Influence of Combination Therapy on the Fluoroquinolone Mutant Prevention Concentration (MPC) in *Pseudomonas aeruginosa*

Matthew Mayer

Supervisor: Dr. George G. Zhanel

A thesis submitted in partial fulfillment of the Honours Thesis

(05.4111/6) Course

Department of Biology

University of Winnipeg

2005

Abstract

Pseudomonas aeruginosa infections are difficult to treat as this organism displays a high level of intrinsic antibiotic resistance. Treatment with fluoroquinolone antibiotics leads to dramatic increases in the Minimum Inhibitory Concentration (MIC), which may be associated with treatment failure. Achieving Mutant Prevention Concentration (MPC) for fluoroquinolones may kill single step resistant mutants, potentiating successful therapy. The fluoroquinolone MPC can theoretically be reduced if combined with a second antibiotic with good activity against *P. aeruginosa*. The MPC's for three clinical isolates of *P .aeruginosa* were established for levofloxacin alone, or in the presence of tobramycin, ceftazidime, piperacillin/tazobactam or azithromycin. The levofloxacin MPC for the strains ranged from 4-8 times their MIC. The levofloxacin MPC was successfully reduced for all three strains in the presence of tobramycin, ceftazidime, and piperacillin/tazobactam, with 2-8 fold decreases. As expected, azithromycin caused no reduction in the MPC, as it is not active against *P. aeruginosa*. These results indicate a decrease in the emergence of *P. aeruginosa* resistance to levofloxacin in the presence of a second antibiotic with activity against the organism, compared to the fluoroquinolone alone, and suggest that dual-drug therapies can limit the emergence of fluoroquinolone resistance in *P. aeruginosa*.

Acknowledgements

A sincere thank you goes out to my supervisor, Dr. George Zhanel, for his guidance, support, and for providing the opportunity and resources to undertake this project. I would also like to thank Heather Smith-Adam, Nancy Laing, and all the other members of the R&D lab for their assistance on a daily basis. Thanks to my committee members, Dawn Rittberg and Dr. Paul Holloway, for their help and input. Additionally, thanks to all my family and friends for their patience and understanding.

Table of Contents

Abstract ii
Acknowledgements iii
Table of Contents iv
List of Tables v
List of Abbreviations Used vi
Introduction 1
1. *Pseudomonas aeruginosa* 1
1.1. Characteristics of the organism: 1
1.2. Clinical significance and pathogenesis: 1
1.3. Antibiotic treatment of *P. aeruginosa* infection: 3
2. Fluoroquinolones 6
2.1. Mode of action 6
2.2. Resistance mechanisms 7
3. Fluoroquinolone Mutant Prevention Concentrations 8
4. Hypothesis and Thesis Objectives 10
Materials and Methods 12
1. Media 12
2. *Pseudomonas aeruginosa* strains 12
3. Antibiotic Stock Solutions 13
3.1. Preparation 13
3.2. Solution quality control 14
4. Minimum inhibitory concentration (MIC) determination 16
5. Mutant Prevention Concentration (MPC) determination 17
5.1. Levofloxacin MPC's 17
5.2. Levofloxacin combination MPC's 19
Results 20
1. Antibiotic sensitivity (MIC's) of *P. aeruginosa* strains 20
2. Mutant prevention concentrations (MPC's) of *P. aeruginosa* strains 20
3. *P. aeruginosa* mutational frequencies 21
Discussion 23
Conclusions 27
References 28

List of Tables

Table 1. Source, purity, and lot #s of antibiotic powders, and their corresponding solvents, used in the preparation of antibiotic stock solutions for all subsequent experiments……. …… 13

Table 2. Minimum inhibitory concentrations (MIC's) of three clinical *P. aeruginosa* isolates determined in triplicate by broth dilution, and established susceptible MIC standards for LVX, TOB, CEF, P/T and AZI against *P. aeruginosa*……. …… 20

Table 3. Levofloxacin MPC's (reported as an MIC multiple) for three clinical *P. aeruginosa* strains grown for 48 hours at 37°C on MH agar supplemented with LVX alone, or LVX in addition to CEF, TOB, P/T, or AZI at their average 24-hr patient serum concentrations…….. …… 21

Table 4. Levofloxacin MPC's (reported in μg/mL) for three clinical *P. aeruginosa* strains grown for 48hrs at 37°C on MH agar supplemented with LVX alone, or LVX in addition to CEF, TOB, P/T, or AZI at their average 24-hr patient serum concentrations……. …… 21

Table 5. Average (n=3) calculated mutational frequencies for three clinical *P. aeruginosa* strains at levofloxacin concentrations, ranging from MIC to MPC, and in the presence or absence of a second antibiotic……. …… 22

List of Abbreviations Used

AZI – Azithromycin

BA – Blood Agar

CEF – Ceftazidime

CF – Cystic Fibrosis

CFU – Colony Forming Units

CSMHB – Cation-supplemented Mueller-Hinton Broth

dH2O – Distilled Water

FQ - Fluoroquinolone

ICU – Intensive Care Unit

LVX – Levofloxacin

MH – Mueller-Hinton

MIC – Minimum Inhibitory Concentration

MPC – Mutant Prevention Concentration

MSW – Mutant Selection Window

NAUTICA – North American Urinary Tract Infection Collective Alliance

NCCLS – National Committee of Clinical Laboratory Standards

P/T – Piperacillin with tazobactam

QC – Quality Control

TOB – Tobramycin

Introduction

1. *Pseudomonas aeruginosa*

1.1. Characteristics of the organism

Pseudomonas aeruginosa is an aerobic, non-sporulating, gram negative bacillus, and the member of the *Pseudomonas* genus most commonly isolated from human infections (Banerjee and Stableforth, 2000). The bacterium is commonly identified in the microbiology lab by its ability to reduce nitrate, grow at 42°C, and produce positive oxidase and catalase reactions (Murray *et al*., 1999). In addition, the organism is readily recognized by the characteristic grape odor, and the production of yellow, green, or blue pigment when grown on solid media (Murray *et al*., 1999).

