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Theoretical and Experimental Studies of the Pharmaco-, Population and Evolutionary Dynamics of Single- and Multi-Drug Therapy for Bacterial Infections

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Abstract

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By Peter Ankomah

The discovery of antibiotics and their use for the treatment of bacterial infections represents one of the major advances of modern medicine. However, despite being available for decades, it is not clear that these drugs are being used in a manner that maximizes their clinical utility; treatment of some bacterial infections is beset by substantial morbidity and high likelihoods of recrudescence and mortality. The goal of this dissertation, in its broadest sense, is to provide quantitative insights to facilitate the design and evaluation of optimal treatment regimens that minimize the likelihood of mortality, the magnitude and term of morbidity and the likelihood of antibiotic resistant bacteria emerging and being transmitted during therapy. To accomplish this, we use a combination of *in vitro* pharmacodynamic experiments, mathematical models and computer simulations to explore the pharmaco-, population and evolutionary dynamics of bacteria under single and multi-drug treatment regimens. As measures of efficacy for different regimens, we examine the relative rates at which they clear infections and their ability to prevent the emergence and ascent of single- and multi-drug resistant bacteria. We conduct these assessments for *Mycobacterium marinum*, a time- and cost-effective surrogate organism for *Mycobacterium tuberculosis* (Chapter 2), *Staphylococcus aureus* and *Escherichia coli* (Chapter 3). We find that for drug combinations, the type of interaction between the component drugs, synergy, additivity or antagonism, can substantially affect the time to clearance of an infection. Save for scenarios in which patients are non-adherent to therapy, the evolutionary advantage of combination therapy in preventing treatment failure due to single-drug resistance, however, prevails regardless of the type of drug interaction. In Chapter 4, we extend the within-host mathematical models of antibiotic therapy developed in the previous chapters by incorporating the contribution of host innate and adaptive immune responses. We explore the properties of this model to determine the relationship between antibiotic dose, dosing frequency and term of therapy on treatment success. We find that under most conditions, high dose treatment for extended periods is more effective than more moderate regimens in increasing the rate of cure, preventing the emergence and ascent of resistance and minimizing potential immunopathology.

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CHAPTER 1

Introduction

1.1 BACTERIAL INFECTIONS AND TREATMENT: A BRIEF HISTORY

Bacterial infections have been an important component of human existence since antiquity (Nelson and Williams, 2007), but their effects on mortality and morbidity became increasingly pronounced as communal societies expanded in size and interconnectedness. *Mycobacterium tuberculosis* provides a particularly germane example; one of the oldest known human pathogens, it was an infectious scourge in Ancient Egypt and had been established in mainland Europe around 2000 BCE (Ryan, 1993). By the 1800's, tuberculosis was responsible for about a quarter of all mortality in Europe (Bloom, 1994). *Yersinia pestis*, the causative agent of the bubonic plague, also cast a black shadow through 14th century Europe, decimating close to half the continent's population (Haensch et al., 2010). Prior to the 20th century, the syphilitic infections caused by *Treponema pallidum* were another major source of global mortality (Nelson and Williams, 2007).

Advances in microscopy and the firm establishment of the germ theory of disease in the 18th and 19th centuries led to rapid improvements in the understanding of infectious disease pathophysiology. A direct corollary of this was enhanced optimism that effective antimicrobial agents could be developed to treat bacterial and other infectious diseases. Serum therapy, the delivery of antitoxins (antibodies) that were isolated from animals exposed to infectious bacterial agents such as *Streptococcus pneumoniae* and *Corynebacterium diphtheriae*, was one of the foremost rational approaches to treatment (Winau and Winau, 2002). However, its logistical impracticality and limited therapeutic

success meant that the search continued for alternative therapies (Magner, 2009). Paul Ehrlich was especially convinced that synthetic 'magic bullets' could be developed to destroy pathogenic agents without harming normal human cells (Ehrlich, 1913). He and his colleagues synthesized the first modern chemotherapeutic agent, Salvarsan, which became the first available treatment for syphilis, despite its substantial side effects (Vecki and Ottinger, 1921). Another class of synthetic antimicrobial agents, the sulfonamides, were discovered in the early 1930's and shown to have some efficacy against streptococci and *Neisseria gonorrheae* (Hager, 2006).

The antibiotic era was officially ushered in with the discovery of penicillin by Alexander Fleming. Penicillin revolutionized antimicrobial therapy because it exhibited high-level cidal efficacy against a number of different bacteria – *Neisseria gonorrheae*, *Treponema pallidum* and many staphylococcal and streptococcal species (Brown, 2005). Streptomycin soon followed and was of particular value because of its activity against *Mycobacterium tuberculosis* (Comroe, 1978). A large number of antibiotic agents were subsequently discovered/developed over a short period of time and effective treatment became available for most bacterial infections (Clatworthy et al., 2007). Optimism about the decline in infectious causes of morbidity and mortality during this period is encapsulated by the unfortunate and oft-parodied pronouncement in 1967 by the then Surgeon-General of the United States, William H Stewart, that it was time to 'close the book on infectious diseases and declare the war against pestilence won' (Garrett, 1994). While the attribution of this quote to him has been challenged (Spellberg, 2008), there is ample evidence of widespread belief among many in the medical community in the

1960's and 1970's that infection no longer posed a significant threat to human health (Fauci, 2001; Petersdorf, 1978).

1.2 BACTERIAL RESISTANCE TO ANTIBIOTICS

This optimistic outlook about infection control was myopic in the extreme, primarily due to the ability of bacteria to evolve resistance to antibiotics. Bacteria acquire resistance via the horizontal transfer of resistance-conferring genes/genetic elements or *through de novo* mutations. The transfer of resistance-conferring genes occurs through DNA uptake from the environment (transformation), phage-mediated DNA transfer (transduction) or through cell-to-cell conjugation. Mutations arise spontaneously during cell division, and some of those may confer resistance and provide an advantage in the face of antibiotic selection pressures. Mutation rates can also be substantially augmented in bacterial strains that exhibit deficiencies in mismatch repair mechanisms (Diacon et al., 2007; Munro et al., 2007; Smith and Romesberg, 2007) resulting in a higher likelihood of such bacteria exhibiting antibiotic resistance (Cirz and Romesberg, 2006; LeClerc et al., 1996; Miller, 1996; Oliver et al., 2000). Moreover, there are indications that antibiotic-mediated stress can also enhance mutation rates and increase the likelihood of resistance evolution (Boshoff et al., 2003; Kohanski et al., 2010; Riesenfeld et al., 1997).

These resistance-conferring genetic changes allow bacteria to evade antibiotic-mediated killing via a myriad mechanisms, including: (i) structural barriers that limit drug access into cells, (Nguyen and Pieters, 2009) (ii) efflux pump-mediated extrusion of antibiotics, (Webber and Piddock, 2003) (iii) enzymatic inactivation of drugs, (Davies and Wright, 1997; Jacoby and Medeiros, 1991) (iv) enzymatic modification to prevent activation of drugs, (Guo et al., 2006) (v) alteration of the drug target, (Hooper, 2000;

Weisblum, 1995) (vi) increased expression of drug target to saturate drug molecules, (Hanaki et al., 1998) and (vii) utilizing alternative metabolic pathways that minimize the effect of drug inhibition (Tenover, 2006).

It should be noted that bacteria can be simultaneously resistant to multiple antibiotics. One means by which this occurs is through the accumulation of multiple mutations, each of which results in resistance to a particular antibiotic (Almeida Da Silva and Palomino, 2011). Resistance genes can also cluster on plasmids such that acquisition of a single plasmid confers multiple drug resistance phenotypes (Nikaido, 2009). In addition, multi-drug efflux pumps that extrude a broad spectrum of antibiotics out of cells also serve as important mediators of multi-drug resistance (Li and Nikaido, 2009).

The forms of resistance highlighted in the preceding paragraphs are all heritable, i.e. borne on genes or accessory genetic elements and transferable to progeny bacteria. Another form of resistance occurs when bacteria are genetically susceptible to antibiotics, but under certain conditions, exhibit a temporary (and reversible) decrease in susceptibility to antibiotics that is non-heritable. One of the major mechanisms leading to this phenotypic form of resistance is bacterial entry into non-replicating or slowly-replicating states. Bacteria can, for instance, cease active growth under conditions of nutrient exhaustion as occurs during the stationary phase of *in vitro* growth, or when a host restricts access to sources of carbon, iron, oxygen, etc (Nathan, 2012). Also, among growing antibiotic-sensitive populations of bacteria, various errors in cellular replication can generate minority subpopulations that undergo temporary periods of non-replication or slowed metabolism (Johnson and Levin, 2013). While in this state of ‘persistence’, these bacteria can survive exposure to otherwise cidal antibiotic concentrations (Bigger,

1944). It is assumed that since antibiotics typically interfere with biomass-building processes, the phenotypic resistance of bacteria in growth stasis follows, at least in part, from their reduced need for building biomass (Nathan, 2012). In addition, persisters and other bacteria in non-replicating states may modify metabolic and transcriptional processes to decrease their susceptibility to antibiotics (Burian et al., 2012; Nguyen et al., 2011). Phenotypic resistance can also be mediated by physical structures such as biofilms (Costerton et al., 1999; Hoyle et al., 1992; Kumon et al., 1994), granulomas (Kjellsson et al., 2012) and abscesses (Wagner et al., 2006) that limit antibiotic access to bacteria within. These structures also provide conducive environments for non-replicating bacteria and usually harbor large populations of these.

Clinically, phenotypic resistance is of no less importance than genotypic resistance, and it has been argued that it may even be the reason why most antibiotics fail (Levin and Rozen, 2006). Aqueous microenvironments with constant nutrient flow and surfaces for attachment are ubiquitous in the human body and promote the formation of microbial biofilms during many infectious bacterial processes (Bell, 2001). Many surgical procedures also involve the temporary or permanent use of catheters, prosthetic joints, valves and other foreign bodies, and the artificial surfaces of these are especially conducive for microbial aggregation. Biofilm growth is a common worry with infections such as cystic fibrosis, endocarditis, osteomyelitis and chronic rhinosinusitis, often lengthening the term of therapy and substantially increasing the probability of treatment failure and/or relapse (Hoiby et al., 2010). Recent lines of evidence have also pointed to persisters playing an important clinical role in extending the length of treatment and

increasing the likelihood of treatment failure for some infections (Lafleur et al., 2010; Mulcahy et al., 2010).

It is worth noting that even during the headily optimistic early days of antibiotic discovery and use, it was readily apparent that resistance could develop and adversely affect treatment outcomes. Once widespread penicillin usage began, resistant strains of *S. aureus*, for instance, began to increase in prevalence until by the late 1960's about 80% of all isolates were resistant to penicillin (Lowy, 2003). The transfer of plasmid-borne broad spectrum beta-lactamases between bacterial species led to resistance to newer generations of beta-lactam antibiotics like methicillin, cephalosporins and carbapenems that were introduced after penicillin (Bush, 2002; Medeiros, 1997; Moosdeen, 1997). Macrolide resistance in staphylococcal isolates also developed rapidly after the introduction of erythromycin in the 1950's, and this phenotype was transferred to *S. pneumoniae* within a decade (Bergstrom and Feldgarden, 2008).

1.3 EPIDEMIOLOGY OF BACTERIAL INFECTIONS AND RESISTANCE

In spite of Stewart's pronouncement, bacterial diseases still rank among the major causes of human mortality and morbidity. In the most recent analysis of the global burden of disease, lower respiratory infections such as pneumococcal and *Haemophilus influenzae* pneumonia, diarrhoeal diseases like cholera, shigellosis and enteropathogenic/enterotoxigenic *Escherichia coli* as well as tuberculosis were among the top ten causes of mortality worldwide (Lozano et al., 2012). Contrary to popular perception, the impact of bacterial infections is not limited to the developing world; it is estimated, for instance, that around 200,000 people acquire a nosocomial infection in the United States each year (Bergstrom and Feldgarden, 2008). These infections are, by some

estimates, the sixth leading cause of mortality in the United States, leading to about 90,000 deaths each year (Bergstrom and Feldgarden, 2008; Peleg and Hooper, 2010).

As has been noted earlier, resistance plays a major role in treatment outcomes of bacterial infections. Relative to infections caused by antibiotic-susceptible bacteria, those caused by resistant bacteria are associated with longer hospitalizations, greater costs and higher mortality rates (Daxboeck et al., 2006; Schwaber et al., 2006). A brief (and by no means comprehensive) survey of resistance epidemiology delineates the scale of the problem. Methicillin-resistant staphylococcus aureus (MRSA) rates, for example, exceed a quarter of all cases of *S. aureus* bacteremia in many areas of Western and Southern Europe (Woodford and Livermore, 2009). In the United States, the corresponding number is around 50% (Draghi et al., 2005; Jones et al., 2008). Most in-hospital MRSA cases exhibit concurrent resistance to fluoroquinolones, macrolides, tetracyclines and aminoglycosides (Woodford and Livermore, 2009). The increasing prevalence of community-acquired MRSA cases poses even more of a challenge to efforts aimed at curbing MRSA spread (Weber, 2005; Zetola et al., 2005). Incidences, though isolated, of vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA) have raised concerns that the glycopeptides, the conventional therapy for MRSA, may also be lost to resistance (Bierbaum et al., 1999; Hiramatsu et al., 1997; Martin and Wilcox, 1997; Ploy et al., 1998; Sievert et al., 2008).

Enterococci are important agents of opportunistic infections, especially in the in-patient setting (Woodford and Livermore, 2009). These bacteria exhibit a high-level of resistance to aminoglycosides and cephalosporins. Vancomycin-resistant Enterococcus (VRE) is increasing in prevalence, raising concern about a potential increase in incidence

of VRSA via horizontal gene transfer. For *S. pneumoniae*, another key pathogenic bacteria, penicillin and macrolide resistance poses major clinical problems (Woodford and Livermore, 2009). Enterobacteriaceae that produce extended spectrum beta-lactamases (ESBL) are another important component of the epidemiological landscape in many places around the world. In Europe, for instance, the average rate of ESBL production in enterobacteria is around 4%, with some countries having rates greater than 70% in their intensive care units (Turner, 2009). Carbapenemase-producing *Klebsiella pneumoniae* are also rising in incidence and could pose a particularly difficult threat in health care settings (Ho et al., 2010).

Then, of course, there is tuberculosis. The World Health Organization (WHO) estimates that close to 4% of the 8.7 million new cases of TB recorded in 2011 were multi-drug resistant (MDR-TB), i.e. resistant to isoniazid and rifampin, the two most potent first-line drugs (WHO, 2012). Approximately 9% of these MDR-TB cases were extensively-drug resistant (XDR-TB), i.e. they exhibited additional resistance to two classes of second-line drugs; a quinolone and at least one of kanamycin, amikacin or capreomycin (WHO, 2012). There have also been worrying reports of pan-resistant TB strains that are not susceptible to any of the current antimycobacterial drugs (Udwadia et al., 2012).

1.4 DESIGN OF ANTIBIOTIC TREATMENT REGIMENS

In order to improve treatment outcomes for bacterial infections, there is the need to design and implement effective antibiotic treatment regimens that minimize: (i) the likelihood of mortality (ii) the term and magnitude of morbidity (iii) the likelihood of relapse (iv) deleterious drug-induced side effects and (v) the likelihood of acquired

resistance and transmission of resistant bacteria. This involves determining the most effective antibiotic for an infection and using it at its optimal dose, dosing frequency and term. This is not a straightforward undertaking, as changing the various parameters of a treatment regimen may affect the goals of therapy in different ways. For instance, *a priori*, increasing antibiotic dose will be expected to increase the rate of bactericidal activity and the likelihood of eradication of the infection. This strategy, however, also increases the potential for serious drug-induced side effects in the patient. With regards the prevention of resistance, high doses are likely to reduce pathogen numbers rapidly and thereby decrease the likelihood of *de novo* resistance evolution. Conversely, it has been suggested that this strategy of 'aggressive chemotherapy' maximizes the selection pressure for any resistant pathogens that are present prior to the initiation of therapy and could promote the ascent and transmission of resistance (Read et al., 2011). For term considerations, the shortest treatment regimens are desirable because they limit antibiotic selective pressure for resistance to the barest minimum necessary (Rice, 2008). However there are always concerns that short courses may also increase the likelihood of recrudescence of the treated infection (Rice, 2008).

It is obvious that well-designed, randomized control trials are needed to help determine optimal therapeutic regimens (Rice, 2008), but these are virtually nonexistent because they are dogged by ethical quandaries and a lack of pharmaceutical industry wherewithal. As precursors to (and unfortunately, in many cases, as stand-in's for) such studies, pharmacodynamic-pharmacokinetic studies and mathematical modeling have been employed to assist in the design of treatment protocols for bacterial infections.

1.4.1 THE PHARMACOKINETIC-PHARMACODYNAMIC (PK-PD) APPROACH

Pharmacodynamics (PD) is the study of the biochemical and physiological effects of drugs on a treated organism or on pathogens in a host (Lees et al., 2004). Antimicrobial pharmacodynamics specifically assesses the relationship between drug exposure and specific microbiological effects such as bacterial killing and/or resistance suppression (Drusano, 2004). Pharmacokinetics (PK), on the other hand, describes the effect of physiological processes in the treated organism on the administered drug. It involves assessments of absorption, distribution, metabolism and elimination of drugs (Drusano, 2004). The PK-PD approach to the treatment of bacterial infections attempts to quantitate the relationship between drug potency against the pathogen, the extent of host exposure to the drugs and the microbiological effects that accompany changes in drug concentration (Mouton et al., 2011). It allows for evaluations of various drugs, doses and dosing schedules to determine the composition of regimens that improve antimicrobial efficacy (Mouton et al., 2011).

PK/PD indices are used as measures of antibiotic efficacy in this approach. The pharmacodynamic component of these indices is usually restricted to a single parameter, the Minimum Inhibitory Concentration (MIC), i.e. the minimum concentration of antibiotic required to prevent *in vitro* growth of a bacterial population over a specified period of time (typically, ~ 18 – 24h). The pharmacokinetic components of these indices describe antibiotics based on whether they exhibit time- or concentration-dependent killing. The cidal effect of time-dependent antibiotics increases with increasing concentration until a maximum kill rate is reached such that further increases in

concentration do not engender any more changes in killing rate. For concentration-dependent antibiotics, killing rates do not plateau with increasing concentrations. PK/PD indices combine the pharmacodynamic and pharmacokinetic elements of antibiotic-bacteria-host interactions to evaluate drug regimens using one of three measures: (i) the time for which drug concentrations are above the MIC (t>MIC), (ii) the ratio of the peak plasma concentration attained by the drug to the MIC (C_{max}/MIC) and (iii) the ratio of the area under the concentration-time curve to the MIC (AUC/MIC) (Van Bambeke et al., 2006). The PK/PD of different classes of antibiotics are considered to be best-described by one of these indices, and comparisons among antibiotics or regimens within these classes are undertaken by quantifying these indices using *in vitro* experimental systems or model organisms, typically neutropenic or other immunocompromised mice (Mouton et al., 2011). A number of studies have provided some evidence to suggest that there is a positive correlation between the values of these PK-PD indices and clinical responses (Ambrose et al., 2001; Bhavnani et al., 2006; Drusano et al., 2004; Forrest et al., 1993; Lodise et al., 2005; Preston et al., 1998; Rayner et al., 2003).

In spite of the aforementioned clinical correlations, it is not clear that the PK-PD approach allows for the determination of truly optimal regimens for treatment of bacterial infections. For one, the use of categorical indices is overly restrictive and may not properly capture the cidal dynamics of all types of drugs (Tam and Nikolaou, 2011). Another major limitation of the approach is its reliance on the MIC as the sole formal PD parameter (Levin and Udekwu, 2010; Mueller et al., 2004). MIC's only measure a limited component of the antibiotic-bacteria interaction, and are designed to assess antibiotic killing under conditions that are optimal for antibiotic action (Udekwu et al., 2009). In

the standard procedure, low densities of exponentially growing bacteria in liquid media are exposed to twofold serial dilutions of antibiotics under ionic conditions in which the drugs are most effective (CLSI, 2005). A number of factors that affect the efficacy of antibiotics are not explicitly considered in this procedure. They include: (i) the physiological state of the bacteria (most antibiotics are ineffective against cells that are not actively growing) (Eng et al., 1991), (ii) the physical structure of the bacterial population (eg. biofilms, cords, granulomas) (Fux et al., 2005; Julian et al., 2010; Parrish et al., 1998), (iii) persistence (subpopulations of non-growing bacteria in exponentially growing cultures) (Lewis, 2010; Wiuff et al., 2005), (iv) the shape of the pharmacodynamic function (the relationship between bacterial killing and antibiotic concentration) (Regoes et al., 2004), (v) the density of the bacteria (as low as a two-order magnitude increase in the density of the initial inoculums used for MIC protocols, for instance, can increase the MIC by up to one hundred-fold) (Davey and Barza, 1987; Soriano et al., 1990; Udekwu et al., 2009) and (vi) post-antibiotic effects (such as the suppression of bacterial growth after brief exposure to antibiotics) (Craig, 1993). Moreover, the MIC is a static parameter that does not consider the relationship between changing antibiotic concentrations and bactericidal activity. Being a simple threshold measure, it does not provide information about the relative cidal effect of sub- and supra-MIC concentrations (Mueller et al., 2004).

An alternative approach to assessing the pharmacodynamics of antibiotics involves the use of classical time-kill experiments that examine the cidal activity of different antibiotic concentrations (Fung-Tomc et al., 2000; Lentino and Strodthman, 1989; Odenholt et al., 2001; Regoes et al., 2004; Udekwu et al., 2009). This more

dynamic approach provides a better picture of the relationship between a range of antibiotic concentrations and bacterial killing. Mathematical models can be used to conduct formal and comprehensive quantitative PD assessments based on concentration-kill results from these experiments. Sigmoid E_{max} pharmacodynamic functions (Equation 1) for characterizing non-linear and saturable dose-responses are often the mathematical tool of choice (Bonapace et al., 2002; Boylan et al., 2003; Corvaisier et al., 1998; Delacher et al., 2000; Hyatt et al., 1995; Kim et al., 2002; Louie et al., 2001; Madaras-Kelly et al., 1996).

$$E = \frac{E_{\max} * C^{\kappa}}{EC_{50}^{\kappa} + C^{\kappa}} \qquad (1)$$

For these functions, the relationship between bacterial killing (E) and antibiotic concentration (C) is summarized by three parameters that represent (i) the maximum kill rate of the antibiotic (E_{max}), (ii) the sigmoidicity of the kill function, i.e. how sensitive the kill rate is to changes in antibiotic concentration (κ) and (iii) the concentration of the antibiotic that achieves a half-maximal kill rate (EC_{50}) (Tam and Nikolaou, 2011). The Hill function is one form of the E_{max} models used in pharmacodynamic modeling (Goutelle et al., 2008). In its general form, it is a four-parameter function that incorporates a baseline response at a drug concentration of 0 in addition to the aforementioned parameters (Equation 2) (Goutelle et al., 2008).

$$E = E_0 + \frac{E_{\max} * C^{\kappa}}{EC_{50}^{\kappa} + C^{\kappa}} \qquad (2)$$

This has the added advantage of demonstrating maximal growth rate differences between different bacterial strains (Regoes et al., 2004) as would result from, for instance, fitness deficits due to resistance (Andersson and Levin, 1999).

