fibrosis patients, including isolates that are refractory to tobramycin by virtue of host resistance mechanisms. In this instance, the use of a tobramycin-benzamil combination or even of benzamil alone might be more successful in eradicating resistant organisms. Furthermore, other additive compounds should be examined in combination assays with aminoglycosides for more effective treatment regimes.


amiloride after inhalation and oral administration in adolescents and adults with cystic fibrosis. Pharmacotherapy 17: 263–270.