The natural habitat of *P. aeruginosa* is moist environments such as soil, water, and vegetation, unlike most bacterial pathogens which have a difficult time growing outside of a host. This provides the bacteria with a great deal of robustness, allowing it to persist and grow on inanimate surfaces including counter tops, sinks, and within hospital equipment such as catheters, disinfectants, dialysis fluid and respiratory breathing apparatus (Banerjee and Stableforth, 2000). This environmental adaptability can result in cross infection in a hospital setting, making *P. aeruginosa* one of the more common nosocomial infections.

1.2. Clinical significance and pathogenesis

P. aeruginosa is not a significant cause of disease in healthy individuals outside of a hospital setting, producing mainly superficial and localized infections (Murray *et al*.,

1999). Common infections from this organism include hair follicle and ear canal infections from swimming in contaminated water. Infections of a more serious nature can still be caused by the bacterium, such as endocarditis in injection drug users, or eye infections in contact lens users with preexisting cornea damage (Murray *et al*., 1999).

P. aeruginosa infections acquired in a hospital setting are a major concern to medical practitioners and public health officials. The bacterium is the leading cause of nosocomial respiratory infections in ICU patients, especially in patients having received a tracheotomy (Rello *et al*., 2003). Other common *P. aeruginosa* nosocomial infection sites include urinary tract infections from contaminated catheters, and life threatening wound or burn infections which can lead to bacteremia (Murray *et al*., 1999). These types of infections occur more frequently in immunocompromised patients, especially those with AIDS.

Respiratory tract colonization by *P. aeruginosa* and related species is nearly ubiquitous in cystic fibrosis (CF) patients, and plays a significant role in CF disease progression and mortality. Chronic *Pseudomonas* airway infection leads to fatal pulmonary dysfunction, and is attributed to roughly 90% of the deaths amongst CF patients (Gilligan, 1991). In North America, *P. aeruginosa* is isolated from the respiratory tracts of 30 to 40% of pediatric CF patients, and 60% of adolescent CF patients (Banerjee and Stableforth, 2000). Aggressive anti-*Pseudomonas* antibiotic therapy is therefore very important in the prolonging of life of individuals suffering from cystic fibrosis.

1.3. Antibiotic treatment of *P. aeruginosa* infection*:*

Bacterial susceptibility to antibiotics is typically assessed by determining the organism's Minimum Inhibitory Concentration (MIC) for a given drug. The MIC is defined as the lowest concentration of an antibiotic that will inhibit the visible growth of a given bacterial species. MIC determination has been standardized in North America by the National Committee of Clinical Laboratory Standards (NCCLS) and is established using a binary dilution series (Log_2), and is reported in μg/mL of antibiotic. MIC's for commonly prescribed antibiotics are routinely reported by clinical microbiology labs upon identification of an infectious bacterial pathogen from a patient. Populations of bacteria are considered to be resistant to a given antibiotic if they are found to have an MIC greater than their species and drug specific interpretive MIC value (National Committee for Clinical Laboratory Susceptibility (NCCLS), 2003). When considered along with the pharmacodynamics and pharmacokinetics of a drug, specifically peak plasma concentrations and drug half-life, MIC values can be used to determine which course of antibiotic therapy is best for the treatment of a clinical infection.

Gram negative bacterial species typically have a higher degree of antibiotic resistance than Gram positive bacteria. This is largely in part due to the presence of a selectively-permeable outer membrane which restricts the entrance of small hydrophobic molecules, including many available antibiotics (Zhanel *et al*., 2004). Additionally, in *P. aeruginosa*, various specific and non-specific efflux pumps are present on the bacterium's inner membrane, limiting the concentration of antibiotic that will accumulate

within the cell. Many antibiotics exhibit a concentration-dependent killing effect on bacteria, making efflux pumps an effective defense mechanism.

Current antibiotic therapy regimes for treating *P. aeruginosa* infections, specifically in CF patients, involve the use of aminoglycosides, β-lactams, or fluoroquinolone class drugs. Aminoglycoside class antibiotics exert a killing effect by binding to bacterial ribosomes and inhibiting bacterial protein synthesis. Tobramycin is an effective and commonly used aminoglycoside for the treatment of *P. aeruginosa* infections in CF patients, but must be administered intravenously and often produces moderate to severe toxicity (Banerjee and Stableforth, 2000).

Useful antibiotic classes based on a β-lactam structure include broad spectrum penicillins, cephalosporins, and carbapenems, all of which inhibit bacterial cell wall synthesis. Piperacillin is broad-spectrum penicillin often used to treat *P. aeruginosa* infections in CF patients, and is typically combined with tazobactam, a β-lactamase inhibitor (Banerjee and Stableforth, 2000). β-lactamase enzymes that rapidly degrade the penicillin β-lactam ring have been a primary bacterial resistance mechanism against this class of drug since the commencement of clinical penicillin use in the 1940s. Because of this, most penicillin derived antibiotics still clinically used are formulated to include a β-lactamase inhibitor in order to increase the drug's effectiveness.

Cephalosporins and carbapenems also contain a β-lactam ring, but are structurally more resistant to β-lactamase degradation. Ceftazidime is a third generation

cephalosporin, with good anti-*Pseudomonas* activity, but is not available in an oral preparation (Banerjee and Stableforth, 2000). Meropenem and imipenem are carbapenems class drugs which are often administered when treating gram-negative bacteria with β-lactamase activity against broad spectrum penicillins, as is often the case with *P. aeruginosa*. Like ceftazidime, these drugs must be administered intravenously, and so are rarely administered outside of a hospital setting (Banerjee and Stableforth, 2000).

Macrolides are a class of antibiotics which block bacterial protein synthesis by binding to the bacterial 50S ribosome subunit (Zhanel *et al.*, 2001). The majority of clinically used macrolides are metabolites (or their synthetic derivatives) biosynthesized by *Streptomyces spp.*, a fungi-like group of soil bacteria. *P. aeruginosa* is intrinsically resistant to the antimicrobial effects of the large and hydrophobic macrolides, which are unable to diffuse through the organism's outer membrane due to the restrictive channel size of membrane porins, and the presence of a water barrier within the channels (Zhanel *et al.*, 2001). Despite their ineffectiveness, azithromycin, a macrolide, is often prescribed to CF patients with *P. aeruginosa* infections as the drug has been shown to prolong patient lifespan by improving lung function, reduced inflammation, and by suppressing the *in vivo* development of the more virulent, mucoid *P. aeruginosa* phenotype (Hansen *et al.*, 2005).