Studies that employ these E_{max}-type models typically restrict their use to the determination of correlations between empirically-determined PK-PD indices for an antibiotic and the maximal antimicrobial effect determined from the model e.g. (Bonapace et al., 2002; Corvaisier et al., 1998; Dalla Costa et al., 1997; Kim et al., 2002; Madaras-Kelly et al., 1996). More comprehensive Hill function-based analyses have been used to demonstrate that antibiotics can differ in the values of all the parameters that make up the pharmacodynamic function (Regoes et al., 2004). Crucially, these analyses also show that antibiotics with the same MIC's but different maximal kill rates or sigmoidicity coefficients can exhibit dramatically different cidal dynamics (Regoes et al., 2004).

1.4.2 MATHEMATICAL MODELS

Mathematical modeling has also been used to explore the design of antibiotic regimens and control programs that maximize infection control and minimize the evolution of resistance during the treatment of bacterial infections. These theoretical studies have been used to examine treatment dynamics at the level of a single infected host and on larger population scales. At the single-host level, models have been used to explore bacterial growth and clearance dynamics under different antimicrobial selection pressures (D'Agata et al., 2008; Gehring et al., 2010; Gerrish and Garcia-Lerma, 2003; Iwasa et al., 2004; Murphy et al., 2008; Roberts and Stewart, 2004). Factors such as

mutation rates to resistance (Gerrish and Garcia-Lerma, 2003), rates of horizontal gene transfer (Gehring et al., 2010), rates of compensatory evolution (Levin et al., 2000; Schulz zur Wiesch et al., 2010), the fitness of resistant bacteria (Cohen and Murray, 2004), timing of treatment onset (D'Agata et al., 2008), duration of treatment (D'Agata et al., 2007) and adherence to therapy (Lipsitch and Levin, 1998) have been shown to have substantial effects on treatment success.

At the epidemiological level, the most important features of an infectious bacterial agent are its rate of spread in the population (described mathematically by the basic reproduction number, which is the number of healthy individuals infected by each infected individual at the beginning of an epidemic), the threshold number of hosts it requires to become established, and the average level of infection (Dobson and Carper, 1996). Models have been used to determine the values of these parameters and how they are affected by various factors (Austin et al., 1999; Cohen and Murray, 2004; Melegaro et al., 2004; Opatowski et al., 2010). For instance, theoretical studies suggest that there may be a quantifiable fitness cost threshold that determines the ability of a resistant pathogen to invade and persist in a population (Cohen and Murray, 2004) and that this threshold is affected by the amount of antibiotic selection pressure present in the community (Austin et al., 1999; Boni and Feldman, 2005). Models have also shown that subgroups of individuals can disproportionately impact the spread of resistance in a community (Andersson et al., 2005; Hotchkiss et al., 2005; Karlsson et al., 2008; Temime et al., 2009) suggesting potential high-yield for control approaches that pay particular focus to these groups.

Theoretical studies have proved particularly useful for assessing the utility of different types of control programs in improving resistance management in hospital settings (Bergstrom et al., 2004; Bonhoeffer et al., 1997; Brown and Nathwani, 2005; Levin and Bonten, 2004; Lipsitch et al., 2000; Sun et al., 2010; Wang and Lipsitch, 2006). The oft-made comparison is between two different strategies: cycling, in which antibiotics are used sequentially on a scheduled basis, and mixing, the heterogeneous use of different antibiotics by different physicians with no regulation of prescription practices. *A priori*, cycling was advocated to be the more effective strategy, but empirical assessments have thus far been inconclusive (Brown and Nathwani, 2005). Mathematical modeling has provided insight to this by showing that the patterned heterogeneity in antibiotic selection pressure generated by cycling does not provide a superior evolutionary advantage over the heterogeneity inherent in non-regulated use (Bergstrom et al., 2004; Bonhoeffer et al., 1997; Levin and Bonten, 2004).

Despite the contributions made by theory, current models have a number of crucial limitations, which, if addressed, could help improve designs of treatment protocols. Particularly glaring is the limited consideration of the host immune defense's contribution to clearance of an infection during treatment (see (D'Agata et al., 2008; Geli et al., 2012; Handel et al., 2009) for exceptions). This is in spite of the fact that antibiotics likely play only a supportive role in the eradication of many infections (Allos, 2001; Garbutt et al., 2012); they serve to decrease bacterial densities to levels at which immune-mediated clearance can occur and thereby minimize the term of the infection and accompanying morbidity as well as the likelihood of mortality. The immune system, however, is not completely benign to the host. Morbidity and mortality due to infections

are as much a consequence of an over-response of the host defenses that leads to immunopathology (Margolis and Levin, 2008) as they are the result of disruptions in normal physiological processes due to bacterial virulence factors. Another limitation of most existing models is their consideration of resistance as an 'all-or-nothing' phenomenon whereby resistant bacteria are regarded as completely unsusceptible to antibacterial action. In reality, resistance represents a continuum of declining susceptibility to drug effect and properly accounting for this in models will likely have a substantial impact on treatment dynamics for resistant bacteria. Moreover, bar a few exceptions (Cogan et al., 2005; Levin and Rozen, 2006; Roberts and Stewart, 2005; Wiuff et al., 2005) resistance is considered only at the genotypic level and most models ignore the effects of different forms of phenotypic resistance, the importance of which has been discussed above.

1.5 COMBINATION ANTIBIOTIC THERAPY

The above consideration of the design of treatment regimens was limited to a simple one antibiotic-one bacteria scenario. However, the simultaneous use of different antibiotics (combination therapy or multi-drug therapy) is also a strategy that is considered when choosing antibiotic regimens to treat bacterial infections. Multi-drug therapy is principally utilized as a means of preventing treatment failure resulting from resistance to single drugs. For a disease like tuberculosis in which resistance to all the major drugs is mediated by single point mutations, resistance to single antibiotics is likely to exist prior to, or develop during the course of therapy. Using combination therapy allows these single-drug resistant bacteria to be eradicated by other drug(s) in the regimen to which they are susceptible (Lipsitch and Samore, 2002). Initial trials of

streptomycin monotherapy for tuberculosis conducted by the British Medical Council, for instance, showed that while many patients could be cured, a substantial proportion of treatment failure resulted from streptomycin resistance (Crofton and Mitchison, 1948). Subsequent studies demonstrated that combining streptomycin with either one of two new antituberculosis agents, thiacetazone and para-aminosalicylic acid decreased rates of treatment failure (Medical Research Council, 1950). As newer agents were added to the TB drug arsenal, there was always a rapid onset of resistance whenever a monotherapeutic regimen was employed (Fox et al., 1999; Manten and Van Wijngaarden, 1969). Eventually, a four-drug regimen was recommended as the most effective strategy to combat resistance and maximize treatment success (Fox et al., 1999).

In addition to this evolutionary virtue, combining antibiotics is also useful for providing broad therapeutic coverage such as in the treatment of polymicrobial infections and under conditions when the etiologic agent is unclear at the start of therapy (Gorbach, 1994). Moreover, multi-drug therapy also has a number of important pharmacological virtues. Antibiotics can be combined at lower concentrations to generate the required cidal effects if single antibiotic use at high concentrations results in intolerable side effects. There is also the potential for synergistic interactions between antibiotics, and consequently, greater cidal activity than would be expected from using single alone.

Combination therapy has its disadvantages though. From a practical perspective, taking more than one drug (likely on different dosing schedules) introduces additional logistical complication into treatment regimens. There is also the potential for antagonistic interactions between drugs, leading to decreased bactericidal efficacy. Moreover, combination therapy increases the selection pressure for resistance and can

favor the emergence of multi-drug resistant bacteria during therapy (Donskey et al., 2000; Michea-Hamzehpour et al., 1987; Rice, 2008).

In order to determine the utility of a multi-drug regimen for treatment, it is essential that the antimicrobial efficacy of the combination be determined. One major way of doing this is by assessing the nature of the interaction between the drugs in a regimen. Combinations may be: (i) additive, (the drugs together generate cidal activity that is equivalent to what would be expected from the activity of the individual drugs); (ii) synergistic (cidal effects of the combination are greater than what would be expected from the activity of the individual drugs); or (iii) antagonistic/suppressive (cidal effects of the combination are less than what would be expected from the independent activity of the constituent drugs). Classifications of synergy or antagonism are based on deviations from a reference/null case of no interaction (additivity), and as such, defining the reference additivity model is crucial for multi-drug pharmacodynamic studies (Greco et al., 1995). There are two predominant pharmacological definitions of additivity; Bliss Independence and Loewe Additivity. Bliss Independence assumes that each constituent drug in an additive combination exerts its cidal action independently of the other drug(s) (Bliss, 1939). Loewe Additivity on the other hand, assumes that drugs in an additive combination operate, functionally, as identical drugs (Loewe, 1928). Thus, for two additive drugs A and B, the Loewe assumption is that the combined effect of equipotent doses of both drugs should be equal to doubling the dose of either drug A or B (Yeh et al., 2009). The ideas behind these definitions of additivity form the basis for various types of drug interaction assessments.

The checkerboard titration method is one of the most common methodologies for evaluating the nature of interactions between antibiotics. It generates a single parameter, the Fractional Inhibitory Concentration (FIC) index which is determined by calculating the ratio of the MIC of each drug in combination to its MIC when used alone and summing these together for all the individual drugs. For example; for two drugs A and B, $FIC = (C_A/MIC_A) + (C_B/MIC_B)$, where MIC_A and MIC_B are the MIC's of drugs A and B individually, and C_A and C_B are the concentrations of drugs A and B in the combinations that generate equivalent inhibitory activity as MIC_A or MIC_B (Hall et al., 1983; Meletiadis et al., 2010). Additivity here is based on Loewe's definition, and an FIC index of 1 represents the case of perfect additivity. In practice, combinations with an FIC between 0.5 – 4 are considered to interact additively, with values greater than 4 representing antagonism and those less than 0.5, synergy (Meletiadis et al., 2010).

Time-kill assays are an alternative means of assessing the pharmacodynamics of drug combinations. They are used to compare the log-fold reduction in viable cell density generated by a combination relative to that generated by the most effective single constituent antibiotic (Lorian, 2005). Combinations that generate greater than two-fold log decreases in cell density are considered synergistic, while antagonistic combinations lead to a two-fold or greater increase in cell density, and additive drugs generate less than a two-fold log difference in viable cell density (Petersen et al., 2006).

Both of these methodologies have their limitations. Checkerboard titrations base their PD assessments solely on MIC assessments, the limitations of which have been discussed in detail above. The FIC index range for additivity is also overly broad, thus making it difficult to determine subtle non-additive drug interactions (O'Shaughnessy et

al., 2006). For time-kill assays, the period over which assessments are conducted, the drug concentrations used and the degree of kill that is indicative of the various types of interactions are nonstandard and vary between studies (Lorian, 2005; Tam et al., 2004). In addition, this method only makes ordinal quantitative distinctions between types of interaction and does not distinguish between quantitatively different levels of interaction within the same class (Tam et al., 2004). Crucially, neither of these measures properly assesses a dose-response relationship between the concentrations of the antibiotics in the combination and bacterial killing.

In order to account for some of these limitations, a few studies have utilized modified E_{max}-based models that allow for the generation of dose-response surfaces for antibiotic combinations (Greco et al., 1990; Lim et al., 2008; Tam et al., 2004; Yuan et al., 2010). Other studies have used sub-lethal (and hence sub-therapeutic) antibiotic concentrations to compare the inhibitory effects on bacterial growth rates engendered by combinations relative to their constituent antibiotics (Wood et al., 2012; Yeh et al., 2006). Generally, however, the pharmacodynamics of multiple antibiotics at clinically realistic and dynamic concentration ranges is still very under-explored. In addition, although a few mathematical models have considered combination therapy as part of their analyses (Bonhoeffer et al., 1997; Lipsitch and Levin, 1997; Lipsitch and Levin, 1998), none of these models has comprehensively assessed the effect of multi-drug PD on treatment dynamics.

Clearly, there is a surfeit of adequately rigorous experimental and theoretical work examining the utility of combination therapy for bacterial infections. For the most part, a number of critical questions remain unanswered. These include: (i) whether there

any generalizable rules about how different classes of antibiotics interact, (ii) what the relationship is between pharmacodynamic interactions of drugs and their evolutionary efficacy, i.e. preventing the emergence of either single- or multi-drug resistance within a treated host, and (iii) under what conditions, and for which (types of) infections multi-drug therapy will be more effective than monotherapy.

1.6 THE QUESTIONS

As the above discussion has shown, antibiotic treatment of bacterial infections is still beset by a substantial amount of problems despite considerable theoretical, epidemiological and experimental efforts. How can we improve on this? One approach is to actively engage in an evolutionary arms race against bacteria by continually developing newer and more effective antimicrobial agents to combat both phenotypic and genotypic resistance. This, however, is a cost- and labour-intensive undertaking, and the serendipity that will be needed for some of these discoveries may not track synchronously with bacterial advancements in the arms race. In addition, the potential financial rewards from developing such drugs are relatively meager, and the current disinterest of pharmaceutical companies in developing antibiotics is likely to continue.

An alternative approach is to determine ways to optimize the use of the current antibiotic arsenal. In order to do this, a number of important questions need to be comprehensively explored. How, for instance, do antibiotics and the immune system collaborate to clear an infection? A better quantitative understanding of this could contribute to the design of treatment protocols that use just the right drug dose, dosing frequency and term to maximize treatment success and minimize selection pressure for resistance. It is also important to know the contribution of various forms of non-inherited

resistance – cells in spatial refugia, cells in growth stasis, etc, to treatment failure and how different antibiotics affect this. Moreover, *a priori*, combination therapy would appear to be an effective strategy for decreasing treatment failure due to *de novo* or pre-existing single-drug resistance. Yet, it is only standard for the treatment of a few infections like tuberculosis. For many other bacterial infections, it is used in an ad-hoc manner with minimal evidence for why particular drug combinations are employed. Regimens are often based on a few case reports and/or 'expert opinion' and their optimality or otherwise is not stringently analyzed. Proper quantitative assessments of the pharmaco-, population and evolutionary dynamics of multi-drug therapy could help improve the utility of this approach for treatment of bacterial infections. The investigations included in this dissertation begin to address some of these critical issues.

1.7 OUTLINE OF THE THESIS AND CHAPTER SUMMARIES

In Chapter 2, I present a jointly theoretical and experimental investigation to explore the pharmacodynamics of multi-drug mycobacterial therapy and its potential impact on treatment success. In spite of the fact that combination therapy is the standard-of-care treatment for tuberculosis, virtually all studies of the pharmacodynamics of TB drugs are restricted to single agents. A major goal of this study was to help mitigate this shortcoming in the literature.

For our experimental organism, we used *Mycobacterium marinum* as a time and cost-effective surrogate for *M. tuberculosis*. *M. marinum* shares over 99% sequence homology with, and exhibits similar intracellular growth, survival mechanisms and other pathogenic behaviors as Mtb (Stamm and Brown, 2004; Stinear et al., 2008). It grows about four times faster *in vitro* than Mtb, and is considered safe for use in BSL2 facilities,

in contrast to Mtb that requires BSL3 containment. *M. marinum* is a natural pathogen of fish, frogs and other ectotherms, but is also capable of causing peripheral granulomatous infections in immunocompetent humans and disseminated disease in immunocompromised people (Stamm and Brown, 2004). Zebrafish (Swaim et al., 2006) and leopard frogs (Ramakrishnan et al., 1997) are being developed as experimental *in vivo* models of *M. marinum* infection, thus providing a potential means of extending our investigations into animal models.

For the PD component of this study, we conducted time-kill experiments using five antimycobacterial drugs singly and in pairs to estimate the parameters and evaluate the fit of Hill-function-based pharmacodynamic models. We found that while Hill functions provided excellent fits for the PD of the single antibiotics, two-drug Hill functions with a unique interaction parameter could not account for the PD of any of the drug pairs. By assuming two antibiotic-concentration dependent functions for the interaction parameter, one for sub-MIC and one for supra-MIC drug concentrations, we were able to generate modified biphasic Hill functions that provided a reasonably good fit for the PD of the drug pairs.

We used Monte Carlo simulations of antibiotic treatment based on the experimentally-determined PD functions to evaluate the potential microbiological efficacy (rate of clearance) and evolutionary consequences (likelihood of generating multi-drug resistance) of these different drug combinations as well as their sensitivity to different forms of non-adherence to therapy. The simulations predicted varying outcomes for different pairs of antibiotics with respect to the aforementioned measures of efficacy. Hence, this investigation confirmed the necessity of multi-drug pharmacodynamic studies

for tuberculosis drugs and showed that drug interactions could have potentially important effects on the efficacy of treatment regimens.

The study I present in Chapter 3 is also a jointly experimental and theoretical study of the pharmaco-, population and evolutionary dynamics of multi-drug therapy. In this investigation, we used other pathogens that can be responsible for chronic infections, the gram-positive bacterium *Staphylococcus aureus* and the gram-negative *Escherichia coli*. For both these bacteria, multi-drug therapy is gaining increasing traction as a useful treatment option for some infections that they generate. For instance, recommended therapy for infective endocarditis caused by *S. aureus*, especially when prosthetic valves are involved, uses at least two drugs simultaneously (Baddour et al., 2005). Further, successful treatment using combination therapy is being used to successfully treat osteoarticular infections and osteomyelitis caused by staphylococcal species (Barberan et al., 2008). Some recent studies have also reported improved results for using combination therapy to treat *E. coli* and other Gram-negative bacteria associated sepsis and bacteremia (Al-Hasan et al., 2009; Kumar et al., 2010; Micek et al., 2010).

After the investigation in Chapter 2 demonstrated the lack of generality of a single, unique parameter model to describe the interactions between drugs, we developed a more general approach to assess drug interactions. In this study, we converted equipotent concentrations of different antibiotics in a drug pair into a single concentration variable. This strategy allowed us to determine the magnitude of cidal activity that was generated by the combination relative to the constituent drugs at comparable concentrations. We assessed antibiotic efficacy in two ways: (i) by conducting time-kill experiments and fitting Hill functions to the exponential phase of antibiotic-mediated

killing, and (ii) by determining the efficacy of the antibiotics and antibiotic pairs in reducing the level of persister subpopulations in the post-exponential phase of killing.

For both bacterial species, we compared the pharmacodynamics of four antibiotics of different classes individually and in pairs to determine whether the combinations were more effective than the individual drugs. Our results provided compelling support for the proposition that the nature and form of the interactions between drugs of different classes needs to be determined empirically and cannot be inferred from the pharmacodynamics or mode of action of the individual drugs. We also found limited correlation between the pharmacodynamic efficacy of drug combinations in the exponential cidal phase and the density of persisters that remain after exposure to cidal concentrations of the antibiotics.

To explore the potential implications of the experimental results for the design and evaluation of multi-drug treatment regimens, we used Monte Carlo simulations of within-host antibiotic treatment that incorporated clinically relevant refuge subpopulations. The results of these simulations suggested that: (i) the form of drug-drug interactions can profoundly affect the rate at which infections are cleared, (ii) two-drug therapy can prevent treatment failure even when bacteria resistant to single drugs are present at the onset of therapy, and (iii) this evolutionary virtue of two-drug therapy is manifest even when the antibiotics suppress each other's activity.

Of note, the 'within-host' mathematical models we used in the aforementioned investigations did not explicitly consider the contribution of the immune system to the eradication of an infection. To address this limitation, we developed a mathematical model that combines innate and adaptive immune responses with the pharmacodynamics

of antibiotics and bacteria, the pharmacokinetics of the drug and the population and evolutionary dynamics of bacteria (Chapter 4). In our analysis of the properties of this model, we gave primary consideration to the relationship between antibiotic dose, dosing frequency and term of treatment on the time before clearance and the likelihood of resistance emerging and/or ascending during therapy. We examined two types of acute infections, one that would be self-limiting and non-lethal and another that would be lethal in the absence of treatment. Our results suggested that under many conditions, high dose treatment for extended periods would be more effective than more moderate regimens in increasing the rate of cure, preventing the emergence and ascent of resistance and minimizing potential immunopathology. We also observed a saturating effect of increasing doses, such that at certain antibiotic concentrations, there were minimal gains to be made with further dose increases. We explored the current status of data in support of predictions and hypotheses made from our analyses and discussed the design of potential empirical studies suggested by our results.

Finally, I briefly summarize the general conclusions of the investigations contained in this dissertation in Chapter 5. I discuss additional questions and lines of inquiry that the studies herein have generated and consider potential future directions.

CHAPTER 2

Two-drug antimicrobial chemotherapy: A mathematical model and experiments with *Mycobacterium marinum*

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2.1 INTRODUCTION

The concurrent use of multiple drugs, which is one of the mainstays of chemotherapy, is useful and in some cases necessary for the successful treatment of diseases such as tuberculosis (TB), HIV/AIDS, malaria and various cancers. Shortly after antimycobacterial agents became available for treating TB, it was recognized that single drug therapy almost invariably led to treatment failure due to the ascent of resistance, but that this could be mitigated by the use of multiple drugs with different modes of action (Medical Research Council, 1948; 1950; 1952; 1953). In its current form, standard tuberculosis treatment consists of a two-month combinatorial course of rifampin, isoniazid, pyrazinamide and ethambutol, followed by a four-month continuation phase of isoniazid and rifampin.

Despite the barrage of antibiotics and long term of combination therapy, *Mycobacterium tuberculosis* (Mtb) strains that are resistant to multiple drugs are an increasingly troubling component of the epidemiological landscape. In 2009, the World Health Organization estimated close to half a million cases of multidrug resistant (MDR) TB (cases in which recovered strains were resistant to the most potent first-line antibiotics, rifampin and isoniazid) (WHO, 2010). By mid-2010, 58 countries had reported at least one case of extensively drug-resistant (XDR) TB (MDR strains that are

additionally resistant to any fluoroquinolone as well as at least one of the injectable drugs capreomycin, kanamycin and amikacin) (WHO, 2010). The important issue is thus: how can the term of tuberculosis chemotherapy and the likelihood of treatment failure due to the evolution of resistance during the course of therapy be reduced?