Introduced in the 1980's, the fluoroquinolone class of antibiotics show a high degree of activity against *Pseudomonas aeruginosa*, and are regularly used to treat

clinical infections of the bacterium (Banerjee and Stableforth, 2000; Zhanel *et al*., 2004). The two fluoroquinolone antibiotics used primarily to treat *P. aeruginosa* infections in North America are ciprofloxacin and levofloxacin. Of the two, ciprofloxacin has better *in vitro* activity against *P. aeruginosa*, but treatment with levofloxacin is considered excellent as well due to higher achievable serum concentrations of the drug. Unlike most other drugs used in the treatment of *P. aeruginosa* infection, ciprofloxacin and levofloxacin can be orally administered (Banerjee and Stableforth, 2000; Kugelberg *et al.*, 2005).

2. Fluoroquinolones

2.1. Mode of action

Clinically used fluoroquinolones can diffuse across Gram-negative bacterial membranes, and act by binding to specific sites on bacterial DNA (Zhanel *et al*., 2002). Once bound, fluoroquinolones are able to inhibit DNA gyrase and DNA topoisomerase IV, two different members of the type II DNA topoisomerase enzyme class. Both DNA gyrase and DNA topoisomerase IV are tetrameric proteins, and are made up of two identical subunits. DNA gyrase is comprised of two "A" and "B" subunits, and DNA topoisomerase IV of two "C" and "E" subunits, coded for respectively by the genes g*yrA*, g*yrB*, *parC* and *parE* (Zhanel *et al*., 2002).

These enzymes play significant roles in DNA replication and transcription by catalyzing DNA supercoiling and relaxation, strand breaking and resealing, and the partitioning of replicated DNA during bacterial binary fission (Zhanel *et al*., 2002). The

A subunit of DNA gyrase breaks and reseals bacterial DNA during supercoiling, but is inhibited by fluoroquinolones from resealing broken DNA strands, leading to cell death. Both subunits of DNA topoisomerase IV are inhibited by fluoroquinolones, leading to irregularities in the DNA partitioning action of the enzyme during cell division (Zhanel *et al*., 2002). Which of the two enzymes is targeted primarily by a fluoroquinolone depends on the specific chemical structure of the drug, and the bacterial species the drug is being used to treat (Alovero *et al*., 2000).

2.2. Resistance mechanisms

In the case of *P. aeruginosa*, the emergence of resistance to fluoroquinolones is due to two primary mechanisms: point mutations in the genes encoding the subunits of the antibiotic's target enzymes, and the over-expression of non-specific or fluoroquinolone specific efflux pumps (Zhanel *et al*., 2004). Single point mutations in the g*yrA*, g*yrB*, *parC* and *parE* genes arise often in bacterial populations as a result of random background mutation, and can translate into functional enzymes with lower fluoroquinolone affinity. These mutations serve as one of the main bacterial resistance mechanisms against fluoroquinolones, and are rapidly selected for in the presence of the antibiotic. Both of these resistance mechanisms can result in drastic increases in fluoroquinolone MIC's, and can arise during the course of antibiotic therapy, resulting in treatment failure. With few other available antibiotics to treat *P. aeruginosa* infections, the appearance of fluoroquinolone resistance is of concern.

3. Fluoroquinolone Mutant Prevention Concentrations

In 2001, Zhao and Drlica proposed the concept of mutant prevention concentrations for the investigation and limitation of fluoroquinolone resistance that results from single point mutations in the genes g*yrA*, g*yrB*, *parC* and *parE*. The mutant prevention concentration (MPC) is defined as the concentration of a fluoroquinolone required to prevent the growth of all single-step fluoroquinolone resistant bacterial mutants. Above the MPC, only bacterial cells with two or more functional point mutations can survive. The MPC is considered to be the upper of boundary of the mutant selection window (MSW); a range of fluoroquinolone drug concentrations for which the growth of bacterial mutants with single point mutations is selected. The lower boundary of the MSW is defined as the organism's MIC; at fluoroquinolone concentrations below the MIC, not enough selective pressure exists to make the growth of bacterial mutants favorable (Zhao and Drlica, 2001).

Single point mutations have been shown to occur randomly in nature at a frequency approximating 1 in 10^6 to 10^8 bacterial cells, with the chances of two simultaneous mutations occurring in a given cell having an approximate frequency of 1 in 10^{12} to 10^{16} (Lipsitch and Levin, 1997). Since bacterial populations in patient infections are often many orders of magnitude smaller than 10^{12} cells, achieving free serum concentrations of fluoroquinolone equal to, or greater than, the pathogen's MPC should theoretically prevent the emergence of resistance due to single point mutations during the course of a patient's antibiotic therapy. This theory has been substantiated by several *in vitro* and *in vivo* animal model studies which have shown that fluoroquinolone mutant

preventing thresholds do exist in both gram-positive and gram-negative bacteria (Dong *et al*., 1999; Sindelar *et al*., 2000; MacGowan *et al*., 2003; Smith *et al*., 2004).

Potential problems with this approach do exist as a pathogen's MPC can be many times higher than its MIC. This potentially puts the MPC in excess safe therapeutic levels of the drug, which can cause toxic and other serious side effects in patients. The clinical potential of MPC's to limit the emergence of fluoroquinolone resistance could be enhanced by discovering ways to lower the actual drug concentration needed to inhibit the growth of single-step resistant mutants. One theorized way of achieving this is by supplementing fluoroquinolone therapy with one or more additional antibiotics (Zhao and Drlica, 2001). The reduction in the fluoroquinolone MPC occurs as a result of increased selective pressure on the organism from the second antibiotic. For this to work clinically, the supplemental antibiotics must have a different mechanism of action, so that a resistance causing mutation against one drug will not simultaneously confer resistance to the other drug. In addition, the two drugs should have similar pharmacokinetic profiles for dual-drug therapy to theoretically work in a clinical setting.

In order for patients to best comply with an antibiotic treatment regime, antibiotics are usually prescribed to be taken between once and three times daily. Because two antibiotics can have drastically different half-lives in the body, a patient may have to take one drug twice a day, and the other three times daily. Poor adherence to dosing schedules can cause the serum concentrations of one antibiotic to fall below effective levels, opening a window of opportunity for a pathogen to evolve resistance against the second drug. Instead of reducing the emergence of resistance, it is possible that dual-drug

therapy can lead to a pathogen developing resistance to two antibiotics instead of just one.

In an *in vitro* model however, it is possible to pre-set the concentrations of the two antibiotics to match the average daily serum concentrations of the drug in a patient who would be properly complying with his or her antibiotic dosing regimes. The average daily serum concentration can be estimated by considering the drug's peak serum concentration and number of drug half-lives between dosing (Thomas, 2003). In 2004, Firsov *et al.* reported that the addition of a second antibiotic, doxycycline, to an *in vitro* dynamic moxifloxacin (a fluoroquinolone) model could prevent the emergence of *Streptococcus pneumoniae* resistance to moxifloxacin at lower concentrations of fluoroquinolone than in the absence of doxycycline. This supports the theory that dual-drug therapy can lower an organism's fluoroquinolone MPC.

4. Hypothesis and Thesis Objectives

The aim of this research was to determine if the *P. aeruginosa* mutant prevention concentration (MPC) of levofloxacin, a fluoroquinolone class antibiotic, is effectively lowered in the presence of a second antibiotic with good activity against *P. aeruginosa*. I hypothesized that the extra selective pressure exerted by a second antibiotic will lower the concentration of levofloxacin needed to inhibit the growth of single-step resistant *P. aeruginosa* cells. In order to test this hypothesis, I carried out the following:

a) Established the MIC of levofloxacin for three clinical *P. aeruginosa* strains using standard laboratory practices.

b) Established the levofloxacin MPC for the same three *P. aeruginosa* strains when approximately 10^{10} cells are grown on agar containing a range of levofloxacin concentrations. A population of this size should be large enough to contain cells with single-step mutations, but smaller than the ~10^{14} needed to have to two-step mutants present.

c) Established the levofloxacin MPC for the three strains when the levofloxacin is supplemented with a second antibiotic, at concentrations approximating average free serum concentrations in adult patients. Ceftazidime, tobramycin, and piperacillin with tazobactam, were tested as representatives of other antibiotics used to treat clinical *P. aeruginosa* infections. Azithromycin was selected as a negative control as the drug is often prescribed to CF patients with *P. aeruginosa* infections, but has no antimicrobial activity against the organism.

Materials and Methods

1. Media

Unless otherwise specified, all experimentation was carried out in cation-supplemented Mueller-Hinton Broth (CSMHB) or on Mueller-Hinton (MH) Agar (BD, Sparks, MD, USA). Mueller-Hinton Broth (BD, Sparks, MD, USA) was prepared according to the manufacturer's instructions. Following sterilization, 0.25mL of sterile Ca^{2+} (10 mg/mL) and 0.125mL of sterile Mg^{2+} (10 mg/mL) solutions were added per 100mL of Mueller-Hinton Broth to prepare CSMHB.

2. *Pseudomonas aeruginosa* strains

Three clinical strains of *Pseudomonas aeruginosa* (46139, 49674, 36375) were selected for the purpose of levofloxacin MPC determination. The strains were previously collected and identified between 2002 and 2003 as part of the ongoing epidemiological study NAUTICA (North American Urinary Tract Infection Collective Alliance). The strains were urinary tract isolates that had been found to be susceptible to levofloxacin during the course of the NAUTICA study.

P. aeruginosa strains were frozen in 50% skim milk powder (BD, Sparks, MD, USA) for use in all experiments. Unless otherwise specified, a sample of skim milk frozen bacteria was subcultured onto MH agar and incubated for 24 hours at 37°C. Isolated colonies were then re-subcultured onto fresh MH agar, and incubated again for 24 hours at 37°C. These plates were then used for inoculation of all experiments pending

the absence of other bacterial contamination on them. All plates were discarded after 24 hours.

3. Antibiotic Stock Solutions

3.1. Preparation

Antibiotics (Table 1) used in all experimentation were originally provided in powdered form and used to prepare 25mL stock solutions with concentrations of 10,000µg/mL. The amount of antibiotic powder (µg) needed was determined using the following formula:

µg = *(desired mL * desired concentration µg/mL) / % purity of drug.*

Powder was weighed to +/- 0.1mg, dissolved in as minimal amount of recommended solvent (NCCLS, 2003) (Table 1) as necessary, and diluted to 25mL with dH2O.

Table 1. Source, purity, and lot #s of antibiotic powders, and their corresponding solvents, used in the preparation of antibiotic stock solutions for all experiments.

Antibiotic	Source	Lot #	Purity	Solvent
Levofloxacin	Biochemika, Buchs, Switzerland	446433/1 (Fluka)	97.7%	0.05M NaOH[a]
Tobramycin	Sigma, St. Louis, MO, USA	109H1278	96.7%	dH2O
Ceftazidime (Pentahydrate)	Sigma, St. Louis, MO, USA	50H-0122	99.8%	1mg/mL Na_2CO_3[b]
Piperacillin (Sodium Salt)	Sigma, St. Louis, MO, USA	043K0522	95.3%	dH2O
Tazobactam (Sodium Salt)	Wyeth-Ayerst, Monmouth Junction, NJ, USA	7E05009-199PTEC	91.1%	dH2O
Azithromycin	Pfizer Inc., Groton, CT, USA	052301-008-05	95.4%	2.5µL/mL Glacial Acetic Acid[b]

a. Fisher Scientific, Fairlawn, NJ, USA, ACS Grade
b. Sigma, St. Louis, MO, USA, ACS Grade

3.2. Solution quality control

Following dilution, antibiotic stock solutions were sterilized by filtration through a 0.2 micron filter, aliquoted into 2.0mL portions, and frozen at -80°C. The remaining 1.0mL portion was immediately used for subsequent quality control testing (described below). Antibiotic stock solutions were thawed immediately prior to using and were discarded rather than being re-frozen.