One approach to improving the efficacy of single drug therapy has been to design treatment regimens based on *in vivo* data of the changes in the concentration of the antibiotic, pharmacokinetics (PK), and *in vitro* data on the relationship between the concentration of the drug and the rate of growth/death of the bacteria, pharmacodynamics (PD) (Ambrose et al., 2007; Craig, 1998; Drusano, 2004; Jacobs, 2001). This PK/PD approach to the rational design of antibiotic treatment regimes has been employed for tuberculosis but almost exclusively for single antibiotics (Diacon et al., 2007; Gumbo et al., 2005; Gumbo et al., 2009; Gumbo et al., 2004; Gumbo et al., 2007a; Gumbo et al., 2007b; Jayaram et al., 2003; Jayaram et al., 2004; Shandil et al., 2007; Wilkins et al., 2008). To extend this approach to the multi-drug treatment regimes clearly needed to prevent acquired resistance, it is necessary to concurrently account for the PD of the different drugs, and most critically, how they interact (Chait et al., 2007; Hegreness et al., 2008; Michel et al., 2008).

Drug interactions are generally classified as antagonistic, synergistic or additive. In the case of bactericidal antibiotics, additive interactions are usually described in one of two ways, ‘Bliss Independence’ and ‘Loewe Additivity’. Bliss Independence asserts that each drug in a combination exerts its killing action independently of the other drugs (Bliss, 1939). For example, if there are two drugs, A and B, and at particular concentrations they kill f_a and f_b ($0 < f_a, f_b < 1$) fractions of a bacterial population in an

hour, at the end of the hour the viable cell density would be reduced to $(1-f_a)(1-f_b)$ of its initial level. For Loewe additivity, the fraction of surviving cells with both drugs would be $1-f_a-f_b$, the constraint being that $f_a+f_b<1$ (Loewe, 1928). Antagonism and synergism can then be defined relative to one of these descriptions of additivity: drugs interact antagonistically if their combined cidal activity is less than would be predicted for an additive drug combination, and synergistically if the cidal activity is more.

Unfortunately, these definitions cannot be readily translated into the PD of two drugs as they do not account for how the rate or extent of killing would vary with the concentrations of the drug. To address this, Greco and colleagues proposed a seminal Emax-based two-drug pharmacodynamic function which assumes that a single parameter can account for the interaction between both drugs (Greco et al., 1990; Greco et al., 1995). If the value of this parameter is zero, then the drugs are additive, with a negative value indicating antagonism and a positive value indicating synergy. Although this and other Emax-based models have been used to characterize the nature of the interactions between different kinds of drugs, including antimicrobials (Deciga-Campos et al., 2003; Ferron and Jusko, 1998; Jonker et al., 2004; Lim et al., 2008; Meletiadis et al., 2007; Tam et al., 2004; Yuan et al., 2010), there has been limited quantitative consideration of how two-drug PD models apply to the design and evaluation of antibiotic treatment regimes for bacteria, particularly those, like tuberculosis, where multiple drug therapy is essential (Lim et al., 2008; Yuan et al., 2010).

In this study, we explore the fit of Hill functions (which subsume Emax models) for the PD of the antimycobacterial antibiotics rifampin, amikacin, clarithromycin, streptomycin and moxifloxacin. We then employ a Hill-function-based variant of the

Greco model to explore the PD of the 10 possible pairs of these drugs. As our experimental organism, we use *Mycobacterium marinum*. In addition to being safer and more convenient to work with, *M. marinum* is a close genetic relative and shares numerous virulence determinants with Mtb. It also recapitulates key immunopathological features of human tuberculosis infection in its natural poikilothermic hosts (Ramakrishnan et al., 1997; Stinear et al., 2008; Swaim et al., 2006).

To explore the potential clinical implications of these theoretical and *in vitro* PD studies, we use Monte Carlo simulations of antibiotic treatment and resistance that incorporate PD functions that best fit our data. Of particular concern in this analysis are: (i) the relative rates at which these different drug combinations clear the simulated infections (their microbiological efficacy) (ii) the likelihood of resistance to the two drugs evolving during the course of therapy (their evolutionary efficacy), and (iii) how that efficacy is affected by different forms of non-adherence to the treatment regime.

2.2 MATERIALS AND METHODS

2.2.1 Bacteria and media: *Mycobacterium marinum* strain ATCC BAA-535 / M was used in all experiments. Bacteria were grown in Middlebrook 7H9 broth (Difco, Detroit, Mich.) supplemented with 0.2% glycerol and 10% albumin-dextrose complex (7H9) at 32°C. Cell densities were estimated by plating on Middlebrook 7H10 agar (Difco) supplemented with 0.5% glycerol and 10% oleic acid-albumin-dextrose complex (7H10) at 32°C.

2.2.2 Antibiotics: Rifampin, amikacin, clarithromycin, streptomycin (Sigma, St. Louis, MO, USA) and moxifloxacin (Bayer, Pittsburgh, PA, USA) were purchased

commercially. Stock solutions were prepared by dissolving the antibiotics in sterile water or methanol, and appropriate dilutions were made in 7H9 broth immediately before use.

2.2.3 Time-kill experiments for generating single-antibiotic Hill functions: Mid-log cultures of *M. marinum* were diluted in fresh medium to obtain a density of approximately $5x10^6$ CFU/mL. 200 μL aliquots of this culture were introduced into wells in a 12-well plate containing 1.8 mL of antibiotic solution. The plates were incubated with shaking at 32°C for 72 h, and samples were taken every 12 h to determine viable CFU's.

2.2.4 MIC determination: Minimum Inhibitory Concentrations (MICs) were estimated using a broth microdilution procedure similar to that recommended by the CLSI(CLSI, 2005) (7H9 was used instead of Mueller-Hinton Broth). Initial inoculating bacterial densities were similar to the densities used to initiate time-kill experiments in order to account for the inoculum effect on MIC demonstrated in Udekwu *et al.* (Udekwu et al., 2009).

2.2.5 Antibiotic-kill experiments for generating two-drug PD functions: Antibiotics were combined to generate solutions that contained 0.1, 0.5, 1.0, 2.0, 5.0 and 10.0 multiples of MIC (xMIC) of each antibiotic. Mid-log cultures of *M. marinum* were diluted in fresh medium to obtain a density of approximately $5x10^6$ CFU/mL. 200 μL aliquots of this culture were introduced into wells in a 12-well plate containing 1.8 mL of antibiotic solution. The plates were incubated with shaking at 32°C for 72 h, and samples were taken at the end of the incubation. The experiment was repeated four times, and gave good quantitative and qualitative replication. We show a representative experiment in the Results section of the manuscript.

2.2.6 Drug interaction modeling: As in Regoes *et al.*, (Regoes et al., 2004) we assume that for single antibiotics, bacterial net growth in the presence of an antibiotic, ψ(A), is dependent on the growth rate of the bacteria in the absence of antibiotics, ψ_{max}, and the death rate due to the antibiotic. The latter is a Hill function, H, composed of the following parameters: ψ_{max}; ψ_{min}, the maximum antibiotic-generated bacterial killing; zMIC, the pharmacodynamic MIC; and κ, which describes the sigmoidicity of the Hill function (Regoes et al., 2004). i.e.:

$$\psi(A_i) = \psi_{\max} - H_i(A_i) \qquad (1)$$

Where

$$H_i(A_i) = \left[\frac{(\psi_{\max} - \psi_{\min(i)}) * \left(\frac{A_i}{zMIC} \right)^{\kappa}}{\left(\frac{A_i}{zMIC} \right)^{\kappa} - \left(\frac{\psi_{\min(i)}}{\psi_{\max}} \right)} \right] \qquad (2)$$

Bacterial net growth rates were determined from the change in bacterial density over the time-kill period, and the pharmacodynamic function was fit to these data using the least square algorithm nls() of R (www.r-project.org) to obtain estimates for the parameters of the Hill function. For two-antibiotic combinations, we incorporated an interaction parameter (α) into the Hill-function mediated killing by both antibiotics. Thus, net bacterial growth rates would be described by the following equation:

$$\psi(A_i, A_j) = \psi_{\max} - H(A_i) - H(A_j) - \alpha * H(A_i) * H(A_j) \qquad (3)$$

and the rate of change in the viable cell density of bacteria, D, treated with combinations of two drugs given by,

$$\frac{dD}{dt} = \psi(A_i, A_j) * D \qquad (4)$$

2.2.7 Estimation of drug interaction parameter (α): By assessing bacterial killing over 72 h when exponentially-growing cultures were challenged with pairwise combinations of antibiotics (A_i and A_j) at different concentrations, we obtained empirical estimates for net bacterial growth rates in the presence of both antibiotics, ψ_{exp}. As the theoretical analyses outlined above generate estimates for ψ_{max}, $H_i(A_i)$ and $H_j(A_j)$, algebraically rearranging the net bacterial growth rate equation gives an equation for determining α:

$$\alpha = \frac{\psi_{exp} - (\psi_{max} - H(A_i) - H(A_j))}{H(A_i) * H(A_j)} \qquad (5)$$

2.2.8 Numerical solutions: To follow the predicted change in the viable cell density of bacteria, we use numerical solutions to the differential equation (4) programmed in Berkeley Madonna™. Copies of this program and other programs used in this study and instructions for their use can be obtained on www.eclf.net/programs.

2.3 RESULTS

2.3.1 Single drug pharmacodynamics: In Figure 2.1 we show the fit of the theoretical single-drug pharmacodynamic function (Equation 1) to the PD data obtained from experiments with five antimycobacterial agents. These data were generated by exposing *M. marinum* to the antibiotics at different concentrations and estimating net bacterial growth/death rates (based on the increase or decrease in the density of viable bacteria) over 72 hours. The analyses of these time-kill data were restricted to 72 hours in order to ensure that bacteria were growing and/or being killed exponentially.

For single antibiotics, the Hill function provides a good fit for the relationship between the concentration of the drug and the growth/death rate of the bacteria (Figure 2.1, see R^2 values). This is also evident in Table 2.1, where we list the estimates of the Hill function parameters for each of the drugs. The maximum growth rates calculated from this

function are very close to that estimated independently (data not shown). Moreover, the estimated zMIC's (MIC's calculated from the Hill functions) and MIC's determined by the CLSI (CLSI, 2005) recommended broth dilution method are, given the factor of two limitation of the latter, coincident. The individual antibiotics exhibited different pharmacodynamic signatures reflected in the varying shapes of the PD function (the parameter κ) and the kill rate parameter ψ_{min}, which ranged from -0.043 to -0.166 h^{-1}.

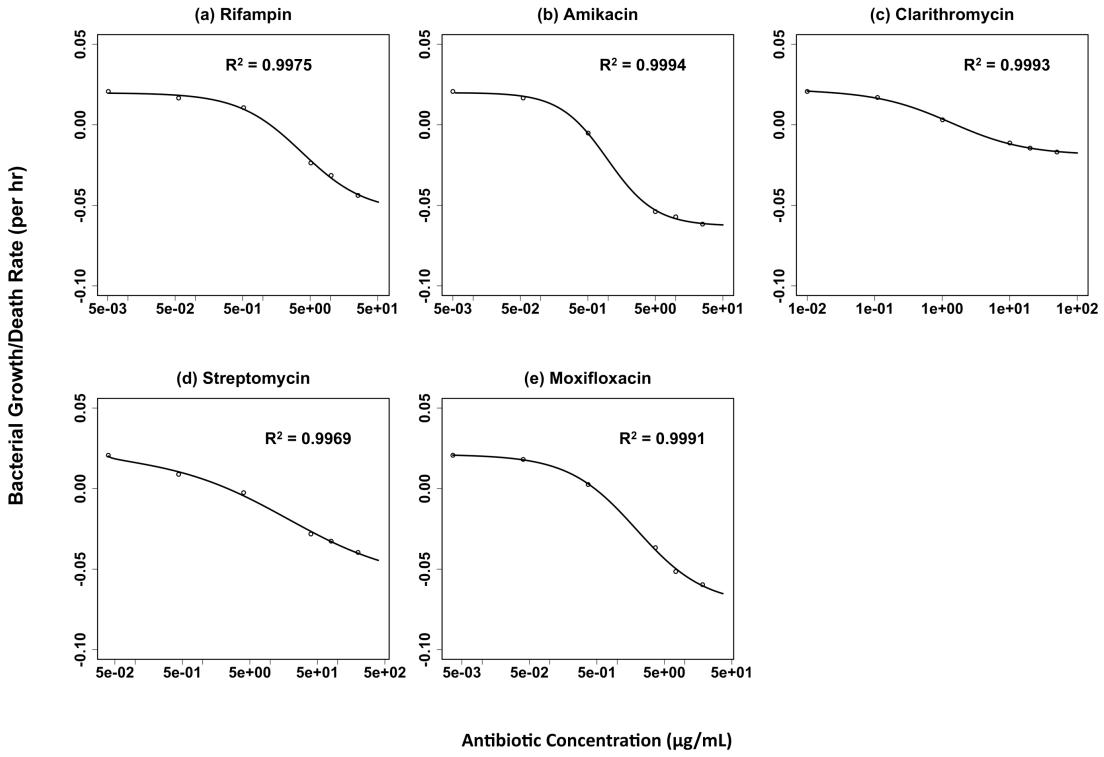

Figure 2.1 Fit of the Hill function to time-kill data for single antibiotics. Adjusted R^2 values determined from an F test are shown. (a) Rifampin, (b) Amikacin, (c) Clarithromycin, (d) Streptomycin, (e) Moxifloxacin.

Table 2.1 Single-drug pharmacodynamic function parameter estimates and standard errors.

Drug	ψ_{max} (h^{-1})	ψ_{min} (h^{-1})	κ	zMIC (mg/L)	MIC (mg/L)
Rifampin	0.0453±0.0018	-0.125±0.0072	0.925±0.17	1.27±0.22	0.512
Amikacin	0.0457±0.0012	-0.145±0.0019	1.23±0.12	0.38±0.029	0.5
Clarithromycin	0.0483±0.00068	-0.0434±0.0013	0.783±0.077	1.58±0.14	1
Streptomycin	0.0465±0.0021	-0.134±0.013	0.508±0.11	2.31±0.50	2
Moxifloxacin	0.0478±0.0015	-0.166±0.0052	0.863±0.091	0.461±0.055	0.37

2.3.2 Two -drug pharmacodynamics: With the PD function parameter estimates for single antibiotics in hand, we proceeded to assess the validity of the two-drug pharmacodynamic function (Equation 3). To accomplish this, we exposed *M. marinum* to combinations of antibiotics, each of which was at some multiple of its respective MIC, and estimated the growth/death rates of the bacteria over 72 hours. Using the differential equation (Equation 4), the estimated single-drug Hill function parameters and different values of α, we compared the observed growth/death rates to those anticipated from the unique α model.

In Figure 2.2 we show the experimentally-observed changes in bacterial growth/death rates generated by different two-antibiotic combinations (curves with markers) together with those predicted from our model for different drug interaction parameters, the α's (curves without markers). Our estimates of these growth/death rates were limited to situations where the density of surviving cells exceeded 10 CFU per ml. Both the experimental and theoretical analyses were conducted for all possible two-drug combinations of the antimycobacterial drugs used in the study.

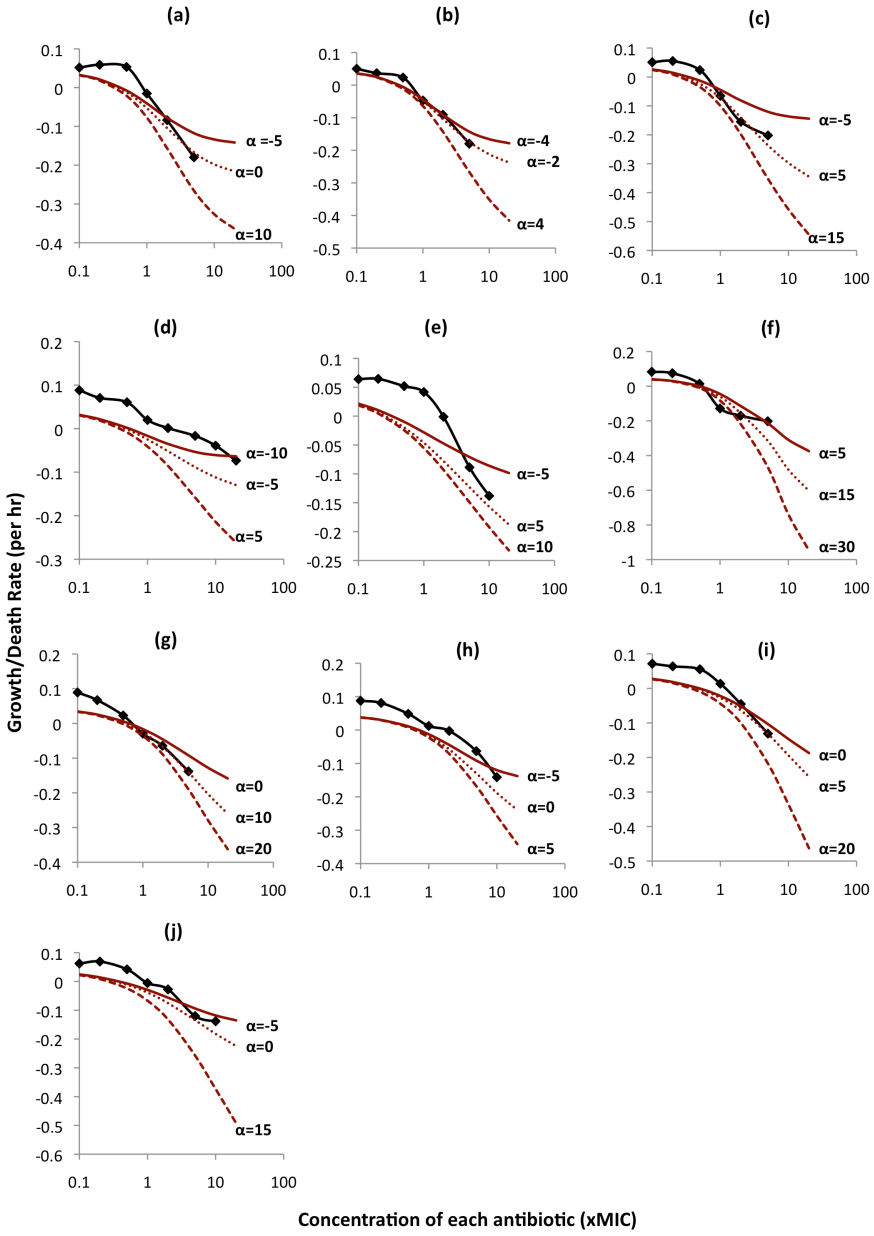

Figure 2.2 Predicted and observed growth/death rates of *M. marinum* exposed to different combinations of two antibiotics. Curves without markers represent predicted theoretical rates, and curves with markers represent observed experimental rates. Values of α represent different degrees of interaction between antibiotics. Positive values indicate synergy, negative values antagonism, and values of zero, additivity. (a) amikacin + clarithromycin (b) amikacin + moxifloxacin (c) amikacin + streptomycin (d) clarithromycin + moxifloxacin (e) clarithromycin + streptomycin (f) rifampin + amikacin (g) rifampin + clarithromycin (h) rifampin + moxifloxacin (i) rifampin + streptomycin (j) streptomycin + moxifloxacin.

For all the drug combinations, it is apparent that a single interaction parameter is insufficient to describe the dynamics over the entire range of concentrations assessed. While the deviation of fit from this single α function varies among antibiotic pairs, in all cases, at lower drug concentrations the observed growth rate is greater than that anticipated from the model. The fit with a single value of α does, however, get somewhat better at higher drug concentrations.

To get a better idea of the relationship between antibiotic concentration and α, we used Equation 5 to separately estimate this interaction parameter for different concentrations of the ten drug pairs (Figure 2.3). For all antibiotic combinations, this interaction became relatively more synergistic with increasing drug concentration. Interactions at sub-MIC concentrations were universally antagonistic, but could be mildly antagonistic, additive or synergistic at supra-MIC concentrations (Figure 2.3 and Table 2A.1 in Appendix). In addition, the rate of change in α from one concentration to the next was much greater at sub-MIC than at supra-MIC concentrations. Interaction coefficients at the larger concentrations only changed to a limited extent and appeared to approach constancy, mirroring the results shown in Figure 2.2. Although not providing a precise fit to these data, if we assume a two-phase interaction function, one for sub- and one for supra-MIC concentrations and use linear regressions to generate the α functions for each phase, a reasonable fit obtains (Figure 2.3 and Table 2A.1 in Appendix).

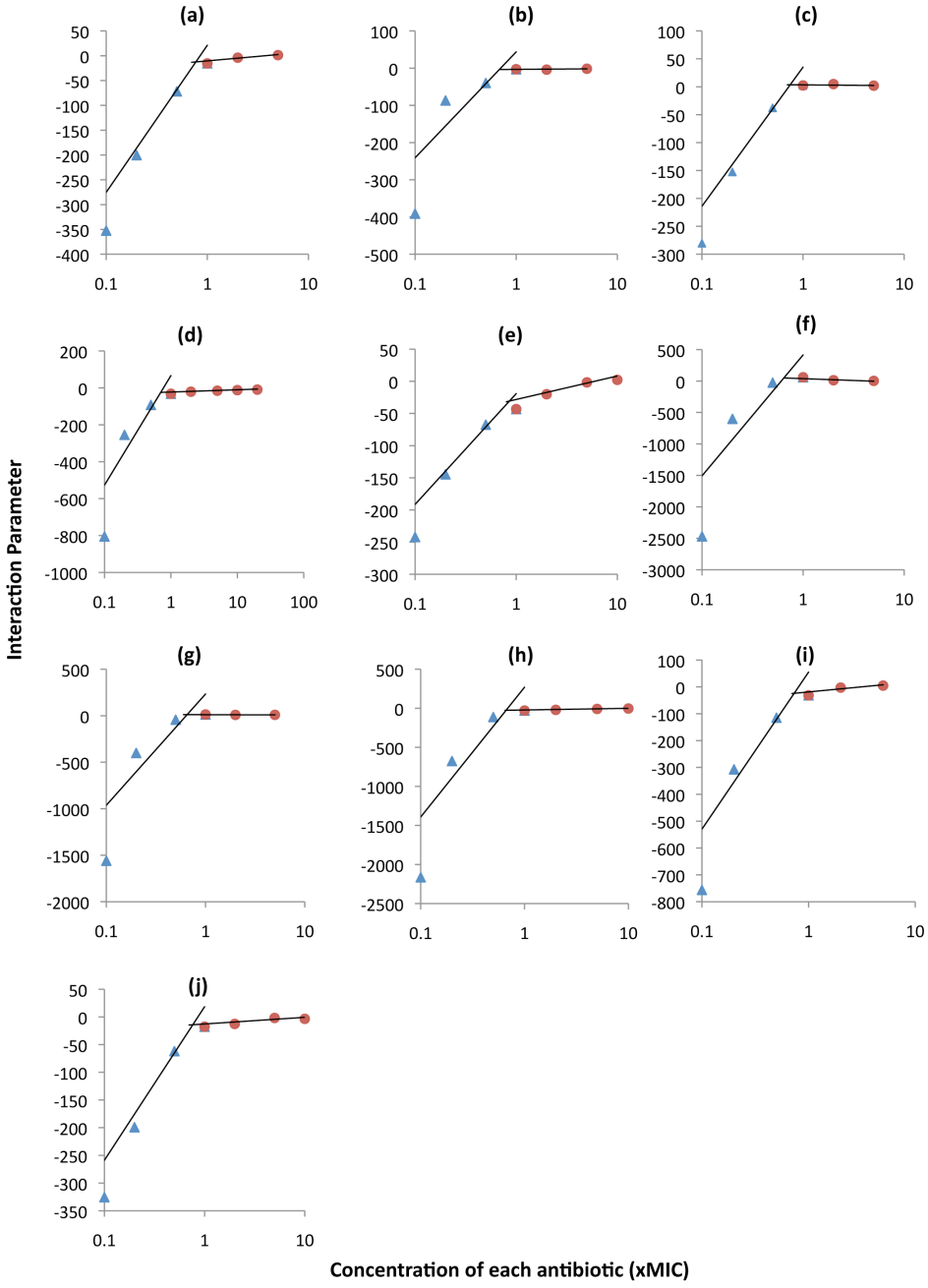

Figure 2.3 The interaction parameter as a function of antibiotic concentration. Independent linear regressions are shown for sub-MIC (triangles) and supra-MIC (circles) concentrations. (a) amikacin + clarithromycin (b) amikacin + moxifloxacin (c) amikacin + streptomycin (d) clarithromycin + moxifloxacin (e) clarithromycin + streptomycin (f) rifampin + amikacin (g) rifampin + clarithromycin (h) rifampin + moxifloxacin (i) rifampin + streptomycin (j) streptomycin + moxifloxacin.