Only stock solutions which had passed quality control testing in accordance with the National Committee for Clinical Laboratory Standards (NCCLS) recommended broth macrodilution method (NCCLS, 2003) were used in subsequent experiments. Briefly, two quality control (QC) bacterial strains with established (NCCLS, 2003) minimum inhibitory concentrations (MIC's) were selected for each antibiotic. A series of sterile test tubes representing each MIC value within the acceptable range for the organism were filled with 1mL of CSMHB. Four tubes representing two values above and two values below the acceptable MIC range were filled with 1mL of CSMHB, with the exception of the tube representing the highest MIC value desired.

A working antibiotic solution of double the drug concentration of the highest desired tube was prepared by diluting the antibiotic stock solution in sterile CSMHB. 1mL of working solution was added to the two tubes with the two highest desired concentrations, resulting in the highest and second highest tubes having twice their target antibiotic concentrations. 1mL was serially transferred from the second highest tube to

the lowest tube, from which 1mL was discarded. This resulted in all tubes having double the desired antibiotic concentration, with 1mL total volume in all tubes save the highest.

Bacterial QC strains were grown on Blood Agar (BA) (Oxoid, Nepean, ON) at 37°C for 24 hours in ambient air (CO_2 for *S. pneumoniae*) before being tested. *P. aeruginosa* (ATCC 27853) was used for levofloxacin, tobramycin and piperacillin with tazobactam. *Escherichia coli* (ATCC 25922) was used for levofloxacin and ceftazidime. *Staphylococcus aureus* (ATCC 29213) was used for ceftazidime, piperacillin with tazobactam, and azithromycin. *Enterococcus faecalis* (ATCC 29212) was used for tobramycin, and *S. pneumoniae* (ATCC 49619) was used for azithromycin.

Isolated colonies from the bacterial QC plates were suspended in 3mL of CSMHB to produce a bacterial suspension with turbidity equal to a 0.5 McFarland Standard (~$1x10^{-8}$ CFU/mL). A 1:100 dilution of the suspension was prepared in CSMHB, and 1mL was added to each of the antibiotic tubes, resulting in a total volume per tube of 2mL, and halving the antibiotic concentration per tube to achieve the desired concentration. In the case of piperacillin and tazobactam, the 1:100 bacterial dilution was prepared in CSMHB containing 8μg/mL of tazobactam, resulting in the desired tazobactam concentration of 4μg/mL per tube. The target bacterial concentration for all tubes was approximately $5x10^5$ CFU/mL

Three control tubes with 1mL of CSMHB were set aside, and 1mL of one of the following was added: sterile CSMHB, 1:100 diluted QC bacteria, or working antibiotic

solution. This was to ensure that any bacterial growth observed in the QC tubes was not due to previous contamination of the CSMHB or antibiotic solutions, and that lack of growth in any QC tubes was not attributable to a non-viable bacterial QC suspension.

A sample of the 1:100 diluted QC bacteria was taken, and further diluted in CSMHB by a factor of 1:1000. A sample of 100µL from the 1:1000 dilution was taken, plated on BA, and incubated for 24 hours at 37°C. Following incubation, QC bacterial colonies were counted, and used to ensure that the concentration of the bacterial inoculums was approximately $5x10^5$ CFU/mL.

All tubes were incubated for 20 hours at 37 °C. MIC's were determined by visibly inspecting the tubes for the presence or absence of bacterial growth. The lowest antibiotic concentration to fully inhibit the growth of the organism was deemed the MIC. If the determined MIC fell within the acceptable QC range for the QC bacteria, the antibiotic solution was considered to be within quality control guidelines and was used for further experimentation.

4. Minimum inhibitory concentration (MIC) determination

MIC's for the three *P. aeruginosa* isolates were therefore determined using the identical broth macrodilution technique described above in the QC of antibiotic stock solutions, according to NCCLS specifications (NCCLS, 2003). Sterile test tubes were prepared with drug concentrations ranging from 0.5 to 256µg/mL. Isolated *P. aeruginosa* colonies on fresh MH plates were used to generate the initial bacterial suspension (0.5 McFarland standard equivalent) which was further diluted and used as described above.

Macrodilution for MIC determination was performed in triplicate for each strain, for each of the five antibiotics used. The QC *P. aeruginosa* (27853) strain was also tested concurrently with each of the three clinical strains using this technique to confirm the accuracy of determined MIC's.

5. Mutant Prevention Concentration (MPC) determination

5.1. Levofloxacin MPC's

Following the preparation and sterilization of 200mL flasks of MH agar according to manufacturer standards, the molten MH agar was placed in a 55°C water bath. Antibiotic stock solutions of levofloxacin were allowed to thaw from -80°C to the point when ice could no longer be visibly detected in the stock tube. Stock solutions of levofloxacin were diluted from 10,000µg/mL to a working solution of 1,000µg/mL. The amount of 1,000µg/mL levofloxacin needed to result in drug concentrations ranging from 0.5 to 128µg/mL in the 200mL flasks of MH agar was added to the molten agar. Each flask was then used to pour 8 agar plates of 25mL with the desired levofloxacin concentration. Plates were allowed to harden and cool until room temperature, and then were stored at 4°C until experimental use. Antibiotic plates unused after 14 days were discarded.