2.3.3 Asymmetric antibiotic concentrations: For convenience, but also to make this approach to evaluating the pharmaco- and population dynamics of two-drug antibiotic treatment readily applicable, we restricted the above PD experiments to situations in which both antibiotics were at the same xMIC concentration. In an effort to explore the robustness of the two-drug PD observed for these cases of symmetric drug concentrations, we performed time kill experiments for three asymmetric (unequal xMIC concentrations) situations: (i) where both antibiotics are below their respective MICs, (ii) where one antibiotic is below its MIC and the other above and (iii) where both are above their MICs.

When both antibiotics are below the MIC, there is antagonism similar to that observed for the symmetric case. This can be seen in Figure 2A.1 (in Appendix), where we present the observed growth rates and those anticipated for situations where there is no interaction between the drugs, $\alpha=0$. As would have been anticipated from the symmetric combination results (Figure 2.2), at sub MICs the drugs together kill at a lower rate than expected were there no interactions between them i.e. they exhibit antagonism. Moreover, the estimated α's for the combination of 0.1 and 0.5 xMIC concentrations of the antibiotics were generally less negative than those calculated for combinations of 0.1-0.1xMIC but more negative than those calculated for the 0.5-0.5 xMIC symmetric cases (Table 2A.2, in Appendix).

Of particular concern in situations where one drug is below the MIC and the other above is that the substantial antagonism observed for below-MIC antibiotic concentrations would be manifest by sub-MIC drugs reducing the efficacy of supra-MIC antibiotics. The results of our experiments indicate that this is not the case (Figure 2A.2,

in Appendix). When combined with a sub-MIC concentration of a second drug, the rate of kill of the supra-MIC drug is no less than that when it is alone and in some cases greater.

To explore the effects of asymmetric concentrations for pairs of above-MIC antibiotics, we compared the observed death rate with that anticipated for no interaction between the antibiotics. The results of these experiments suggest that there is either no interaction between the antibiotic pairs or there is the mild antagonism or synergy observed for the symmetric drug concentration experiments (Figure 2A.3, Appendix). In sum, the results of these experiments with asymmetric drug concentrations are consistent with that anticipated from the symmetric concentration experiments depicted in Figure 2.3.

2.3.4 Predicted dynamics of treatment: To evaluate how the pharmacodynamics estimated above would be manifest in a treatment regime, we use a simulation of the within-host population dynamics of bacteria in a two-drug therapy regime for tuberculosis. In Figure 2.4, we present a diagram of the model used for the analysis (equations for the model can be found in Protocol 2A.1 in the Appendix). In designing this model and in choosing the dosing parameters, bacterial densities and PD parameters, we tried to mimic that which would be appropriate for mycobacterial chemotherapy. The structure of our model is based on that suggested by D. Mitchison (Mitchison, 1979). It assumes two compartments, one in which the bacteria are actively proliferating and the other where they are dividing slowly and thereby responding differently to antibiotics (Lipsitch and Levin, 1998; Warner and Mizrahi, 2006). This compartment difference in antibiotic susceptibility is reflected in the pharmacodynamic Hill functions, such that the

maximum and minimum rates of growth/death are proportional to the rate of replication in the two compartments. The idea is that the slowly dividing subpopulation is relatively refractory to killing by the antibiotics, as would be the case for latent or persister cells in a tuberculosis infection.

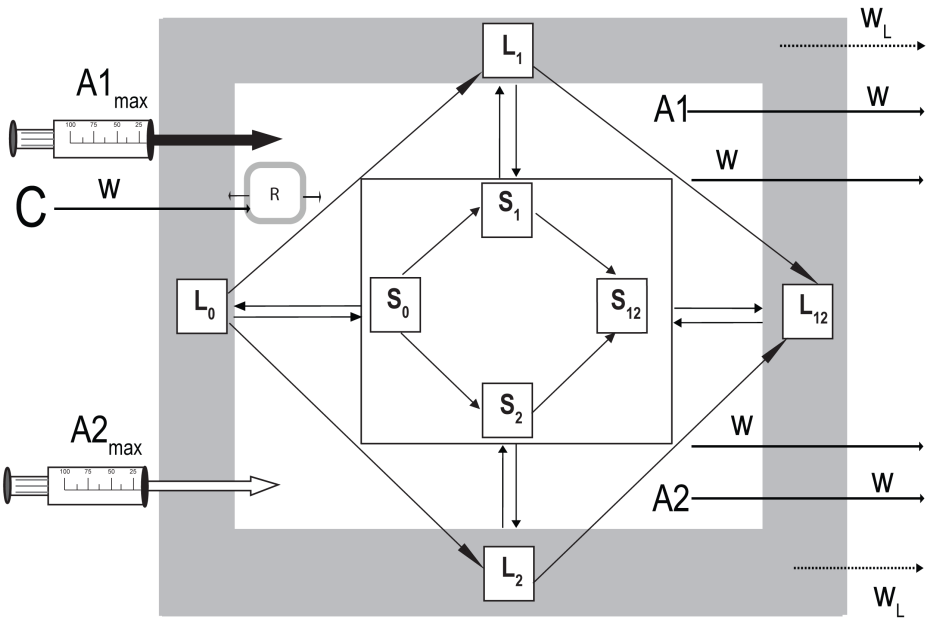

Figure 2.4 Two-compartment population and evolutionary dynamic model of two-drug antibiotic therapy. Main (active) compartment: S0, bacteria susceptible to both antibiotics; S1, bacteria resistant to antibiotic 1; S2, bacteria resistant to antibiotic 2, S12, bacteria resistant to both antibiotics. Latent (refractory) compartment: L0, bacteria susceptible to both antibiotics; L1, bacteria resistant to antibiotic 1; L2, bacteria resistant to antibiotic 2, L12, bacteria resistant to both antibiotics. C, reservoir resource concentration; R, internal concentration of the limiting resource; A1 and A2, internal concentrations of the antibiotics; A1max and A2max, concentration of antibiotics added periodically; w, flow rate of resources into and out of the compartments; w_L, flow rate of latent population from the latent compartment.

We allow for four states of the bacteria, one that is susceptible to both drugs, S0 and L0 (S and L for rapidly- and slowly-dividing populations respectively), S1 and L1 for those resistant to drug 1, S2 and L2 for cells resistant to drug 2, and S12 and L12 for cells that are resistant to both drugs. These variables are both the densities (cells/ml) of

bacteria in these states as well as their state designations. By resistance we are assuming that these bacteria are totally refractory to the drugs, with MICs at least 100X that of the susceptible cells. Resistance also engenders a 5% fitness cost which is manifest as a 5% lower maximal growth rate of bacteria in those states. This assumed cost is in the range of what has been observed for *M. marinum* mutants resistant to the antibiotics considered in this study (Table 2A.3, in Appendix). We allow migration at rates f_{ls} (from latent to susceptible) and f_{sl} (from susceptible to latent) cells per hour, representing either a physical or a physiological translocation between the compartments.

Resources for bacterial growth enter and are removed from the habitat (host) at a constant rate, w ml per hour. The bacteria, however, are removed from the habitat at two rates, w for S0, S1, S2 and S12, and w_L for L0, L1, L2, and L12, where $w > w_L$. For the pharmacodynamic functions, we use the two-drug Hill functions with the biphasic model for the interaction coefficient described above. For pharmacokinetics we assume that a fixed dose A1max and A2max of each drug is added every T hours. In addition to washout at rate w, both drugs also decay at a rate d mg/L per hour. In these simulations we assume that at the onset of treatment, the sensitive population is initially at a density of $S0=5x10^7$ in the main compartment (Shimao, 1987) and $L0= 5x10^4$ cells per ml in the refractory compartment.

As would be anticipated for hosts infected with numbers of bacteria that exceed the reciprocal of the mutation rates, we assume that there are minority populations of bacteria resistant to single antibiotics, S1, S2, L1 and L2, with a relative frequency of 10^{-3} to the corresponding susceptible population (Pyle, 1947). We also allow resistance to single drugs to evolve during the course of the simulations at rates proportional to the

product of the number of individuals of each ancestral state and a mutation rate. The actual generation of mutants occurs in a semi-stochastic manner, via a Monte Carlo routine. At each time step (Δt) in the finite step size (Euler) simulation, the probability that a mutant would be generated is the product of the number of individuals of the genotype, Δt and the mutation rate μ. When the random number is less than this product, a mutant is added to the noted population, e.g. when S1 is generated from S0, a bacterium is added to the S1 state and one removed from the S0 state. We use step sizes of Δt so that the probability of a mutant being added at a particular time interval is always less than 1. For these simulations, μ takes values in the range of that estimated from fluctuation experiments for different antibiotics and *M. marinum* (Table 2A.3, in Appendix). There are no doubly resistant cells, S12 and L12 at the start of the simulations, but they can evolve by mutation from the single resistant states.

In Figure 2.5, we follow the changes in density of the different bacterial populations in the main compartment (5a) and in the refractory compartment (5b). The PD parameter values used in this simulation are those in the range estimated in our experiments for the combination of rifampin (A1) and amikacin (A2). These antibiotics are inoculated every 24 hours at a concentration of 5X their respective MICs and decline in concentration due to flow and a decay rate, d=0.075 per hour. With these parameters, the overall densities of the sensitive and single-resistant populations continue to decline during the course of the simulation. In the main compartment this decline is punctuated by oscillations in density reflecting the waxing and waning of the antibiotic concentration, with net decline each hour. The single resistant populations are cleared earlier than the sensitive for two reasons: their lower initial densities and their lower

fitness relative to the sensitive bacteria. This interpretation was confirmed by running simulations in which single resistant populations were at higher initial densities and had lower fitness costs (data not shown). Under these conditions, their resistance to single antibiotics does not make up for this fitness cost.

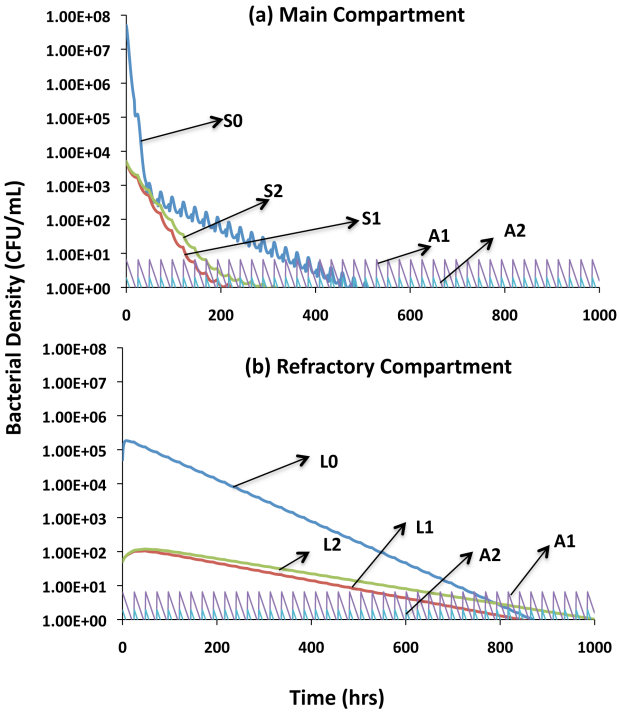

Figure 2.5 Clearance dynamics for different subpopulations in the main and refractory compartments of the PD/PK model. Parameters used are those observed for the rifampin (A1) + amikacin (A2) combination. In these simulations, w and w_L are, respectively, 0.02 and 0.002 per hour; $f_{ls}=f_{sl}=0.001$; the antibiotic decay rate is $d=0.075hr^{-1}$ and the maximum and minimum bacterial growth rate for each subpopulation in the latent compartment is 10% of those in the active. (a) Main compartment, (b) Refractory compartment.

In the refractory compartment, the rate of change in cell density is lower and the oscillations are not manifest to the same extent as in the main compartment. This occurs because the replication and washout rates are lower, as is the rate of kill by the antibiotics. As a result of continuous migration of cells from and to the slower-growing population, the rate of decline in the density of cells in the main compartment is reduced

whilst that in the refractory compartment increased relative to what would obtain were they the sole compartments or not connected. Said another way, the existence of a refractory compartment prolongs the term of therapy.

To compare the relative efficacy of different combinations of antibiotics, we ran these simulations with the estimated PD parameter values obtained for the different combinations of drugs. In addition to simulations with symmetric antibiotic concentrations for the two drugs, we also conducted these simulation experiments with asymmetric antibiotic concentrations. The former were initiated with 5xMIC of both drugs and the latter with 5xMIC of one antibiotic and 2xMIC of the other. As a result of flow and decay, the asymmetric drug concentration simulations include periods where both drugs are above the MIC, one above and one below, and both below. The interaction coefficients used in these simulations are those estimated from the corresponding symmetric and asymmetric concentration experiments. As our measure of the efficacy of treatment, we considered the time until the total density of bacteria was less than one (time to clearance). The results of these simulations are presented in Table 2.2. While in some runs doubly resistant mutants emerged, ascended and thereby precluded clearance, these were not included in the Table 2.2 clearance data. The frequencies of runs in which double resistance emerged are considered separately.

Although mutation is a stochastic process, there was effectively no between-run variation in the time before clearance. For eight out of the ten combinations, clearance occurred in less than 1600 hours. The rifampin + amikacin combination was the most effective, leading to clearance in 1080 hrs. The combinations of clarithromycin + moxifloxacin and clarithromycin + streptomycin took substantially longer to clear the

bacteria; compared to the rifampin + amikacin combination, the clarithromycin + moxifloxacin combination took some 4 times longer, with the clarithromycin + streptomycin combination taking approximately 11 times longer. This is what would be anticipated from the relative pharmacodynamics of the different drug combinations (Figure 2.2).

Table 2.2 Relative efficacy of antibiotic combinations in clearing bacteria during simulated infections.

	Time to Clearance (hours)	
Antibiotic Combination	Antibiotics at symmetric xMIC concentrations	Antibiotics at asymmetric xMIC concentrations
Rifampin + Amikacin	1080	2785
Rifampin + Clarithromycin	1527	2521
Rifampin + Streptomycin	1433	2396
Rifampin + Moxifloxacin	1453	2642
Amikacin + Clarithromycin	1428	2568
Amikacin + Streptomycin	1315	2452
Amikacin + Moxifloxacin	1090	2690
Clarithromycin + Streptomycin	11668	13035
Clarithromycin + Moxifloxacin	4530	5793
Streptomycin + Moxifloxacin	1422	2257

As in the symmetric case, the majority of the antibiotic combinations in the asymmetric simulations cleared the infection over a relatively similar period, i.e. <2800 hours. The reason that the average time to clearance is greater for the asymmetric concentrations is because there is a lower peak concentration for one of the two drugs, rather than equal peaks. While clarithromycin + streptomycin and clarithromycin + moxifloxacin remained the least effective drugs, the most effective combination was streptomycin + moxifloxacin rather than rifampin + amikacin. Compared to streptomycin + moxifloxacin, clarithromycin + moxifloxacin and clarithromycin + streptomycin took, respectively, approximately 2.5 and 6 times longer to clear the infection.

2.3.5 The evolution of multiple resistance: What is the relationship between the PD of the antibiotics and the likelihood of mutants resistant to both drugs emerging? To address this question, we separately performed 1000 simulation experiments using three sets of parameters reflecting the 'extreme' conditions of relative efficacy for the symmetric combinations: rifampin + amikacin, clarithromycin + moxifloxacin and clarithromycin + streptomycin. The aggregate results from these simulation experiments are presented in column one of Table 2.3.

As can be seen, the two-drug resistant population emerged in only a few runs. Although the relative number of runs in which resistance emerged for the different drug combinations is what would be anticipated from the clearance data in Table 2.2, the differences were not statistically significant ($p \sim 0.525$). With these parameters, the frequency of two-drug resistance emerging was low and was roughly the same for all three pairs of drugs.

2.3.6 Non-adherence: In a number of epidemiological studies, non-adherence to the prescribed treatment regime has been associated with adverse therapeutic outcomes (Burman et al., 1997), longer terms of treatment and acquired drug resistance (Espinal et al., 2001; Pablos-Mendez et al., 1997). In practice, non-adherence takes a number of forms and depends on a variety of factors such as organization of treatment and care (access to services, length, drug-type and other requirements for therapy, support services, etc) individual interpretations of illness and wellness, drug side effects and the social context in which therapy is undertaken (Munro et al., 2007). How does non-adherence contribute to the amount of time required for microbiological cure and the likelihood of multi-drug resistance emerging within a host during the course of

treatment? How sensitive are different drug combinations to the adverse outcomes of non-adherence? To address these questions, we considered three broadly-inclusive types of non-adherence that we call random, thermostat (Lipsitch and Levin, 1998), and drug holiday (described below). To explore the relationship between the PD of the drug combinations and the frequency of non-adherence with respect to the generation of the double resistant mutants, we conducted 1000 runs for each of the three aforementioned drug combinations and the different non-adherence scenarios. The results of these simulations are presented in Table 2.3.

Table 2.3 Percent of 1000 runs in which multi-drug resistant mutants emerged by 1000 hours.

		Random Non-adherence			
Antibiotic Combination	Complete Adherence	10% Non-adherence	20% Non-adherence	Thermostat Non-adherence	Extended Drug Holiday Non-adherence
Rifampin + Amikacin	0.8	1.2	1.4	100	1.7
Clarithromycin + Moxifloxacin	1.2	2.1	3.9	1.3	5.2
Clarithromycin + Streptomycin	1.3	2.6	4.1	1.7	5.8

2.3.6.1 Random non-adherence: We model this scenario in the following manner: At each dosing period there is a probability P ($0 \leq P \leq 1$) that both drugs will be taken and a corresponding probability (1-P) that neither will be taken. To simulate this we use a Monte Carlo routine where if the random number, $r \leq P$, the drugs are administered, but if $r>P$ that dosing period is skipped. In Figure 2.6(a), we illustrate this process for a single run where two-drug resistance emerges. Non-adherence is reflected in a hiatus in the dosing and a rise in the density of all the bacterial populations. There are periods, such as

between 600 and 648 hours, where consecutive doses are missed. This results in a substantial rise in the density of bacteria and thereby an increase in the likelihood of a doubly resistant mutant being generated.

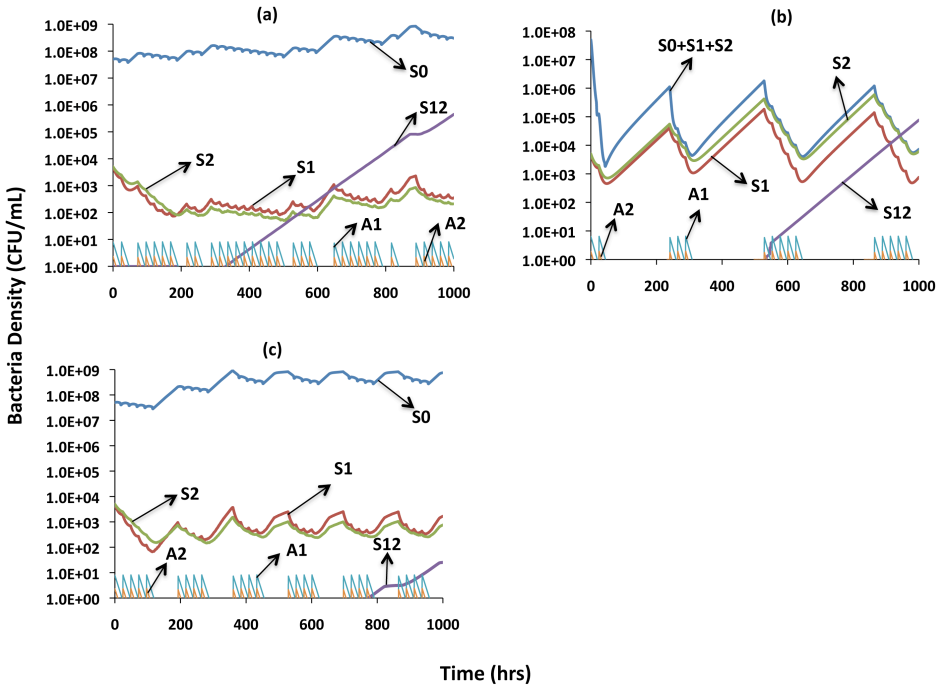

Figure 2.6 Dynamics of non-adherence with therapy. Changes in the absolute concentrations of the antibiotics and densities of bacteria: S0- sensitive to both drugs, S1- resistant to drug A1, S2- resistant to drug A2, and S12- resistant to both A1 and A2. (a) Random non-adherence: Parameters used are those estimated for clarithromycin + streptomycin, assuming a 20% probability of non-adherence at each dosing. (b) Thermostat non-adherence: Parameters used are those estimated for rifampin + amikacin. (c) Drug holiday non-adherence: Parameters used are those estimated for clarithromycin + moxifloxacin. These figures represent runs in which double resistance (S12) emerged. The relative frequencies of this outcome are shown in Table 3. See the text for descriptions of these different modes of non-adherence.

With 10% random non-adherence (P=0.9), there was no significant difference among drug combinations in the probability of resistance arising (p~0.073) (Table 2.3, Column 2). With 20% random non-adherence (P=0.8) there was a highly significant drug combination effect, p~0.001 (Table 2.3, Column 3). The likelihood of multiple resistance arising with 20% non-adherence was negatively related to the microbiological efficacy of

these different drug combinations. The relationship between the probability of a doubly resistant population emerging for different levels of random non-adherence was also directly related to the microbiological efficacy of the drug combinations. For the rifampin + amikacin combination, there was no significant difference among the 0, 10% and 20% non-adherence regimes ($p \sim 0.435$). For the other two pairs, there were significant $p<0.001$ relationships between the frequency of non-adherence and the likelihood of double resistance emerging.

2.3.6.2 Thermostat non-adherence: We simulate this by incorporating a situation in which treatment ceases when the density of the rapidly growing population falls below 10^4 and doesn't commence again until the density exceeds 10^6. The situation we are mimicking is one in which patients cease taking their antibiotics when they are feeling better (the bacterial densities are low enough not to be symptomatic) and do not take the drugs again until the density is high enough to be symptomatic. We illustrate this situation in Figure 2.6(b) with a run in which two-drug resistance emerged.

In column 4 of Table 2.3, we summarize the results of 1000 simulations of thermostat non-adherence for the three drug combinations. With respect to our measure of microbiological efficacy, the thermostat non-adherence scenario seems paradoxical. Two-drug resistance emerged far more frequently in the runs with the most microbiologically effective drug combination, indeed in all 1000 runs. The reason for this is that the more effective drug combination reduced the density more rapidly than the less effective drug combinations. As a result there were far more frequent periods where drugs were not taken and the single-resistant populations ascended to high-enough densities where two-drug resistant mutants were produced with a very high probability.

Under the parameter conditions of this simulation, the non-adherence threshold was never crossed in any of the 1000 simulations for either of the two less effective drugs.

2.3.6.3 Drug Holidays: We model this scenario in the following manner: Both drugs are taken for 4 consecutive dosing periods, at which time neither drug is taken for the subsequent 3 dosing periods. This regime continues throughout the duration of simulated treatment. We are mimicking a situation where holidays are imposed because the drugs may be costly, limited in their availability or induce debilitating side effects that are alleviated by terminating treatment for an interval. In Figure 2.6(c) we illustrate this situation for a run where two-drug resistance emerged. As noted in the last column of Table 2.3, the overall frequency of double resistance was on the order of 5% and similar for the two microbiologically less effective drug combinations. For the most effective drug combination, relative to complete adherence, the drug holidays doubled the likelihood of two-drug resistance emerging.

2.4 DISCUSSION

With few exceptions, studies of the pharmacodynamics (PD) of antibiotics and bacteria have been restricted to single drugs (Diacon et al., 2007; Gumbo et al., 2005; Gumbo et al., 2009; Gumbo et al., 2004; Gumbo et al., 2007a; Gumbo et al., 2007b; Jayaram et al., 2003; Jayaram et al., 2004; Shandil et al., 2007; Wilkins et al., 2008). Some infections, particularly those that are long-term like tuberculosis, require multiple antibiotics for treatment to be effective. It follows then, that for the rational design of treatment protocols for these infections, multidrug PD analyses are necessary.

Our results indicate that Hill functions provide an excellent fit for the single-drug PD for *Mycobacteria marinum* and each of the five antibiotics considered in this study,

amikacin, clarithromycin, moxifloxacin, rifampin and streptomycin. On the other hand, if, as is assumed in the classical model of Greco and colleagues (Greco et al., 1990; Greco et al., 1995), the interactions between drugs is expressed as a single parameter with a constant value, two-drug Hill function models do not fit the PD observed for any of the 10 pairs of drugs considered. In all cases, at lower antibiotic concentrations the interactions between the drugs is antagonistic; they are less effective together than anticipated from their action alone. As the antibiotic concentrations increase, this drug-drug interaction becomes relatively more synergistic and approaches constancy. To address this phenomenon, we allow for two phases of the drug-drug interaction, one for low (sub-MIC) and one for high (supra-MIC) concentrations with an antibiotic concentration-dependent function for the interaction term. Albeit not as convenient as a unique parameter, these functions can be readily estimated from time-kill data. Most importantly, the biphasic drug interaction Hill function models thus generated provide quantitatively accurate analogues of the PDs of all 10 pairs of antibiotics examined.

It has been hypothesized that there are subpopulations of bacteria within an infected TB host that exhibit differential growth rates and, by extension, variable susceptibility to antimycobacterial agents (Dickinson et al., 1972; Grosset, 1980; Mitchison, 1979; Zhang and Mitchison, 2003; Zhang et al., 1999). Here, we develop a simple mathematical model that accounts for this within-host bacterial heterogeneity by assuming that there are two ‘compartments’, one that houses rapidly-growing and the other slowly-growing bacteria. The model incorporates the possibility of non-adherence to therapy, which is considered to be one of the major contributory factors to TB treatment failure (Burman et al., 1997; Pablos-Mendez et al., 1997; Wares et al., 2003).

Our computer simulations of tuberculosis chemotherapy employing the empirically estimated biphasic Hill functions suggest that there can be substantial differences among drug combinations in treatment efficacy, as measured by the time to clearance. Of the ten antibiotic pairs we consider, rifampin + amikacin is the most effective and streptomycin + clarithromycin the least, with some eleven-fold difference in the time before clearance. With the parameters used in our semi-stochastic model of treatment and assuming different probabilities for the occurrence of random non-adherence, either complete adherence or limited non-adherence to the therapeutic regime would not be manifest as a significant difference among drug combinations in the likelihood of the generation and ascent of two-drug resistant mutants. However, with greater rates of non-adherence, the likelihood of two-drug resistance emerging becomes increasingly dependent on the drug combination employed. The emergence of two-drug resistance due to random non-adherence is more likely for less microbiologically effective drug combinations than those that are more effective.

With externally imposed regular drug holidays, the likelihood of emergence of two-drug resistance is also inversely proportional to the microbiological efficacy of the antibiotic combination. Our results suggest that quite a different situation obtains when the drug holidays depend on the bacterial load, as is the case for thermostat non-adherence. Under the parameter conditions used in our simulations, the most microbiologically effective drug combination almost invariably leads to the emergence of two-drug resistance. As a result of the enhanced efficacy, the time required to reduce the bacterial densities to below a non-symptomatic threshold is decreased for the more effective antibiotic combination. Consequently, in the course of therapy this threshold

and the resulting drug holidays are reached and manifest more frequently for the more effective drug combinations than the less effective. During these holidays, intermediates resistant to single antibiotics can reach high enough densities for the single drug resistant clones to acquire the second mutation needed for two-drug resistance. It is easy to write-off this paradoxical result as an artifact of the model because of the extraordinary frequency of two-drug resistance emerging in our simulations. On the other hand, this outcome is not entirely unreasonable if indeed patients go off treatment when they are no longer symptomatic but remain infected. While we are not championing the validity of this potential downside of effective chemotherapy, we believe it may warrant further consideration.

This jointly theoretical and experimental study raises important as well as intriguing issues about the interactions between antibiotics of different classes and how these interactions are affected by their concentrations. Our results, however, provide no information about the physiological, molecular and other processes underlying these interactions. What are these processes? It is clear that answering this question is not going to be trivial. As Yeh and Kishony argue, intuitive deductions about the type of interactions between drugs based on the metabolic pathways of action of their individual action are, at best, simplifications (Yeh and Kishony, 2007). Antibiotic action is pleiotropic and not limited to structural or metabolic alterations to a particular target. As such, the resulting cellular death or growth cessation upon antibiotic use can be due to multiple factors. Although there is evidence that antibiotics of different types kill by a common non-specific mechanism, the production of hydroxyl radicals (Dwyer et al., 2007; Kohanski et al., 2007; Kohanski et al., 2008), the rates of kill vary among drugs

and their concentrations in ways that cannot be predicted from their respective targets and mode of action.

Particularly intriguing is the antagonistic interaction observed at lower (sub-MIC) concentrations among all the antibiotic pairs studied. Why? How? We know that antibiotics at both sub- and supra-MIC concentrations affect mycobacterial transcription patterns in a variety of ways and can lead to a number of physiological and biochemical stress responses (Goh et al., 2002; Wilson et al., 1999). Some of these responses have been observed to reduce antimicrobial activity through actions such as antibiotic efflux, ribosomal protection, etc (Colangeli et al., 2005; Colangeli et al., 2007; Michele et al., 1999; Morris et al., 2005). One possible explanation is that at sub-MIC concentrations for two drugs, these stress responses make the bacteria more refractory to antibiotic activity, but the drugs do not generate enough cidal activity to overcome this refractoriness – a phenomenon that would manifest as pharmacodynamic antagonism.

To paraphrase the statistician George Box, 'All models (and model systems) are wrong, some are useful' (Box, 1987). We endorse this perspective and of course believe our model and model system are useful. However, we see this utility restricted to its potential to evaluate, *in vitro*, the efficacy of different antibiotic combinations for clinical applications. Our models are not intended to be quantitatively exact analogs of tuberculosis chemotherapy but rather to generate a framework within which questions relevant to TB treatment could be approached. They were designed in the tradition advocated by Richard Levins (Levins, 1966), to maximize reality and generality at the loss of precision. Thus, even though the pharmacodynamic parameters are directly estimated and drug doses simulated in clinically realistic range (Hall et al., 2009), the

time scale in these simulations do not reflect the actual time course of tuberculosis chemotherapy and dosing schedule.

We elected to do the experimental work on this project with *M. marinum* because we are particularly interested in multi-drug treatment of tuberculosis. As a model for Mtb, *M. marinum* has its virtues and limitations. In addition to being more convenient to work with than Mtb, *M. marinum* infections in fish and amphibians demonstrate key elements of Mtb infections in humans (Ramakrishnan et al., 1997; Swaim et al., 2006). Of particular import is the formation of epitheloid granulomas with lymphocytic involvement (Tobin and Ramakrishnan, 2008). Thus, using either fish or amphibians, it should be possible to evaluate, *in vivo*, the predictions of our models. *M. marinum* is also limited as a model for multi-drug treatment of Mtb primarily because of its natural resistance (relatively high MICs) to some the first line antibiotics used to treat tuberculosis, in particular isoniazid, ethambutol and pyrazinamide. While one of the antibiotics used in this study, rifampin, is a first line tuberculosis drug, the others are only used in cases where first line drugs fail.

Albeit simple, our TB chemotherapy model incorporates some, but clearly not all of the complexity of a *M. tuberculosis* infections and their treatment. It accounts for the subpopulation heterogeneity that has been postulated for these infections (Dickinson et al., 1972; Grosset, 1980; Mitchison, 1979; Zhang and Mitchison, 2003; Zhang et al., 1999) and the effects of that heterogeneity on the PD of the antibiotic treatment. On the other hand, this model does not formally account for the third subpopulation suggested by the recent observation that some Mycobacteria in macrophages induce efflux pumps that make them tolerant to antibiotics (Adams et al., 2011). At a pharmacodynamic level, this

phenomenon is, however, somewhat subsumed in our model by the presence of a subpopulation of bacteria that is less susceptible to the antibiotics than another segment of the population. Additionally, while our model takes into account three forms of the non-adherence that is considered to be one of the major contributory factors to TB treatment failure (Burman et al., 1997; Pablos-Mendez et al., 1997; Wares et al., 2003), it certainly does not incorporate all of the nuances of non-adherence.

We are unaware of other studies that have combined experimental work on the PD of multiple drugs with a quantitative consideration of the potential clinical implications of these PDs. There have been investigations of the PD of multiple antibiotics that have employed a fitting approach for a quantitative description of the interactions between drugs (Meletiadis et al., 2003; Meletiadis et al., 2007). Similar to that observed here, some of these studies provide evidence that the interactions between antibiotics can vary with their concentrations (Berenbaum et al., 1983; Hegreness et al., 2008; Meletiadis et al., 2007). Nevertheless, to our knowledge, this quantitative relationship has not been taken into account in the design of treatment programs; the interactions between different antibiotics are simply described as additive, synergistic or antagonistic, but without consideration of how this relationship changes with antibiotic concentration. The models we develop and the experimental methods we employ in this study can be used for any combinations of bactericidal antibiotics and bacteria that can be grown *in vitro*. Whether the biphasic interaction phenomenon observed with *M. marinum* and the five drugs considered would be manifest with other bacteria and drug combinations remains to be seen.

2.5 APPENDIX

The following appendix contains supplementary information for the above investigation. All the figures, tables and protocols that follow have been pre-referenced in the text of the chapter.

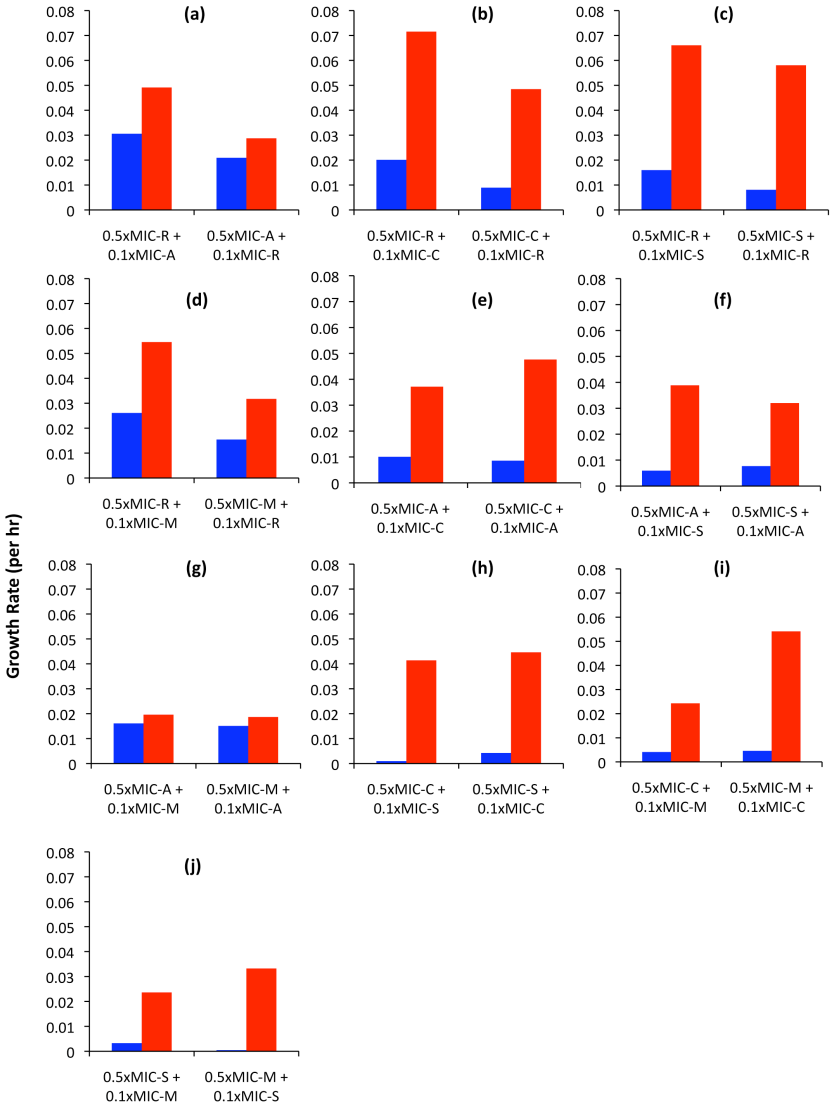

Figure 2A.1 Predicted and observed growth rates of *M. marinum* exposed to asymmetric sub-MIC antibiotic concentrations. Blue bars represent predicted rates anticipated from the Hill functions under the assumption that the drugs are acting

additively. Red bars represent the growth rates observed for the noted concentrations. Multiples-of-MIC concentrations at which antibiotics are combined are indicated. R-rifampin; A-amikacin; C-clarithromycin; S-streptomycin; M-moxifloxacin. (a) amikacin + clarithromycin (b) amikacin + moxifloxacin (c) amikacin + streptomycin (d) clarithromycin + moxifloxacin (e) clarithromycin + streptomycin (f) rifampin + amikacin (g) rifampin + clarithromycin (h) rifampin + moxifloxacin (i) rifampin + streptomycin (j) streptomycin + moxifloxacin.

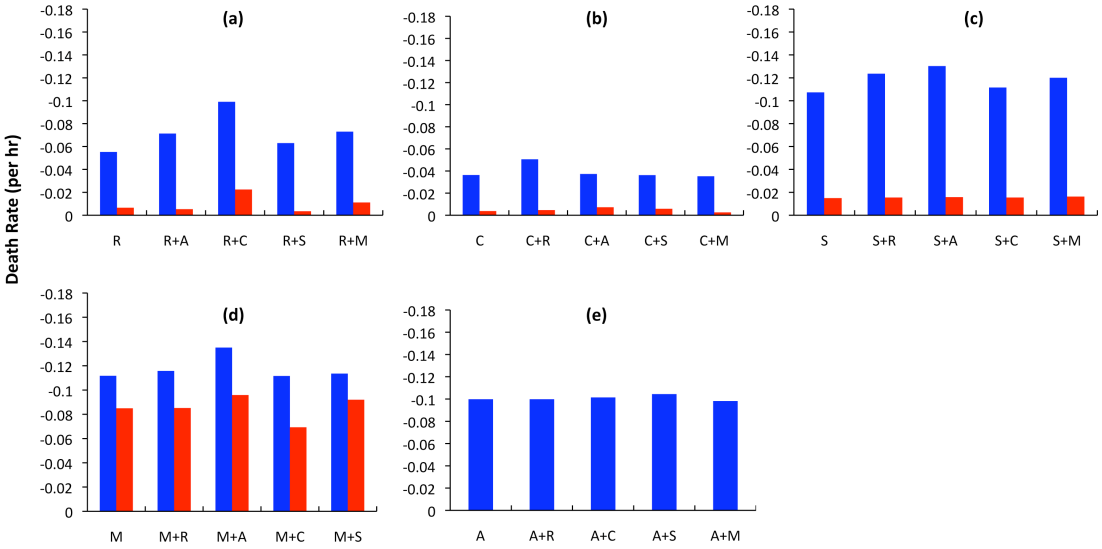

Figure 2A.2 Predicted and observed growth/death rates of *M. marinum* exposed to sub- and supra-MIC antibiotic combinations. Growth/death rates observed for single drugs in comparison to that observed with those drugs in combination with a sub-MIC concentration of second antibiotic. Red bars represent combinations of antibiotics at 2xMIC and 0.1xMIC; blue bars represent combinations of antibiotics at 5xMIC and 0.5xMIC. For amikacin, only the lower (2xMIC+0.1xMIC) concentration results are presented. At the higher concentrations the extent of kill exceeded the limit of detection. R-rifampin; A-amikacin; C-clarithromycin; S-streptomycin; M-moxifloxacin. (a) rifampin (b) amikacin (c) clarithromycin (d) streptomycin (e) moxifloxacin.

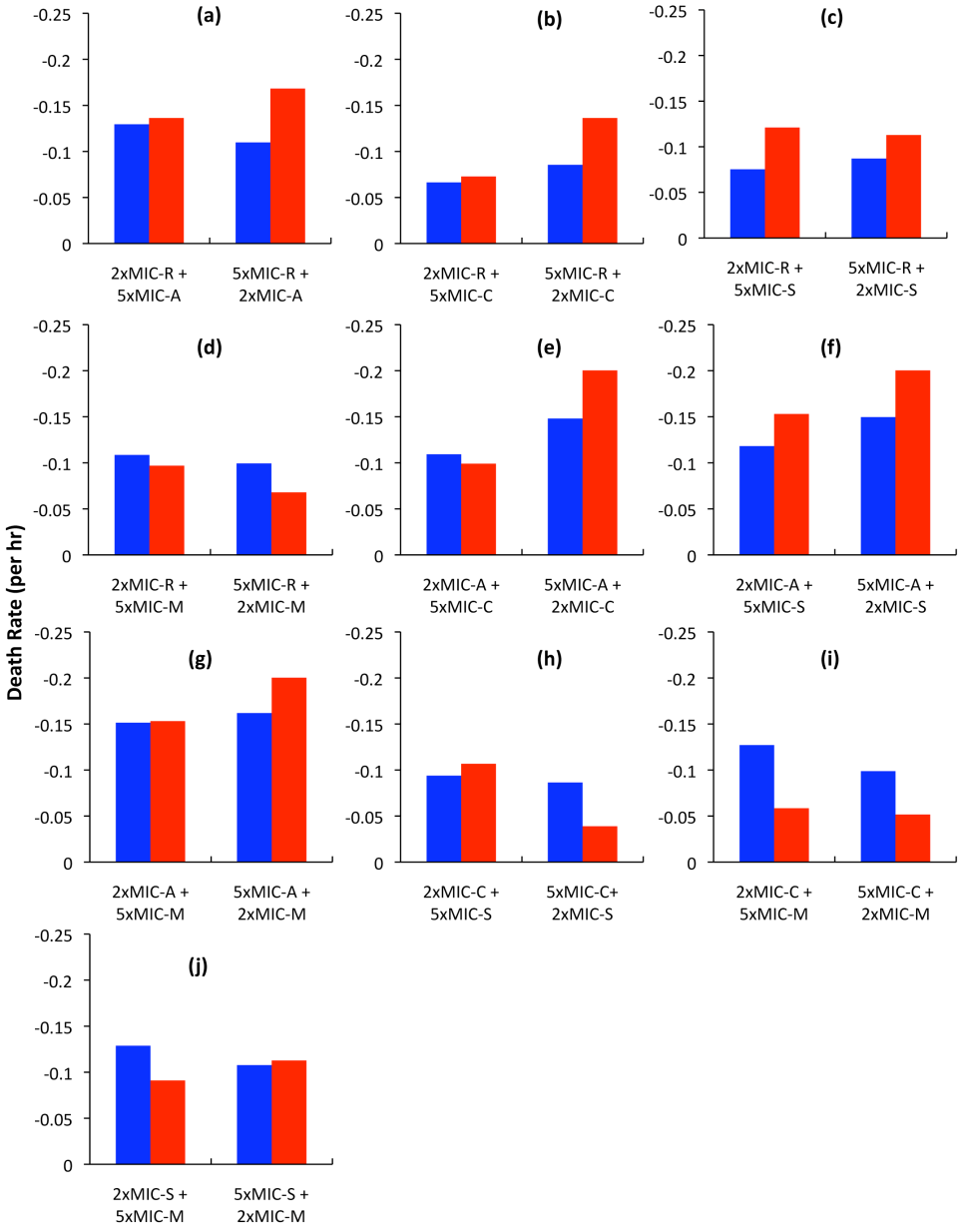

Figure 2A.3 Predicted and observed death rates of *M. marinum* exposed to asymmetric supra-MIC antibiotic concentrations. Blue bars represent predicted rates anticipated from the Hill functions under the assumption that the drugs are acting additively. Red bars represent the growth rates observed for the noted concentrations. Multiples-of-MIC concentrations at which antibiotics are combined are indicated. R-rifampin; A-amikacin; C-clarithromycin; S-streptomycin; M-moxifloxacin. (a) amikacin + clarithromycin (b) amikacin + moxifloxacin (c) amikacin + streptomycin (d) clarithromycin + moxifloxacin (e) clarithromycin + streptomycin (f) rifampin + amikacin (g) rifampin + clarithromycin (h) rifampin + moxifloxacin (i) rifampin + streptomycin (j) streptomycin + moxifloxacin.