A heavy bacterial suspension of *P. aeruginosa* cells from a fresh MH agar plate was prepared in 3mL CSMHB and incubated for 3 hours at 37°C to achieve the desired concentration of approximately equal to $1x10^{10}$ CFU/mL. The suspension was then serially diluted in CSMHB to $1x10^{-10}$. A 100µL sample from the $1x10^{-5}$ to the $1x10^{-10}$ diluted tubes was plated on MH agar and incubated for 24 hours at 37°C. Only results

from experiments where the undiluted CFU concentration was found to fall between $5x10^9$ and $5x10^{11}$CFU/mL were used. To determine this, the colonies grown on the MH plates inoculated with samples from the six diluted tubes were counted, and used to calculate the cell density in the undiluted bacterial suspension with the following formula:

*CFU/mL = # colonies / (0.1mL plated * tube dilution)*

A 100µL sample from each of the undiluted ($1x10^0$) through $1x10^{-5}$ diluted tubes was plated on the MH agar previously prepared with levofloxacin at concentrations of 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32, 64, and 128µg/mL. In the case of isolates 49675 and 36375 where the levofloxacin MIC was 1.0µg/mL, samples plated on the 0.5µg/mL plates were discarded and not analyzed. Plates were incubated at 37°C for 48 hours, and mutant colonies were counted to determine the number of mutant CFU/mL with the formula:

*Mutant CFU/mL = # mutant colonies / (0.1mL plated * tube dilution)*

Mutational frequencies were calculated as follows:

Mutational frequency = mutant CFU/mL / undiluted tube CFU/mL

The levofloxacin MPC for each organism was considered the concentration at which fewer then 10 mutant colonies grew, even when samples plated were from the undiluted bacterial suspension. Mutational frequencies were not calculated at the MPC, as the frequency was smaller than the inverse of the bacterial concentration in the undiluted cell suspension, and at best could only be approximated. Since MPC is typically reported as a multiple of an organism's MIC, the concentration at which mutational frequency fell to 0 was divided by the strain's levofloxacin MIC. All

levofloxacin MPC experiments were performed for each of the three *P. aeruginosa* strains in triplicate.

5.2. Levofloxacin combination MPC's

MH agar plates prepared with levofloxacin concentrations ranging from 0.5 to 128μg/mL were also supplemented with one of the secondary drugs per experiment: Tobramycin (TOB), ceftazidime (CEF), piperacillin and tazobactam (P/T), or azithromycin. Secondary antibiotics were diluted from frozen stock solutions (10,000μg/mL) to a working solution of 1,000μg/mL. The working solutions were added to the MH agar concurrently with the levofloxacin, and were diluted to respective concentrations of 6 (TOB), 32 (CEF), 128/4 (P/T), or 0.4 (AZI) μg/mL. The concentrations used for secondary antibiotics were chosen to reflect the average 24 hour serum concentrations in healthy adults. Concentrations of the second antibiotics were held static in all plates per combination experiment, regardless of the levofloxacin concentration.

Levofloxacin combination MPC's were determined in an identical manner as for levofloxacin MPC's alone. Again, all experiments were performed in triplicate for each bacterial strain.

Results

1. Antibiotic sensitivity (MIC's) of *P. aeruginosa* strains

The MIC's for levofloxacin (LVX), tobramycin (TOB), ceftazidime (CEF), piperacillin with tazobactam (P/T), and azithromycin (AZI) were established for the clinical *P. aeruginosa* isolates 46139, 49674, and 36376 by macrodilution using antibiotic solutions found to be within NCCLS QC guidelines (NCCLS, 2003). All three isolates were found to be susceptible to LVX, TOB, CEF, and P/T but resistant to AZI (Table 2).

Table 2. Minimum inhibitory concentrations (MIC's) for three clinical *P. aeruginosa* isolates determined in triplicate by broth dilution, and the organism's established susceptible MIC standards for LVX, TOB, CEF, P/T and AZI.

Isolate	**Antibiotic MIC's (µg/mL)**				
	LVX	**TOB**	**CEF**	**P/T**	**AZI**
46139	0.5	1.0	2.0	4.0/4.0	> 256
49674	1.0	0.5	4.0	4.0/4.0	> 256
36375	1.0	1.0	1.0	16/4.0	> 256
Susceptible MIC[1]	≤ 2	≤ 4	≤ 8	≤64/4	Not Established[2]

1. Resistant breakpoints for *P. aeruginosa* established by NCCLS
2. No AZI resistance breakpoint MIC defined as bacterium is intrinsically resistant

2. Mutant prevention concentrations (MPC's) of *P. aeruginosa* strains

The MPC's of levofloxacin were found to be 8 times the MIC for strains 46139 and 49674, and 4 times the MIC for strain 36375 (Table 3). This equates to LVX concentrations of 4.0, 8.0 and 4.0 µg/mL respectively for the three strains (Table 4).

A reduction in levofloxacin MPC's of the three *P. aeruginosa* strains was observed in all levofloxacin drug combinations, except with AZI, where no decreases were found (Table 3). Decreases in MPC's ranged from 8-fold for 46139 when either

CEF or P/T was used with LVX, to 2-fold for 36375 when TOB was used (Table 3). Reduction in levofloxacin MPC's for the three *P. aeruginosa* strains was the same when either CFT or P/T was used (Table 3). No antagonism was observed with any drug combination.

Table 3. Levofloxacin MPC's (reported as an MIC multiple) for three clinical *P. aeruginosa* strains grown for 48hrs at 37°C on MH agar supplemented with LVX alone, or LVX in addition to CEF, TOB, P/T, or AZI at their average 24-hr patient serum concentrations.

Isolate	Combination Levofloxacin MPC's (MIC Multiple)				
	Alone	With CEF	With TOB	With P/T	With AZI
46139	8	1	2	1	8
49674	8	2	1	2	8
36375	4	1	2	1	4

Table 4. Levofloxacin MPC's (reported in µg/mL) for three clinical *P. aeruginosa* strains grown for 48hrs at 37°C on MH agar supplemented with LVX alone, or LVX in addition to CEF, TOB, P/T, or AZI at their average 24-hr patient serum concentrations.