Table 2A.1 Linear Regression Parameters for the Biphasic Antibiotic Interaction Function.

Antibiotic Combination	Sub-MIC Antibiotic Concentrations			Supra-MIC Antibiotic Concentrations		
	Slope	Intercept	R^2	Slope	Intercept	R^2
Amikacin + Clarithromycin	329.22	-308.12	0.79	3.606	-15.676	0.77
Amikacin + Moxifloxacin	316.26	-272.49	0.52	0.4221	-3.9734	0.53
Amikacin + Streptomycin	276.93	-241.77	0.77	-0.2866	3.8084	0.15
Clarithromycin + Moxifloxacin	659.53	-592.83	0.57	0.9247	-24.867	0.64
Clarithromycin + Streptomycin	191.33	-210.46	0.74	4.3471	-35.223	0.71
Rifampin + Amikacin	2135.8	-1720.5	0.54	-11.794	56.529	0.65
Rifampin + Clarithromycin	1328.8	-1095.5	0.54	-0.6755	12.103	0.34
Rifampin + Moxifloxacin	1845.8	-1576	0.57	2.6737	-27.569	0.85
Rifampin + Streptomycin	649.7	-594.83	0.66	7.5399	-29.97	0.67
Streptomycin + Moxifloxacin	308.13	-289.7	0.79	1.4934	-15.582	0.65

Table 2A.2 Value of interaction parameter at different combinations of sub-MIC concentrations.

	Interaction Parameter			
Antibiotic Combination[1]	0.1xMIC + 0.1xMIC	0.1xMIC(Antibiotic A) + 0.5xMIC (Antibiotic B)	0.5xMIC (Antibiotic A) + 0.1xMIC (Antibiotic B)	0.5xMIC+0.5xMIC
Rifampin + Amikacin	-2470	-97.1	-355.8	-25.7
Rifampin + Clarithromycin	-1559.6	-326.6	-259.2	-42.7
Rifampin + Streptomycin	-756.1	-403.2	-195.4	-115
Rifampin + Moxifloxacin	-2163.9	-164.5	-248.1	-112.5
Amikacin + Clarithromycin	-352.3	-290.5	-79.3	-71.8
Amikacin +Streptomycin	-280.4	-176.4	-74.7	-37.9
Amikacin + Moxifloxacin	-390.9	-32.5	-17.9	-40.0
Clarithromycin + Streptomycin	-242.1	-77.2	-61.3	-67.5
Clarithromycin + Moxifloxacin	-805	-118.2	-68.3	-92.6
Streptomycin + Moxifloxacin	-325.6	-60.6	-67.3	-61.8

[1] First Antibiotic named in the combination is Antibiotic A

Protocol 2A.1 Differential equations used for simulation of the mathematical model.

$$\frac{dS0}{dt} = g_{S0}S0 - wS0 - 2\mu S0 - f_{sl}S0 + f_{ls}L0$$
$$\frac{dS1}{dt} = g_{S1}S1 - wS1 + \mu S0 - \mu S1 - f_{sl}S1 + f_{ls}L1$$
$$\frac{dS2}{dt} = g_{S2}S2 - wS2 + \mu S0 - \mu S2 - f_{sl}S2 + f_{ls}L2$$
$$\frac{dS12}{dt} g_{12}S12 - wS12 + \mu S1 + \mu S2 - f_{sl}S12 + f_{ls}L12$$
$$\frac{dL0}{dt} = g_{L0}L0 - w_L L0 - 2\mu L0 + f_{sl}S0 - f_{ls}L0$$
$$\frac{dL1}{dt} = g_{L1}L1 - w_L L1 + \mu L0 - \mu L1 + f_{sl}S1 - f_{ls}L1$$
$$\frac{dL2}{dt} = g_{L2}L2 - w_L L2 + \mu L0 - \mu L2 + f_{sl}S2 - f_{ls}L2$$
$$\frac{dL12}{dt} = g_{L12}L12 - w_L L12 + \mu L1 + \mu L2 + f_{sl}S12 - f_{ls}L12$$

where μ per cell per unit time is the mutation rate for change from cells of the noted to the resistant state, e.g. S0--> S1.

[Growth Rates]

$$g_{S0} = \psi_{\max} - H(A1) - H(A2) - \alpha(A1, A2)H(A1)H(A2)$$
$$g_{S1} = \psi_{\max}(1 - s_1) - H(A2)$$
$$g_{S2} = \psi_{\max}(1 - s_2) - H(A1)$$
$$g_{S12} = \psi_{\max}(1 - s_{12})$$
$$g_{L0} = \psi_{\max L} - H(A1) - H(A2) - \alpha(A1, A2)H(A1)H(A2)$$
$$g_{L1} = \psi_{\max L}(1 - s_1) - H(A2)$$
$$g_{L2} = \psi_{\max L}(1 - s_2) - H(A1)$$
$$g_{L12} = \psi_{\max L}(1 - s_{12})$$

where s_1, s_2 and s_{12} $(0 \leq s_x \leq 1)$ are the selection coefficients (fitness costs of resistance) and $\alpha(A1,A2)$ is the antibiotic concentration–dependent drug-drug interaction coefficient (see text).

CHAPTER 3

The Pharmaco –, Population and Evolutionary Dynamics of Multi-Drug Therapy: Experiments with *S. aureus* and *E. coli* and Computer Simulations

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3.1 INTRODUCTION

The simultaneous use of multiple anti-microbial agents is standard for the treatment of long-term infectious diseases like tuberculosis and HIV/AIDS (Connolly et al., 2007; Thompson et al., 2010). Multiple drugs are also used to treat polymicrobial infections and in situations where the etiologic agent of an infection is unknown at the start of therapy (Gorbach, 1994). Increasingly, this "combination therapy" is being used for the treatment of other chronic bacterial infections like endocarditis, osteoarticular infections and osteomyelitis as well as sepsis (Baddour et al., 2005; Barberan et al., 2008; Micek et al., 2010).

The motivation for treating with multiple, rather than single drugs, has both evolutionary and pharmacological components. Theoretically, if multiple drugs with different modes of action are used for treatment, bacteria resistant to each single drug, if present, will remain susceptible to the other drugs. Hence, multi-drug therapy would be less likely to be thwarted by the evolution of resistance than monotherapy. This intuitively appealing evolutionary reason for combination therapy is supported by evidence (Cappelletty et al., 1995; den Hollander et al., 1997; Johnson and Thompson,

1986; Johnson et al., 1985; Kang and Rybak, 1995; Kang et al., 1994; Mclaughlin et al., 1983; Michalsen and Bergan, 1981) as well as logic. From a pharmacodynamics (PD) perspective, there are at least two potential virtues for combination therapy. The drugs can be synergistic in their action and provide greater cidal activity than single drugs at comparable doses. Combining drugs can also result in increased antimicrobial activity without elevating single-drug concentrations to levels that engender debilitating side effects. In some situations, the *in vitro* synergy of multiple treating drugs is positively correlated with bactericidal activity and clinical outcome (Anderson et al., 1978; Dejongh et al., 1986; Klasters.J et al., 1972; Klastersky et al., 1976a; Klastersky et al., 1976b; Lau et al., 1977) and, at the same time, antagonistic interactions between drugs *in vitro* can negatively impact therapeutic success (Jawetz et al., 1951; Johansen et al., 2000; Lepper and Dowling, 1951).

As appealing as the reasons for multi- rather than single drug therapy may be, the clinical utility of combination therapy remains equivocal for many infections (Dellit et al., 2007). One of the reasons for this is the relative dearth of sufficient answers to a number of fundamental questions. How does one know whether a specific combination therapy regimen will be more or less effective than monotherapy for a specific infection? How does one quantify the pharmacodynamics of multiple drugs? Are there generalizable rules about how drugs of different classes interact? Under what conditions will the collective activity of multiple drugs exceed their individual activity? How do the pharmacological interactions between drugs in combination affect the emergence of resistance during the course of therapy?

Although these questions have been addressed in various ways, at this juncture the answers obtained are restrictive. Checkerboard titrations and time kill assays seem to be the most popular *in vitro* methods to evaluate the form of interactions between antibiotics (synergy, antagonism, suppression or additivity). The checkerboard assay generates a single parameter, the Fractional Inhibitory Concentration (FIC) index as a measure of the efficacy of drug combinations relative to their respective individual efficacies measured by the Minimum Inhibitory Concentration, MIC (Hall et al., 1983). Time-kill assays express the efficacy of drug combinations in terms of the log-fold reduction in viable cell density generated by these combinations relative to the most active single agent over an arbitrary time period (Lorian, 2005). Neither of these measures of the combined action of drugs provides information about the functional relationship between the concentrations of these drugs and the rate at which the target bacteria are killed (Regoes et al., 2004). The dynamics of antibiotic-mediated killing by pairs of drugs with the same FIC index and/or log-fold reductions in viable cells can differ profoundly and these single parameter measures may not provide an adequate picture of the cidal properties of drug combinations for the design of antibiotic treatment regimens. Another limitation of this single interaction parameter approach is that it fails to account for the changes in the form of the interaction with changing concentrations of the drug, pharmacokinetics (Ankomah and Levin, 2012; Berenbaum et al., 1983; Hegreness et al., 2008; Meletiadis et al., 2007).

The relationship between the concentration of single bactericidal antibiotics and the rate of growth or death of bacteria during the initial exposure can be fit to Hill functions (Ankomah and Levin, 2012; Regoes et al., 2004), but at this juncture it is

unclear how these or other pharmacodynamic functions can account for the complication of the interactions between drugs. To our knowledge, there is no *a priori* way to quantitatively predict how multiple drugs will interact from their single drug pharmacodynamics. Although there have been some compelling analyses of the pharmacodynamics of multiple antibiotics and bacteria, with few exceptions e.g. (Ankomah and Levin, 2012; Lim et al., 2008) these have been restricted to low and often sub-MIC and thereby sub-therapeutic concentrations of these drugs (Wood et al., 2012; Yeh et al., 2006).

Finally, there is the phenomenon of persistence. Antibiotic-mediated killing is a biphasic process: the rate of bactericidal activity during *in vitro* time-kill experiments declines with time and approaches zero. Depending on the drug employed, a substantial fraction of genetically susceptible but phenotypically resistant bacteria, the persisters, survive (Bigger, 1944; Lewis, 2010). A comprehensive consideration of the pharmacodynamics of combination therapy would also provide information about how multiple drugs affect the level of persistence. Bar two recent exceptions (Allison et al., 2011; Hofsteenge et al., 2013), all studies of persistence of which we are aware have focused solely on single drugs.

In this report we develop, illustrate and evaluate a procedure that addresses these quantitative questions of the pharmaco- and evolutionary dynamics of multi-drug antibiotic therapy. Using *in vitro* experiments with *Staphylococcus aureus* and *Escherichia coli,* we determine the functional relationship between the concentrations of four antibiotics of different classes (singly and in pairs) and the rate of growth/kill of these bacteria during the exponential phase of their confrontations with these drugs.

Using this method, we are able to explore the pharmacodynamics of multiple drugs at supra- as well as sub-MIC concentrations. We also evaluate the relationship between cidal concentrations of these antibiotic combinations and the density of persisters surviving exposure to the drugs. To explore the potential clinical implications of the experimental PD results, we employ a mathematical model of multi-drug therapy that allows for the evolution of resistance to the treating drugs. Using computer simulations with parameter values in the ranges of those estimated from the experimental analyses, we explore the effects of two-drug PD efficacy on the rate of clearance of infections and the emergence of single- and multi-drug resistance.

3.2 MATERIALS AND METHODS

3.2.1 Bacterial Strains and Growth/Sampling Media: Experiments involving *E. coli* were conducted using strain 018:K1:H7 (designated CAB1) that was originally isolated from a child with meningitis and supplied by Craig A. Bloch (Bloch et al., 1989). This strain has been used in previous studies of the within-host pharmacodynamics of antibiotic and phage treatment (Bull et al., 2002; Regoes et al., 2004; Wiuff et al., 2005). The experiments involving *Staphylococcus aureus* were conducted using strain Newman which was isolated from a patient with osteomyelitis and generously provided by Dr. William Shafer. Bacteria were grown in 10 mL of Lysogeny Broth (LB) (*E. coli*) or Mueller-Hinton II (MHII) broth (*S. aureus*) in 50-mL Pyrex flasks at 37°C with aeration and shaking (200 rpm). Viable cell densities in bacterial cultures were determined by plating dilutions (made in 0.85% saline) on LB Agar.

3.2.2 Antibiotics: For experiments involving *E. coli*, 10 µg/mL stock solutions of ciprofloxacin, ampicillin, tobramycin and tetracycline were diluted in fresh LB to

appropriate concentrations for each experiment. Antibiotic stocks used in the *S. aureus* experiments were prepared to a final concentration of 10 µg/ml for ciprofloxacin, gentamicin and oxacillin while vancomycin was prepared to a final stock concentration of 15 µg/ml. Dilutions of requisite antibiotics were made fresh in MHII broth to the appropriate concentrations for each experiment. All antibiotics were procured from Mediatech, Inc. (Herndon, Va.) and Sigma-Aldrich (St. Louis, Mo.).

3.2.3 MIC Determination: Minimum Inhibitory Concentrations (MIC) for *E. coli* CAB1 and *S. aureus* Newman were estimated using the broth microdilution procedure recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2005).

3.2.4 Antibiotic Time-kill Experiments: Overnight cultures of *E. coli* CAB1 were diluted 1:2000 into fresh LB to initiate exponential growth, and were allowed to grow to a final density of approximately 1 x 10^7 cells per mL before antibiotics at desired concentrations were added. For single drug experiments, 0, 0.2x, 0.5x, 1x, 2.5x, 5x and 10 multiples of MIC (xMIC) were added to each culture, and for dual drug time kill experiments, pairs of antibiotics were combined to generate solutions that contained 0, 0.2x, 0.5x, 1x, 2.5x, 5x and 10xMIC of each antibiotic. The cultures were sampled to estimate viable cell densities every 10 min for the first 1 h, every 30 min for the next 2 h, and at 6h. Overnight *S. aureus* Newman cultures were diluted to a final concentration of ~ 1x10^7 bacteria per ml in fresh MHII media and incubated for 1 hour at 37°C shaking at 200 rpm to ensure entry into the exponential growth phase. Cultures were then inoculated with 0, 0.1x, 0.5x, 1x, 2.5x, 5x and 10xMIC of each antibiotic individually and then in pairs of equal concentrations for the dual treatment. Viable cell densities were estimated every 10 minutes for the first hour and then every 30 minutes for the next 5 hours.

3.2.5 Level of Persistence Experiments: In order to assess the level of persistence, we conducted late-term time kill experiments using 10 independent replicate cultures for each drug and drug pairing. Experiments were initiated as described in the aforementioned time-kill assays, but sampling was done at a single time point - 6 h for *E. coli* and 22 h for *S. aureus*. Sampling at these time points has been previously shown to provide good estimates for persisters in a culture (Dorr et al., 2010; Johnson and Levin, 2013). We also confirmed that, with the protocol used, there were no drug carryover effects on plating efficiency.

3.2.6 Pharmacodynamic Functions: As in Regoes *et al*. (Regoes et al., 2004), we use a four-parameter Hill function-based pharmacodynamic function (Equation 1) to characterize the exponential phase death rate engendered by the antibiotic(s) singly and in pairs,

$$H(A) = \left[\frac{(\psi_{\max} - \psi_{\min}) * \left(\frac{A}{MIC} \right)^{\kappa}}{\left(\frac{A}{MIC} \right)^{\kappa} - \left(\frac{\psi_{\min}}{\psi_{\max}} \right)} \right] \qquad (1)$$

where ψ_{max} is the maximum bacterial growth rate in the absence of antibiotics, ψ_{min} is the maximum death rate generated by the antibiotic, κ describes the sigmoidicity of the Hill function, the *MIC* is the pharmacodynamic minimum inhibitory antibiotic concentration, and *A* is the antibiotic concentration. In this study, the concentrations of single antibiotics are presented as multiples of the MICs as estimated by standard CLSI serial dilution procedures. For pairs of drugs, A is equal to the sum of equal multiples of the component single drug CLSI estimated MICs. For both single and two drugs, we use exponential phase time kill data for different multiples of the CLSI MICs and the procedure in

(Regoes et al., 2004) to generate Hill functions and estimate their parameters. Thus for each single drug, we have two estimates of MIC, that obtained by serial dilution and the realized MIC (rMIC) estimated from the Hill function. For pairs of drugs we only have single estimate of the minimum inhibitory concentration, that obtained by fitting the Hill function, rMICs.

For single drugs and for drug pairs, net bacterial growth rates under antibiotic action are described by the following respective equations:

$$\psi(A_i) = \psi_{\max} - H_i(A_i) \qquad (2)$$

$$\psi(A_i, A_j) = \psi_{\max} - H_{i,j}(A_i + A_j) \qquad (3)$$

3.3 RESULTS

3.3.1 Multi-drug pharmacodynamics in theory: We open this section with an *a priori* consideration of the pharmacodynamics of two drugs for qualitatively different forms of interactions between these drugs. As our measure of the concentrations for pairs of drugs, in theory and in practice, we use a single variable xCU (x multiples of "Cidal Units"), which is calculated as the sum of equal multiples of the MICs of each single drug. For example, if the MIC of drug A is 1 μg/mL and that of B 2μg/mL, for the pair, 2xCU is the combination of 1μg/mL of A and 2.0 μg/mL B. Implicit in this measure is a null assumption of Loewe's additivity (Loewe and Muischnek, 1926) which assumes that the magnitude of the killing effect of additive multiple drugs is proportional to that which would result from the sum of equipotent concentrations of each drug separately. For instance, under this assumption, the combination of 0.5xMIC each of two additive drugs,

xCU=1, would be equal to 1xMIC of each of the antibiotics on their own (Yeh et al., 2009).

Using the xCU's as measures of the concentrations of single and pairs of drugs and a method similar to that used in Regoes *et al.* (Regoes et al., 2004) (See Materials and Methods), it is possible to fit Hill functions to the rate of bacterial killing during the exponential phase of kill. In Figure 3.1, we illustrate the form of the Hill functions that would be anticipated for single drugs (A or B) and qualitatively different types of two drug interactions (A+B).

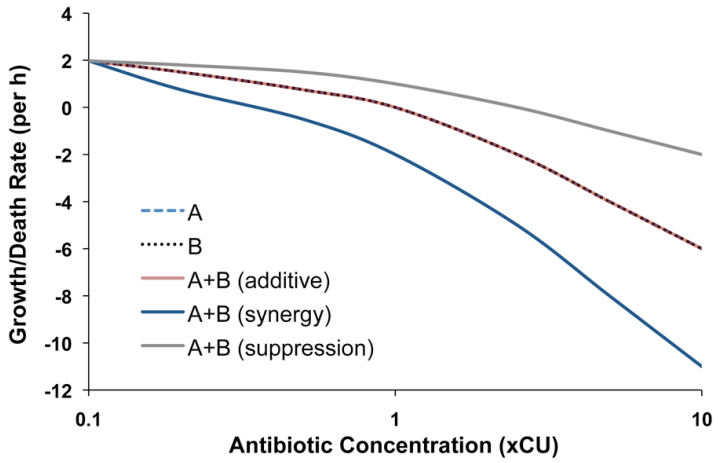

Figure 3.1 Anticipated single and two-drug Hill functions for qualitatively different types of drug interactions. Hill functions of single antibiotics (A or B) and combinations (A+B) representing synergy, additivity and suppression are shown. The growth and death rates used for these illustrations are in the range of those observed experimentally.

In this idealized case, if (i) the drugs are additive at each concentration, the rate of kill generated by the two drugs together is identical to that of each of the drugs alone; (ii) the drugs are suppressive, their combined rate of kill is less than that of each of the single drugs alone, and (iii) the drugs are synergistic, their combined rate of kill is greater than that for the individual drugs. It should be noted that in this illustration, per our

assumption of Loewe additivity, the single drug Hill functions are identical and the same as that for a purely additive drug combination. In generating Figure 3.1, we assumed a directly proportional relationship between antibiotic concentration and the rate of kill engendered. In theory, more complex relationships between drug concentration and rate of antibiotic-mediated killing can occur, and as seen from the below experimental results, do obtain.

3.3.2 Multi-drug pharmacodynamics in practice: We performed time-kill experiments using single and two-drug combinations to determine the relationship between the concentrations of these drugs and the rate of kill of the target bacteria (Figures 3A.1-3A.4, in Appendix). Ampicillin, ciprofloxacin, tobramycin and tetracycline were used in the *E. coli* experiments and oxacillin, vancomycin, ciprofloxacin and gentamicin in experiments with *S. aureus*. For both single and multiple drugs, we observed biphasic cidal dynamics; an exponential decline in bacterial survival followed by a leveling off period with minimal cidal activity.

We fit Hill functions to the concentration-dependent rate of kill of bacteria during the exponential phase of killing in our experiments, between 0 and 1 hour for *E. coli* and between 0 and 4 hours for *S. aureus*. We estimated the Hill function parameters for each of the four single antibiotics and six pairs of antibiotics used in the time-kill experiments with both bacteria. As the equivalent of the pharmacodynamic, Hill function estimate of the MIC for single drugs, we determined the analogous Hill function estimate for pairs of drugs, which we call the realized MIC, rMIC. The estimates of these parameters are available in Tables 3A.1 and 3A.2 (in Appendix).

In Figures 3.2 and 3.3, we show the PD functions for all two-drug combinations together with the corresponding single-drug PDs for the component antibiotics. For *E. coli* there was no detectable cidal activity at antibiotic concentrations less than 0.1xCU and we use 0.1xCU as the minimum concentration (Figure 3.2). Since we observed cidal activity at lower drug concentrations for *S. aureus* (a consequence of lower rMIC's), we use 0.01xCU as the minimum concentration (Figure 3.3).