Isolate	Levofloxacin MPC's (µg/mL)				
	Alone	With CEF	With TOB	With P/T	With AZI
46139	4.0	0.5	1.0	0.5	4.0
49674	8.0	2.0	1.0	2.0	8.0
36375	4.0	1.0	2.0	1.0	4.0

3. *P. aeruginosa* mutational frequencies

Mutational frequencies for the three *P. aeruginosa* isolates where found to be highest at levofloxacin concentrations equal to their MIC values. Mutational frequencies were found to decrease stepwise with each higher levofloxacin concentration, until they could no longer be calculated at the isolates' determined MPC (Table 5). Mutational frequencies were substantially lower at a given levofloxacin concentration in experiments when the media had also been supplemented with CEF, TOB, or P/T (Table 5). When

AZI was supplemented as the second antibiotic, mutational frequencies were found to be somewhat higher than those resulting from LVX alone (Table 5).

Table 5. Average (n=3) calculated mutational frequencies for three clinical *P. aeruginosa* strains at levofloxacin concentrations, ranging from MIC to MPC, and in the presence or absence of a second antibiotic.

Isolate	Levofloxacin Concentration (n x MIC)	Average Mutational Frequency (n=3)				
		LVX Alone	With CEF	With TOB	With P/T	With AZI
46139	1x	1.31×10^{-6}	< inoculum^{-1}	7.46×10^{-10}	< inoculum^{-1}	2.22×10^{-4}
	2x	4.42×10^{-8}		< inoculum^{-1}		1.50×10^{-5}
	4x	1.48×10^{-8}				1.74×10^{-7}
	8x	< inoculum^{-1}				< inoculum^{-1}
49674	1x	7.25×10^{-5}	6.43×10^{-7}	< inoculum^{-1}	1.04×10^{-8}	9.21×10^{-6}
	2x	4.79×10^{-6}	< inoculum^{-1}			1.15×10^{-6}
	4x	1.26×10^{-7}				3.79×10^{-8}
	8x	< inoculum^{-1}				< inoculum^{-1}
36375	1x	8.61×10^{-5}	< inoculum^{-1}	1.66×10^{-7}	< inoculum^{-1}	2.86×10^{-3}
	2x	8.97×10^{-6}		< inoculum^{-1}		2.54×10^{-5}
	4x	< inoculum^{-1}				< inoculum^{-1}

Discussion

The results from this thesis demonstrate that the levofloxacin MPC for three levofloxacin susceptible *P. aeruginosa* clinical isolates can be successfully reduced when the drug is co-administered with a second antibiotic that works via a different mode of action, to which the organism is susceptible (Table 3). This phenomenon was observed across all classes of antibiotic tested which possessed anti-*Pseudomonal* activity, and resulted in MPC's that were within one doubling dilution of each other (Table 3). A reduction in mutational frequencies at equivalent levofloxacin concentrations was also found in the presence of the second antibiotic, with the exception of azithromycin (Table 5). These results suggest that the decrease in levofloxacin MPC is happening as a result of the added selective pressure from the presence of a second, effective antibiotic.

Clinical administration of two or more antibiotics in the treatment of infections is usually rationalized with the knowledge that multiple antibiotics often exert additive or synergistic effects, increasing the likelihood of pathogen eradication. The data shown in this thesis suggests that combination therapy might not only provide a greater likelihood of pathogen killing, but also a greater likelihood of resistance prevention. Peak serum concentrations in a typical once daily 750mg oral levofloxacin adult dosing regime is approximately 10μg/mL. When this is considered with the drug's half life of 7-8 hours, the average daily serum concentration is approximately 4.0μg/mL, which does approach the MPC's of the three clinical *P. aeruginosa* isolates used in this experiment. With combination therapy, these MPC values could potentially be lowered to below achievable levofloxacin serum concentrations in adult patients. If found to be effective clinically as well as *in vitro*, combination therapy is a new technique which can be used in order to

reduce the prevalence of drug-resistant *P. aeruginosa*, and perhaps other pathogens, in a hospital setting.

Further work should be done to investigate the effect of combination therapy on fluoroquinolone MPC's in less susceptible strains of *P. aeruginosa*. The three *P. aeruginosa* strains examined in this thesis were intentionally selected because of their high susceptibility to levofloxacin. Since many *P. aeruginosa* infections are nosocomial, many clinical isolates have already been exposed to antibiotics, and as a result are less susceptible to them. These strains would potentially have higher MIC's, making their MPC's more difficult to reach using standard antibiotic therapy in the absence of a second antibiotic. However, it is very possible that strains that have decreased susceptibility to fluoroquinolones have larger mutant selection windows. In other words, the susceptible strains used in these experiments were found to have MPC's of 8, but other *P. aeruginosa* strains with higher MIC's could be found with MPC's of 16 or higher. A levofloxacin susceptible isolate with an MIC of 2.0μg/mL and an MPC of 16 would require drug concentrations of 32μg/mL to theoretically prevent the emergence of single-step fluoroquinolone resistant mutants. Under these circumstances, it would be difficult to apply the MPC concept in a clinical setting without combination therapy when treating anything but the most susceptible *P. aeruginosa* infections. Strains with higher initial levofloxacin MPC's should also therefore be tested using the same procedures described in this thesis to see if combination therapy could be used as a tool for *P. aeruginosa* strains with varied susceptibilities.

Certain limitations in the model tested in this thesis warrant further experimentation before proceeding with combination therapy in the course of patient infection treatment. These experiments utilized a static model; drug concentrations of each antibiotic incorporated into the organism's growth media were fixed at pre-selected concentrations. Pharmacodynamic models are often used to better assess how drug and bacteria interact in an environment similar to that of the human body, where drugs are administered once or twice daily and eliminated from the system at rates equal to their *in vivo* half lives, resulting in a constant rise and fall of drug concentration. *In vitro* pharmacodynamic models have already been used to investigate the effects of a dynamic system on fluoroquinolone MPC's for both *S. pneumoniae* (Firsov *et al.,* 2004) and *P. aeruginosa* (MacGowan *et al*., 2003), but the effect of combination therapy on *P. aeruginosa* MPC's has not been examined in this way. In addition, animal models should be used to study this effect *in vivo*.