For *E. coli*, combining ampicillin with any drug yielded a greater rate of kill than ampicillin alone at comparable concentrations. The ampicillin+ciprofloxacin (Figure 3.2a) and ampicillin+tetracycline (Figure 3.2b) combinations were generally intermediate in efficacy between the component single antibiotics (a qualitative result we designate as antagonism), while the ampicillin+tobramycin combination (Figure 3.2e) exhibited synergy at most concentrations. When used in combination, tetracycline diminished the cidal activity of the two most efficacious antibiotics, ciprofloxacin and tobramycin. In combination with ciprofloxacin a suppressive interaction prevailed (Figure 3.2c), while for the tobramycin+tetracycline combination, the two drugs together exhibited the same efficacy as tetracycline alone (Figure 3.2f). The combination of tobramycin with ciprofloxacin exhibited synergistic interactions at concentrations below approximately 5xCU. At greater concentrations than this, the single antibiotic tobramycin was more effective than when used in combination (Figure 3.2d).

For *S. aureus*, most antibiotic combinations were either intermediate in efficacy between the individual drugs or generated cidal activity equivalent to that of the more effective of the constituent drugs (Figures 3.3a,b,d,e). We observed suppressive interactions at higher concentrations when vancomycin was combined with either

ciprofloxacin (Figure 3.3c) or oxacillin (Figure 3.3f). Indeed, for the latter combination, the two individually bactericidal drugs became bacteriostatic. It is also worth noting that save for the representative beta-lactams, the maximal death rates exhibited in the *S. aureus* experiments for all drugs/drug pairings were substantially lower than those observed in the *E. coli* experiments.

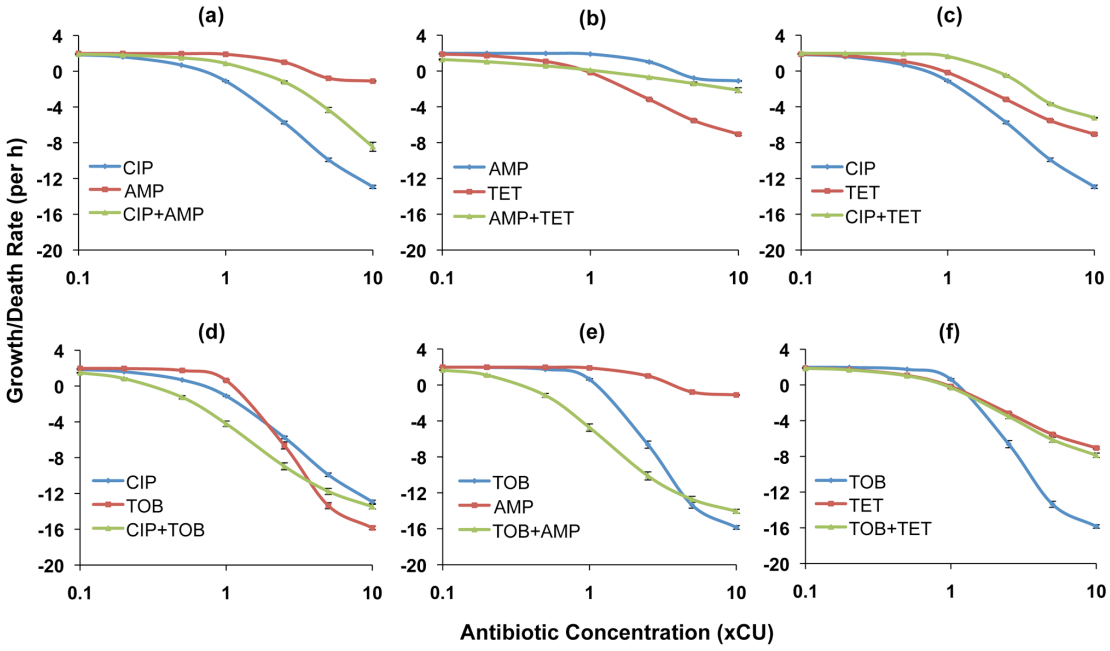

Figure 3.2 Hill functions for two-drug combinations and the constituent individual antibiotics (*E. coli*). Each graph shows the Hill functions for a drug combination and the constituent single drugs with drug concentrations normalized as multiples of Cidal Units (xCU). Error bars represent the standard errors for the growth/death rate at each antibiotic concentration. (a) ampicillin (AMP), ciprofloxacin (CIP), and ampicillin+ciprofloxacin (b) ampicillin, tetracycline (TET), and ampicillin+tetracycline (c) ciprofloxacin, tetracycline, and ciprofloxacin+tetracycline (d) ciprofloxacin, tobramycin (TOB), and ciprofloxacin+tobramycin (e) tobramycin, ampicillin, and tobramycin+ampicillin (f) tobramycin, tetracycline, and tobramycin+tetracycline.

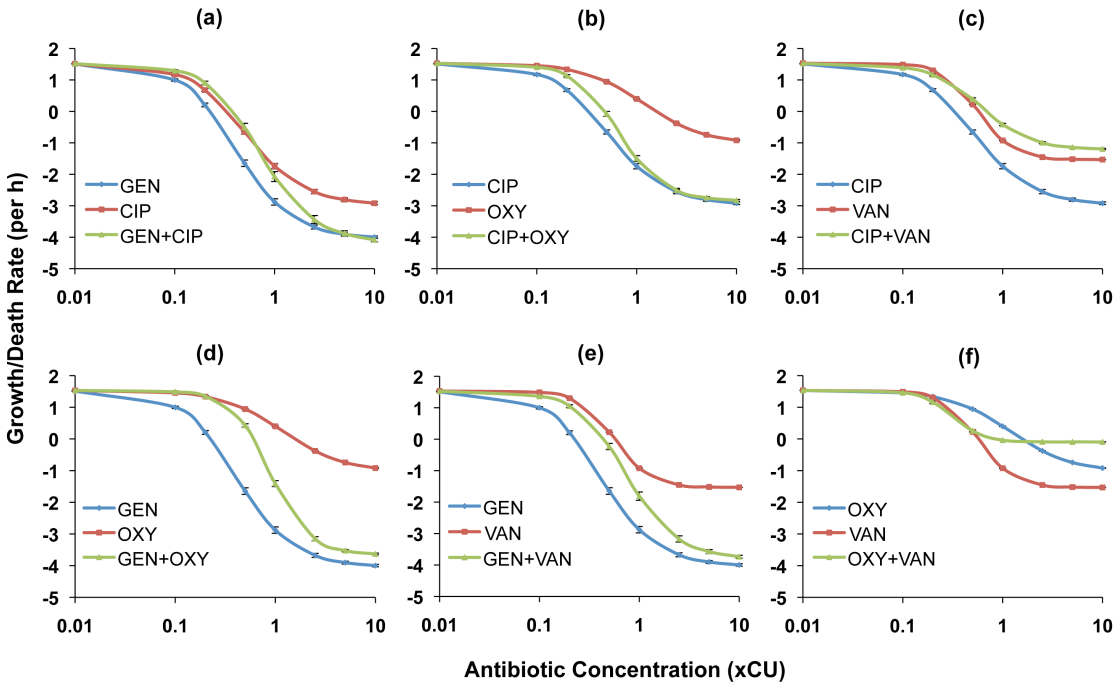

Figure 3.3 Hill functions for two-drug combinations and the constituent individual antibiotics (*S. aureus*). Each graph shows the Hill functions for a drug combination and the constituent single drugs with drug concentrations normalized as multiples of Cidal Units (xCU). Error bars represent the standard errors for the growth/death rate at each antibiotic concentration. (a) ciprofloxacin (CIP), gentamicin (GEN), and ciprofloxacin+gentamicin (b) ciprofloxacin, oxacillin (OXY), and ciprofloxacin+oxacillin (c) ciprofloxacin, vancomycin (VAN), and ciprofloxacin+vancomycin (d) gentamicin, oxacillin, and gentamicin+oxacillin (e) gentamicin, vancomycin, and gentamicin+vancomycin (f) oxacillin, vancomycin, and oxacillin+vancomycin.

3.3.3 Persistence: Hill functions provide good fits for the initial exponential phase of time-kill curves but not for the second phase during which the rate of killing declines and the viable cell population is dominated by persisters. In an effort to examine how two-drug therapy affects levels of persisters, we extended our analysis to the relationship between single and two-drug treatment regimens and the density of persisters present after exposure to the drugs. In Figures 3.4 and 3.5, we show persistence levels for drug combinations and the component single antibiotics of each combination. The average

CFU's and standard errors for ten independent replicate cultures of 2.5x, 5x and 10xCU treatments sampled at 6h for *E. coli* (Figure 3.4) and 22h for *S. aureus* (Figure 3.5) are shown.

For *E. coli*, similar densities of persisters were observed for ciprofloxacin and ampicillin used individually as well as in combination (Figure 3.4a). Tetracycline used on its own resulted in the highest level of persistence among all the antibiotics studied. When combined with ampicillin, the density of persisters observed was similar to that generated by tetracycline alone. This result occurred despite the observation that treating with the other antibiotic in the combination, ampicillin, led to a lower level of persistence compared to tetracycline (Figure 3.4b). Combining ciprofloxacin and tetracycline, however, led to lower levels of persistence than equivalent concentrations of tetracycline (Figure 3.4c). Among all the antibiotics, tobramycin was the most effective in reducing the level of persisters. We recovered persisters only at 2.5xCU in treatments with tobramycin. When combined with ciprofloxacin, the combination was more effective than ciprofloxacin used singly and just as effective as tobramycin alone (Figure 3.4d). Combining tobramycin with ampicillin (Figure 3.4e) and tetracycline (Figure 3.4f), on the other hand, decreased the efficacy of tobramycin.

In the *S. aureus* experiments, gentamicin and ciprofloxacin used singly resulted in lower levels of persistence than oxacillin and vancomycin (Figures 3.5a,f). Strikingly, cultures exposed to the presumptively cidal 2.5xCU of oxacillin had, by 22 hours, grown to the same densities as antibiotic-free controls (Figure 3.5b). This result can be attributed to a decline in the effective concentration of this drug, rather than mutations to resistance (Johnson and Levin, 2013). However, combinations of 1.25xMIC of oxacillin with

1.25xMIC of any of the other drugs exerted a cidal effect, and the cultures did not grow (Figures 3.5b,d,f). When gentamicin was present in the drug pair, for all combinations of two drugs the level of persistence was at least as low as when gentamicin was used alone (Figures 3.5a,d,e). Combinations involving ciprofloxacin generated densities of persisters either equivalent to that generated by ciprofloxacin alone (Figures 3.5a,b) or intermediate between those generated by the individual antibiotics (Figure 3.5c).

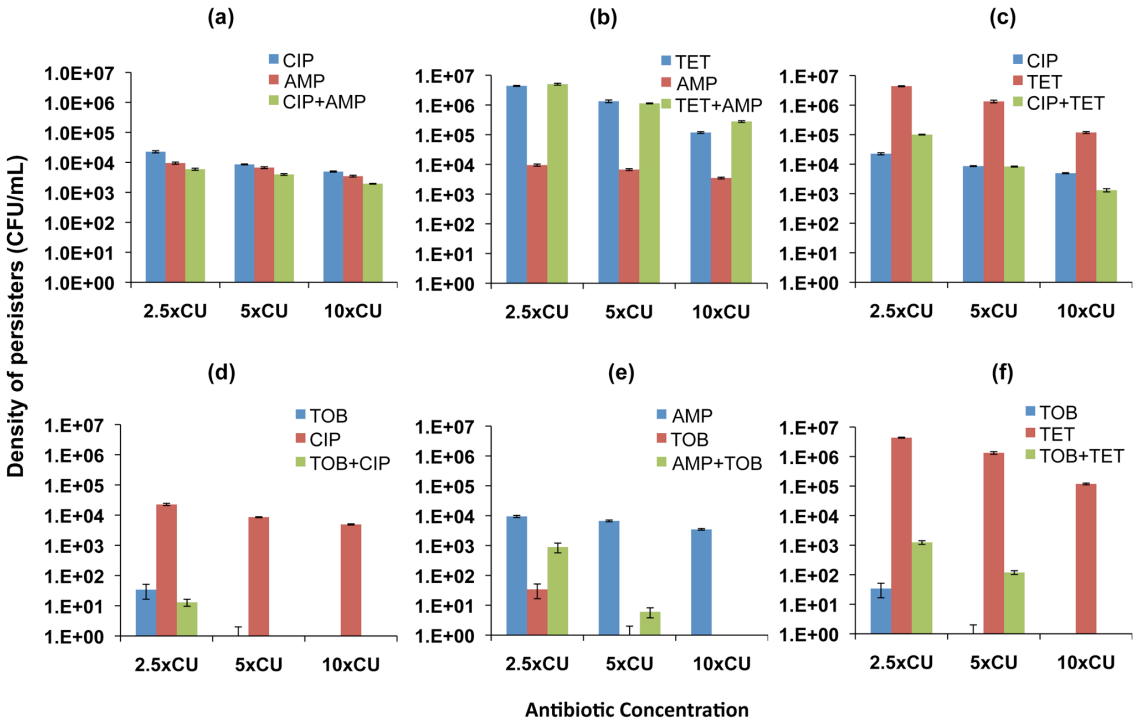

Figure 3.4 Density of persisters for two-drug combinations and the constituent individual antibiotics (*E. coli*). Viable cell densities of *E. coli* following 6 hours of exposure to equivalent cidal concentrations of single drugs and two-drug combinations (mean and standard error for 10 independent cultures shown). (a) ampicillin (AMP), ciprofloxacin (CIP), and ampicillin+ciprofloxacin (b) ampicillin, tetracycline (TET), and ampicillin+tetracycline (c) ciprofloxacin, tetracycline, and ciprofloxacin+tetracycline (d) ciprofloxacin, tobramycin (TOB), and ciprofloxacin+tobramycin (e) tobramycin, ampicillin, and tobramycin+ampicillin (f) tobramycin, tetracycline, and tobramycin+tetracycline.

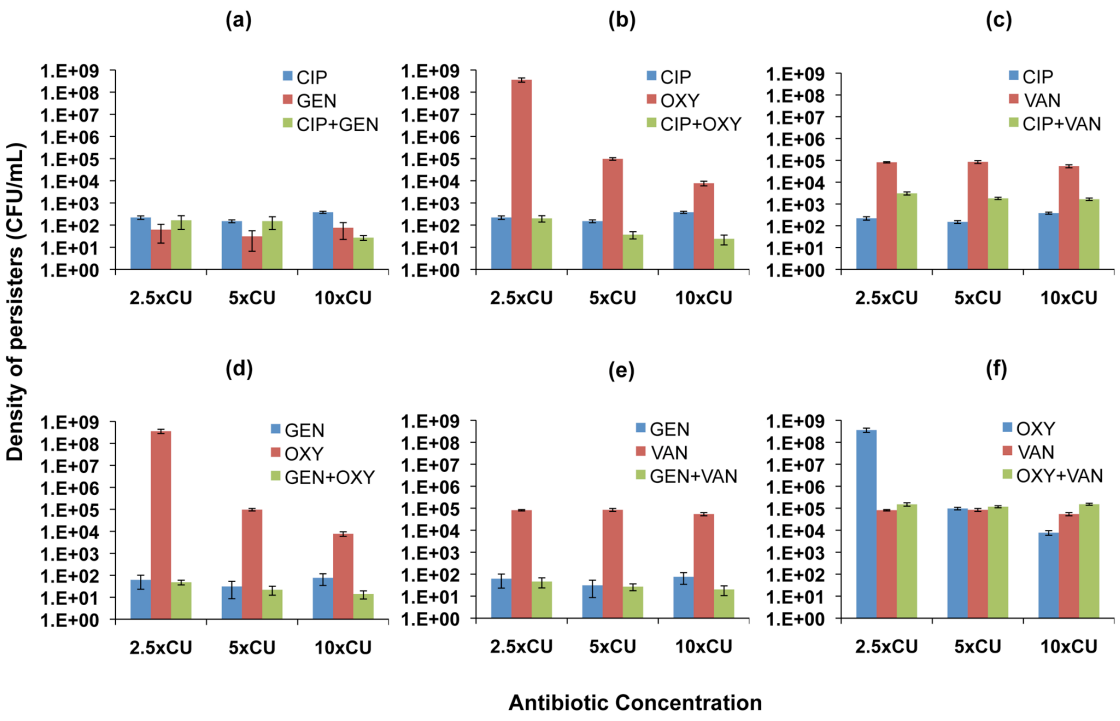

Figure 3.5 Density of persisters for two-drug combinations and the constituent individual antibiotics (*S. aureus*). Viable cell densities of *S. aureus* following 22 hours of exposure to equivalent cidal concentrations of single drugs and two-drug combinations (mean and standard error for 10 independent cultures shown). (a) ciprofloxacin (CIP), gentamicin (GEN), and ciprofloxacin+gentamicin (b) ciprofloxacin, oxacillin (OXY), and ciprofloxacin+oxacillin (c) ciprofloxacin, vancomycin (VAN), and ciprofloxacin+vancomycin (d) gentamicin, oxacillin, and gentamicin+oxacillin (e) gentamicin, vancomycin, and gentamicin+vancomycin (f) oxacillin, vancomycin, and oxacillin+vancomycin.

3.3.4 Potential Clinical Implications: What are the implications of the preceding pharmacodynamic results for the design and evaluation of antibiotic treatment regimens and the emergence of antibiotic resistance? To begin to address these questions we use a simple mathematical model of the within-host pharmacokinetics, population and evolutionary dynamics of bacteria undergoing multi-drug therapy.

3.3.4.1 The Model: The model used here is a variant of that used in (Levin and Udekwu, 2010). It considers two antibiotics with concentrations and designations, A and B, and

two subpopulations of bacteria; one that is actively replicating and one that is not (the persisters), with densities and designations, S and P, respectively. Bacteria can be of one of four different genotypic resistance profiles: they can be susceptible to the action of both antibiotics, susceptible only to A or B and resistant to the other, or resistant to both. Note though, that any bacterium in a persister state exhibits a phenotypic refractoriness to antibiotic action regardless of its genotypic resistance profile.

Persisters are generated from S cells in a stochastic manner which we simulate via the following Monte Carlo procedure: the maximal rate of persister production is set at f per cell per hr, and if f*S*Δt is greater than the value of a rectangularly-distributed random number between 0 and 1, then one individual is lost from the S population and one gained by the P population. The step size of an Euler simulation, Δt, is chosen so that the probability of generating a persister is less than 1. The transition from persisters back to growing cells is simulated in a similar fashion, with a maximal rate of g per cell per hour, where $g<f$. Single- and two-drug resistant bacteria are also generated via a similar Monte Carlo procedure, with maximal rates of mutant production μ_A and μ_B, representing mutation rates to resistance for antibiotics A and B respectively.

We represent the pharmacodynamics of both single and combined antibiotic action (i.e. treating with Antibiotic A, B, or both) with a Hill function, as per the preceding experimental analyses. For pharmacokinetics, we assume regular antibiotic input of A_{max} and B_{max} µg/mL every T hours. The effective concentration of these drugs decline at rates d_A and d_B µg/mL per hour. Net bacterial growth depends on the efficacy of antibiotic cidal action as well as on the availability of a limiting resource of

concentration R µg/mL. We assume a continuous flow of this resource from a reservoir where it is maintained at a concentration C µg/mL. This resource enters the host at a rate w per mL per hour, which is the same rate at which antibiotics, bacteria, resources and wastes are washed out. The rate of bacterial replication is a monotonically increasing function of R with a half-saturation coefficient of k_m µg/mL (Monod, 1949). Conversion of resources into bacterial cells occurs at a conversion efficiency of e µg/cell. For the numerical analysis of the properties of this model, computer simulations, we use Berkeley Madonna™. Copies of the program can be obtained from www.eclf.net/programs.

The standard values and/or ranges of the parameters and variables considered in our numerical analysis of the properties of this model are presented in Table 3.1. We note here that this simple mathematical model is not intended as a quantitatively precise analogue of a specific disease and treatment process but rather to provide a schema for assessing the potential clinical implications of our *in vitro* pharmacodynamic results. Whenever possible, the parameter values used are in the range of those estimated from the experimental analyses. Parameters not specific to this study are within the range of those used in other pharmacodynamic and pharmacokinetic studies of antimicrobial therapy (Levin and Udekwu, 2010; Regoes et al., 2004; Wiuff et al., 2005).

Table 3.1 Values and ranges for variables and parameters used for generating numerical solutions.

Variable/Parameter	Description	Value or range considered*
Variables		
A, B	Antibiotic concentration (μg/mL)	0 – 10
S_X	Density of planktonic bacteria sensitive to both antibiotics, x=0; resistant to A, x=RA; resistant to B, x=RB; and resistant to A and B, x=RAB (cells per mL)	$1\text{-}10^{10}$
P_X	Density of persisters sensitive to both antibiotics, x=0; resistant to A, x=RA; resistant to B, x=RB; and resistant to A and B, x=RAB (cells per mL)	$1\text{-}10^{10}$
R	Concentration of the limiting resource (μg/mL)	0-1000
Parameters		
ψ_{max}	Maximum hourly growth rate of replicating bacteria	(1.5)
ψ_{miny}	Maximum hourly death rate of antibiotic y, where y=A, B and AB (A+B)	-1 – -15
MIC_y	Minimum Inhibitory Concentration of antibiotic y, where y=A, B and AB (A+B) (μg/mL)	(1)
κ_y	Hill coefficient of antibiotic y, where y=A, B and AB (A+B)	(1)
w	Hourly washout rate	(0.2)
f	Hourly rate at which S is converted into P	10^{-2} or 10^{-5}
g	Hourly rate at which P is converted into S	10^{-3} or 10^{-6}
C	Reservoir resource concentration (μg/mL)	(1000)
e	Efficiency of resource conversion into cells (μg/cell)	$(5\text{x}10^{-7})$
k_m	Concentration of resource at half maximal growth (μg/mL)	(0.25)
A_{max}, B_{max}	Antibiotic concentration added at each dosing period (μg/mL)	(5)
d_A, d_b	Antibiotic decay rate (h^{-1})	(0.1)
T	Time between doses (h)	(12)
μ_A, μ_B	Mutation rate (mutations per cell division)	10^{-8}

* Values in parentheses are the standard values used for numerical analysis of the model.

3.3.4.2 Single and multi-drug therapy and the contribution of persistence levels: We open this consideration with sample simulations involving single- (Figure 3.6a) and dual-

drug therapy (Figure 3.6b) to explore the contributions of persistence to the term of therapy and the emergence of resistance. Figure 3.6a shows that with single-drug therapy, when mutants resistant to the treating drug are present they ascend to high levels and generate concomitant levels of resistant persisters. Since resistance to the second drug is generated by mutation, the large numbers of bacteria resistant to the treating drug can allow for the generation of a minority population of bacteria resistant to both drugs. With two-drug therapy the bacteria resistant to single drugs will be eradicated due to their susceptibility to the other antibiotic (Figure 3.6b). Populations of these single-drug resistant bacteria do not grow to high enough densities to generate persister populations that can influence the clearance dynamics.