Drugs from the carbapenems and polymyxin classes, both of which show activity against wild-type *P. aeruginosa* strains, were not tested in this experiment. Additionally, only one drug type from each of the other antibiotic classes tested was selected for the combination therapy experiments. Further experimentation should be carried out with a carbapenem and polymyxin as well as additional penicillins, aminoglycosides, and cephalosporins to ensure that the reduction of fluoroquinolone MPC observed during experimentation was not limited to the three drugs selected. A larger sample size of *P. aeruginosa* strains should also be examined as well

In conclusion, the results from this thesis demonstrated that the levofloxacin MPC's of three *P. aeruginosa* strains (46139, 49674, 36375) are reduced 2-8 fold in the presence of ceftazidime, tobramycin and piperacillin with tazobactam, but not azithromycin. These results show that combination therapy could potentially be used in the clinical treatment of *P. aeruginosa* infections in order to limit the prevalence of fluoroquinolone resistance in a bacterial species that already posses a high degree of intrinsic antibiotic resistance. Future research needs to be conducted using additional antibiotics, less susceptible *P. aeruginosa* strains, and other experimental models, to confirm the apparent therapeutic benefits of combing fluoroquinolones with other antibiotics established by this work.

Conclusions

1. The levofloxacin MPC's of the three *P. aeruginosa* isolates were established as being between four and eight times their respective MIC values.
2. In a static *in vitro* model, the combination of levofloxacin with a second antibiotic with a different mechanism of action, to which the three *P. aeruginosa* strains were susceptible, caused a 2-8 fold reduction in their MPC's.
3. Combination therapy could potentially be used in a clinical setting to limit the emergence of fluoroquinolone resistance in levofloxacin susceptible *P. aeruginosa* strains through the reduction of the organism's MPC.
4. These findings warrant further research using additional models, less susceptible *P. aeruginosa* strains, and additional secondary antibiotics, to further validate combination therapy as a means to limit the prevalence of fluoroquinolone resistance in *P. aeruginosa* clinical infections.

References

Alovero FL, Pan XS, Morris JE, Manzo RH, Fisher LM. 2000. Engineering the specificity of antibacterial fluoroquinolones: benzenesulfonamide modifications at C-7 of ciprofloxacin change its primary target in *Streptococcus pneumoniae* from topoisomerase IV to gyrase. Antimicrobial Agents and Chemotherapy 44(2):320-325.

Banerjee D and Stableforth D. 2000. The treatment of respiratory pseudomonas infection in cystic fibrosis: what drug and which way? Drugs 60(5):1053-1064.

Dong Y, Zhao X, Domagala J, Drlica K. 1999. Effect of fluoroquinolone concentration on selection of resistant mutants of *Mycobacterium bovis* BCG and *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 43(7):1756-1758.

Firsov AA, Vostrov SN, Lubenko IY, Portnoy YA, Zinner SH. 2004. Prevention of the selection of resistant *Staphylococcus aureus* by moxifloxacin plus doxycycline in an in vitro dynamic model: an additive effect of the combination. International journal of antimicrobial agents 23(5):451-456.

Gilligan PH. 1991. Microbiology of airway disease in patients with cystic fibrosis. Clinical microbiology reviews 4(1):35-51.

Hansen CR, Pressler T, Koch C, Hoiby N. 2005. Long-term azitromycin treatment of cystic fibrosis patients with chronic *Pseudomonas aeruginosa* infection; an observational cohort study. Journal of Cystic Fibrosis 4(1):35-40.

Kugelberg E, Lofmark S, Wretlind B, Andersson DI. 2005. Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. The Journal of antimicrobial chemotherapy 55(1):22-30.

Lipsitch M and Levin BR. 1997. The population dynamics of antimicrobial chemotherapy. Antimicrobial Agents and Chemotherapy 41(2):363-373.

MacGowan AP, Rogers CA, Holt HA, Bowker KE. 2003. Activities of moxifloxacin against, and emergence of resistance in, *Streptococcus pneumoniae* and *Pseudomonas aeruginosa* in an in vitro pharmacokinetic model. Antimicrobial Agents and Chemotherapy 47(3):1088-1095.

Murray PR and American Society for Microbiology. 1999. Manual of clinical microbiology. 7th ed. Washington, D.C.: ASM Press.

National Committee for Clinical Laboratory Standards. 2003. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically: Approved Standard M7-A6. Wayne, PA: NCCLS.

Rello J, Lorente C, Diaz E, Bodi M, Boque C, Sandiumenge A, Santamaria JM. 2003. Incidence, etiology, and outcome of nosocomial pneumonia in ICU patients requiring percutaneous tracheotomy for mechanical ventilation. Chest 124(6):2239-2243.

Sindelar G, Zhao X, Liew A, Dong Y, Lu T, Zhou J, Domagala J, Drlica K. 2000. Mutant prevention concentration as a measure of fluoroquinolone potency against Mycobacteria. Antimicrobial Agents and Chemotherapy 44(12):3337-3343.

Smith HJ, Walters M, Hisanaga T, Zhanel GG, Hoban DJ. 2004. Mutant prevention concentrations for single-step fluoroquinolone-resistant mutants of wild-type, efflux-positive, or ParC or GyrA mutation-containing *Streptococcus pneumoniae* isolates. Antimicrobial Agents and Chemotherapy 48(10):3954-3958.

Thomas G. 2003. Fundamentals of medicinal chemistry. New York, N.Y.: J. Wiley & Sons.

Zhanel GG, Hoban DJ, Schurek K, Karlowsky JA. 2004. Role of efflux mechanisms on fluoroquinolone resistance in *Streptococcus pneumoniae* and *Pseudomonas aeruginosa*. International journal of antimicrobial agents 24(6):529-535.

Zhanel GG, Dueck M, Hoban DJ, Vercaigne LM, Embil JM, Gin AS, Karlowsky JA. 2001. Review of macrolides and ketolides: focus on respiratory tract infections. Drugs 61(4):443-498.

Zhanel GG, Ennis K, Vercaigne L, Walkty A, Gin AS, Embil J, Smith H, Hoban DJ. 2002. A critical review of the fluoroquinolones: focus on respiratory infections. Drugs 62(1):13-59.

Zhao X and Drlica K. 2001. Restricting the selection of antibiotic-resistant mutants: a general strategy derived from fluoroquinolone studies. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 33 Suppl 3:S147-56.