We explore the combined roles of exponential-phase cidal dynamics and persistence with a consideration of two extreme cases: (i) a worst case scenario in which the two antibiotics interact suppressively and also lead to a high level of persistence (Figure 3.6c) and (ii) the best case scenario of synergistic antibiotics that lead to a low level of persistence (Figure 3.6d). We differentiate between the types of drug interaction by using different values for the maximal death rate that drug combinations engender. To account for the observation that different combinations of drugs generate different levels of persistence, we modulate the persister generation and loss parameters, f and g, such that increased efficacy for drug combinations in terms of reducing the level of persistence leads to lower values of these parameters. Values of the conversion parameters are chosen such that densities of persisters are in the range of those we observed in our experimental results. To address the fact that most infections are only treated when the number of bacteria is sufficiently great to cause symptoms, and that resistance can be

acquired by mutation or horizontal gene/genetic element transfer from the existing flora, in our simulations we assume that at the onset of treatment there are already minority populations of cells resistant to each antibiotic (Drlica, 2003). We also assume that there is a minority population of persister cells present prior to the initiation of therapy.

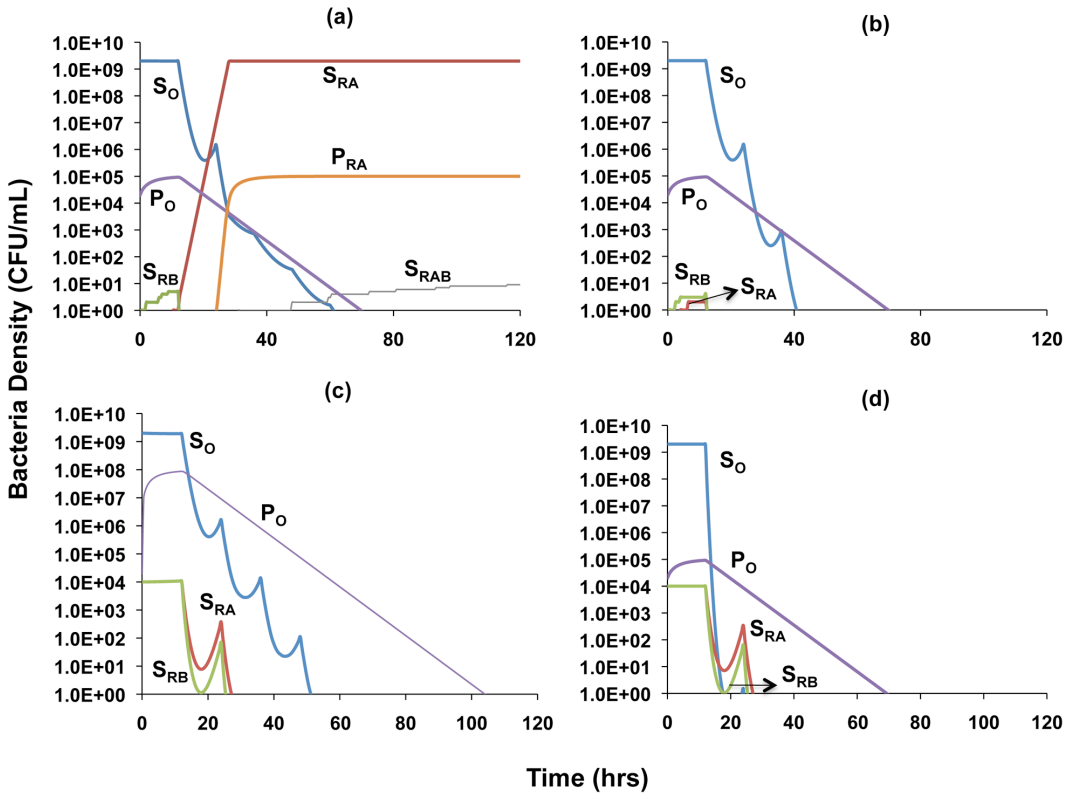

Figure 3.6 Simulation of the population dynamics of actively replicating and persister bacteria under antibiotic treatment. Unless otherwise noted, parameter values used for the simulations are the standard values in Table 1. (a) Clearance dynamics under single antibiotic treatment, assuming low level persistence (A_{max} = 0, B_{max}=10, f=10^{-5}, g=10^{-6}, ψ_{minA}=0, ψ_{minB}=-5) (b) Clearance dynamics under dual antibiotic treatment, assuming additive drug interactions and low level persistence (f=10^{-5}, g=10^{-6}, ψ_{minA}=-5, ψ_{minB}=-5, ψ_{minAB}=-5) (c) Clearance dynamics under dual antibiotic treatment, assuming suppressive drug interactions and high level persistence (f=10^{-2}, g=10^{-3}, ψ_{minA}=-10, ψ_{minB}=-5, ψ_{minAB}=-2) (d) Clearance dynamics under dual antibiotic treatment, assuming synergistic interactions and low level persistence (f=10^{-5}, g=10^{-6}, ψ_{minA}=-10, ψ_{minB}=-5, ψ_{minAB}=-15).

As can be seen by comparing Figures 3.6c and 3.6d, synergistic interactions between antibiotics and a low level of persistence serve to decrease the time to clearance of the infection. Evidenced by the similarities in the decline slopes of the P populations in Figures 3.6c and 3.6d, it is worth noting that the rate of clearance of the persister population with synergistic antibiotics is similar to that with suppressive drugs. However, the synergistic antibiotics are able to eradicate the persister population more rapidly by more efficiently reducing the numbers of the sensitive population that replenishes lost persister cells. Mutants simultaneously resistant to both drugs do not arise because the number of cells in the populations resistant to single drugs and their persisters remain too low to generate doubly resistant mutants.

3.3.4.3 The contribution of a spatial refuge: The above situation, where the entire population is exposed to the same level of the antibiotic is an idealized one that may be met in flasks, but is unlikely in patients. For many infections, perhaps the majority, antibiotics will not have complete access to the infecting population of bacteria. Some bacteria may be in abscesses, empyema or embedded as non/slowly-dividing cells in biofilms (Davies, 2003; Wagner et al., 2006). To account for this, we extend the model to allow for another population of bacteria, B, which occupy a spatial refuge and are thereby less responsive to the antibiotics than the planktonic population. Bacteria in this subpopulation are generated deterministically from both S and P cells at a rate of f_b per hour and return to the actively replicating population at a rate of g_b per hour. We assume that bacterial growth rate is decreased in the refuge and that bacterial susceptibility to antibiotics is proportional to their growth rate (Brown et al., 1988). As such, the decrease in maximal growth in the refuge population (ψ_{maxb}) is paralleled by an equivalent

quantitative increase in the MIC of antibiotics in that compartment. Resources enter this refuge and the bacteria within are washed out at rate w_b per hour ($w_b<w$). We show a schematic of this two-compartment model in Figure 3.7. The complete set of equations is available in Text 3.A1 (in Appendix).

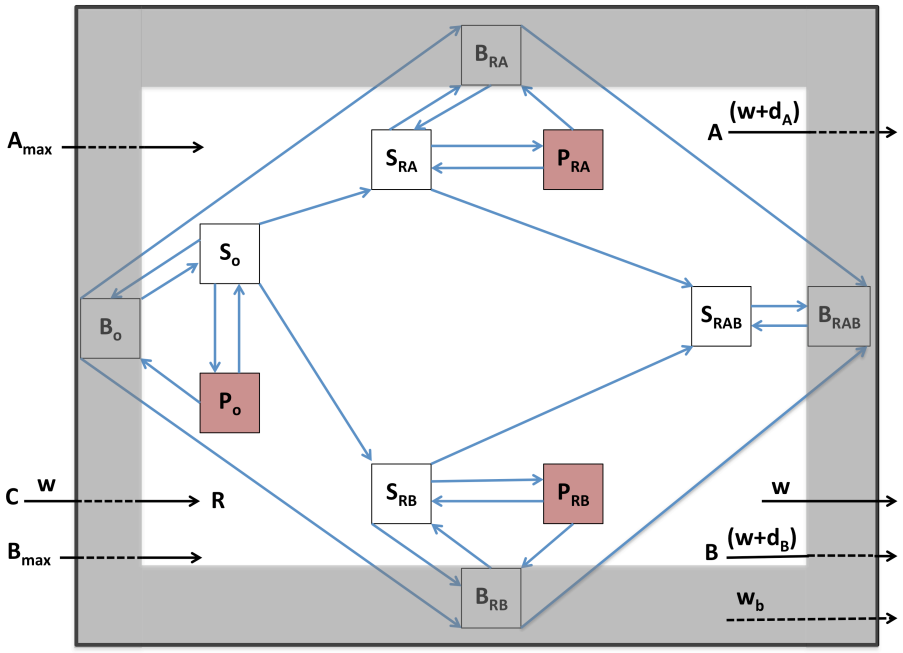

Figure 3.7 Schematic diagram of the population and evolutionary dynamic model of two-drug therapy. S_x, actively-growing bacteria; P_x, persisters; B_x, bacteria in spatial refuge; x=O, sensitive to both antibiotics; x=RA, resistant to antibiotic A; x=RB, resistant to antibiotic B; x=RAB, resistant to both antibiotics. C, resource reservoir; R, internal concentration of resource; A_{max} and B_{max}, concentration of antibiotic periodically added; A and B, internal concentration of antibiotics, d_A and d_B, antibiotic decay rates; w, flow rate, main compartment; w_b, flow rate, spatial refuge.

We consider the role of the refuge with simulation runs using the same parameters and initial conditions as in the single compartment simulation, Figures 3.6c and 3.6d, but now allow bacteria to migrate to a refuge at the same rates at which persisters are formed.

Contrary to the results shown in Figure 3.6, the infections are not cleared, and susceptible bacteria in both the refuge and the planktonic compartment oscillate around constant densities (Figures 3.8a and 3.8b). This result obtains because for both physiological (decreased replication rate) and spatial (reduced antibiotic access) reasons, bacteria in the refuge are more refractory to antibiotics than a more transient planktonic persister subpopulation which continually reverts to a rapidly growing state. It should be noted though, that the infections can be cleared by either increasing antibiotic dose or decreasing the rate of migration of cells into the refuge (Figure 3A.5, in Appendix).

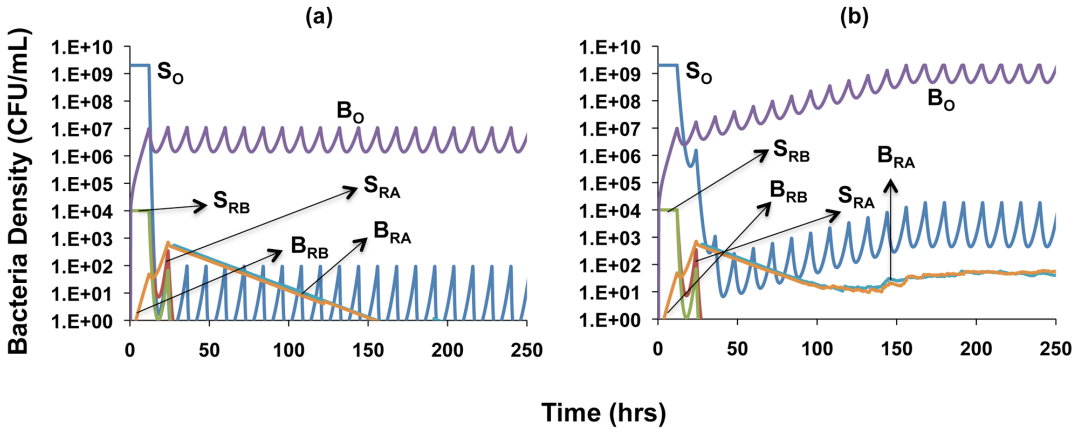

Figure 3.8 Simulation of the population dynamics of actively replicating and spatial refuge bacteria under antibiotic treatment. Unless otherwise noted, parameter values used for the simulations are the standard values in Table 1. For subpopulations in the spatial refuge, ψ_{maxb}=0.5, w_b=0.05, f_b =10^{-5}, g_b =10^{-6}, MIC_A=3, MIC_B=3, MIC_{AB}=3. (a) Clearance dynamics under dual antibiotic treatment, assuming synergistic drug interactions (ψ_{minA}=-10, ψ_{minB}=-5, ψ_{minAB}=-15) (b) Clearance dynamics under dual antibiotic treatment, assuming suppressive drug interactions (ψ_{minA}=-10, ψ_{minB}=-5, ψ_{minAB}=-2).

A comparison of Figures 3.8a and 3.8b shows an effect of the type of interaction between antibiotics. The susceptible cells are maintained at a lower density when the drug interaction is synergistic (Figure 3.8a) than when it is suppressive (Figure 3.8b).

Also, while the single-drug resistant mutants are eliminated under synergistic interactions (Figure 3.8a), they are maintained when the interaction is suppressive (Figure 3.8b). Under the latter conditions, the population of susceptible cells is maintained at a high enough density to continually generate single-drug resistant mutants. However, since the single-drug resistant bacteria remain susceptible to the activity of the other drug, we do not record any instances of dual-drug resistance in these simulations regardless of whether interactions are synergistic or suppressive.

3.4 DISCUSSION

The rational design of multi-drug antibiotic therapy requires information about the pharmacodynamics of the component drugs individually and in combination as well as how those drugs will affect the population and evolutionary dynamics of the target bacteria. In this study, we use *in vitro* pharmacodynamic experiments with *E. coli* and *S. aureus* to explore the pharmacodynamics of single and pairs of antibiotics of different classes. Using mathematical models and computer simulations, we explore how the observed pharmacodynamics will affect the microbiological course of therapy and evolution of resistance. Here we briefly summarize these theoretical and experimental results and discuss their potential implications for multi-drug therapy.

3.4.1 Pharmacodynamics: We use Hill functions to characterize the relationship between the concentrations of single and pairs of drugs and the rates of kill of the target bacteria during the initial, exponential, phase of exposure. The concentrations of both single and pairs of drugs are expressed as single variables, multiples of cidal units. These cidal units are, for single drugs, equivalent to multiples of Clinical and Laboratory

Standards Institute (CLSI) (CLSI, 2005) estimates of their MICs; for pairs of drugs, they are sums of equipotent concentrations of the two drugs (equal multiples of their respective CLSI MICs). This formulation allows a comparison of the cidal/inhibitory activities of drugs in combination with those of their component single drugs at equivalent concentrations. Using this method we characterize and quantify the form of the interaction between pairs of drugs, synergy, antagonism, suppression or additivity.

Our experimental results illustrate the necessity of comprehensive empirical PD assessments for drug combinations rather than attempting to predict their interactions *a priori* or based on single interaction parameters. In experiments with *E. coli*, drug combinations exhibited concentration-dependent synergy, antagonism and suppression in ways that, for most combinations, could not have been predicted from current understanding of the mechanisms of drug action. For example, it is generally assumed and seemingly reasonable to anticipate that when mixed with drugs that are bacteriostatic, like chloramphenicol, antibiotics that require cell division for their action, like the beta lactams, will not be as effective in killing bacteria than when they are alone (Jawetz and Gunnison, 1952). Unfortunately, the classification of antibiotics as bactericidal or bacteriostatic is not as clear in practice as is often alluded to (Pankey and Sabath, 2004). For example, in our *E. coli* experiments, tetracycline, which is often classified as bacteriostatic (Lorian, 2005), was clearly bactericidal at higher concentrations, more so than ampicillin, which is a member of the presumptively bactericidal beta-lactam family of drugs. The combination of tetracycline and ampicillin was more effective in killing bacteria than ampicillin alone, albeit less so than tetracycline on its own. On the other

hand, combinations of tetracycline with ciprofloxacin or tobramycin were less effective than either of these drugs alone.

For *S. aureus* we only observed antagonistic and suppressive interactions for all six pairs of drugs considered. With two exceptions, the efficacy of the combinations of drugs was intermediate between that of the most and least bactericidal. The exceptions are noteworthy; vancomycin in combination with either ciprofloxacin or oxacillin exhibited suppressive interactions. Most dramatically, the combination of vancomycin and oxacillin had virtually no bactericidal activity. This is a good illustration of the point we made earlier, that based on the PD of these single drugs we could not have predicted how they would interact in combination.

It is clear from single drug studies that the level of persistence depends on the antibiotics and their concentrations (Johnson and Levin, 2013). While the present experiments support this interpretation, they are also consistent with the proposition that there is no way to predict how two drugs will interact to determine the level of persistence. What is clear from our results is that the density of persisters with two-drug combinations will be no greater than that of the single drugs alone. For most combinations, the density of persisters was intermediate between that of the two antibiotics or at a level similar to that observed for the component drug that generated a lower level of persistence. This suggests that the component antibiotics determine the lower and upper limits for the density of persisters when drugs are combined. Interestingly, there is limited correlation between the pharmacodynamic efficacy of combinations in the exponential, cidal, phase of the encounter between the bacteria and

drugs and the level of persistence. As suggested earlier for the kill phase of the pharmacodynamics, the physiological and molecular reasons for this are unclear.

3.4.2 Population and evolutionary dynamics and potential implications for treatment: Our mathematical and computer simulation model of the pharmaco-, population and evolutionary dynamics of bacteria undergoing dual drug therapy illustrates how the interactions between drugs affect the microbiological course of treatment. Drug combinations that exhibit suppressive interactions in either the rate of kill and/or level of persistence will require more time to clear an infection than synergistic drugs. From the perspective of treatment, persistence is a refuge from the cidal action of the antibiotics. If that refuge is small, i.e. the persistence level is low, it will have little effect on the rate of clearance. On the other hand, a high level of persistence serves as a substantial refuge that continually re-seeds the treated population and lengthens the term of therapy. Our analysis suggests that in general, while persisters may retard the rate at which bacteria are cleared, they are unlikely to prevent clearance. This, however, should not be interpreted to suggest that persistence cannot lead to treatment failure, since the magnitude of morbidity and the probability of mortality increases with the term of the infection. Lengthier treatment durations can also increase the likelihood of patient non-adherence and thus increase the probability of exposure to sub-therapeutic concentrations of antibiotics. Recent work by two of the authors (PJTJ and BRL) suggests that these sub-MIC concentrations can enrich bacterial populations for existing persisters and also promote the generation of persisters and thereby increase their density in treated populations (Johnson and Levin, 2013). Most importantly, there is evidence from clinical studies that supports the proposition that in addition to delaying

clearance, persistence may also lead to treatment failure (Chao and Rubin, 2010; Lafleur et al., 2010; Lewis, 2010; Mulcahy et al., 2010).

In addition to subpopulations of bacteria that are physiologically refractory because they are not growing or growing slowly, there are also subpopulations that, for spatial or other reasons, are less accessible to antibiotics than the dominant population. In our simulations we show that the presence of these refugia can prevent clearance by treatment regimens that lead to clearance in their absence. This has in fact been observed for chronic infections with physically-structured subpopulations of bacteria, such as endocarditis and osteomyelitis, and also for catheter and other foreign-body associated infections (Costerton et al., 1999). As with persistence, our models indicate that treatment with synergistic combinations of drugs can improve the microbiological course of treatment, i.e. reduce the densities of bacteria in chronic infections relative to suppressive combinations.

A traditional reason for using multiple, rather than single, antibiotics is to prevent the ascent of bacteria resistant to single antibiotics. The results of our simulations support this interpretation of the evolutionary utility of two-drug therapy. Although in our simulations mutants resistant to single drugs were initially present at low frequencies, these cells were either cleared or remained minority populations. Further, with the parameters employed, two-drug resistance never emerged. The reason for the latter is that the populations of single-drug resistant bacteria and their corresponding persister and refuge subpopulations remained in check by the drug to which they were susceptible. They did not grow to high enough numbers to generate multi-drug resistance via mutation. This evolutionary benefit of two-drug therapy obtained even when the drugs

suppressed each other's activity. Indeed, there exists some experimental evidence to suggest that antagonistic and suppressive drug combinations may be even more efficient than synergistic combinations in preventing evolution of multi-drug resistance (Hegreness et al., 2008). When interactions are synergistic, evolution of resistance to one of the drugs aborts the enhancing effect of the other, whereas with antagonistic interactions single-drug resistance removes the suppressive effect on the drug to which those mutants are susceptible (Chait et al., 2007; Hegreness et al., 2008).

Of note though; while in the absence of refugia two-drug therapy can lead to the clearance of minority populations of single-drug resistant bacteria, this need not be the case when there are refugia. As a consequence of these refugia, the number of bacteria sensitive to both antibiotics can remain sufficiently large to continually seed the population with mutants resistant to single drugs. Whether or not this will occur depends on the nature of the two-drug interactions. Suppressive drugs, because they lead to greater densities of susceptible cells, are more likely to allow for the continuous repopulation of single–drug resistance by mutation.

3.4.3 Caveats and Limitations: At best, *in vitro* pharmaco- and population dynamics experiments and mathematical modeling and simulation studies of the sort presented here can only provide a rational and necessarily quantitative base for the design of antibiotic treatment protocols. The within-host model we use here, for instance, does not explicitly consider the contribution of the innate or adaptive immune systems to clearance. Ultimately the evaluation of these protocols has to be made in treated animals where the immune system contributes to the clearance of the infection and, alas, the pathology (Margolis and Levin, 2008).

The approach we have used in both the experimental and modeling elements of this study have been phenomenological, they do not incorporate or address the physiology and molecular mechanisms of action of single antibiotics or interactions between antibiotics in inhibiting the growth and killing their target bacteria. We justify this approach in two ways: First from the practical perspective of antibiotic treatment, the phenomenology considered, the relationship between the concentrations of single and multiple antibiotics in inhibiting the growth and killing the bacteria is more important than an understanding of the mechanisms responsible. Second, despite all that is known about the targets of antibiotic action and how they are related to the molecular structure of these compounds, we still know relatively little about how antibiotics inhibit the growth of and kill bacteria, see for example (Kohanski et al., 2007). Similarly, in our consideration of persisters we assume that these bacteria are generated stochastically, and do not explicitly account for deterministic mechanisms such as stress responses (Vega et al., 2012; Wu et al., 2012b) that can also contribute to persister generation. This approach has the virtue of simplifying the model while still maintaining its quantitative integrity, since the levels of persisters generated in the simulations are equivalent to those observed experimentally.

For convenience and tractability, in our model we treated susceptibility and resistance as discrete states with different pharmacodynamic properties. In reality bacterial susceptibility and resistance to antibiotics is a continuum that depends not only on the specific target of the drug, but also the rates at which cells take up and remove these compounds, e.g. via efflux pumps. In some cases, single mutations in regulatory loci or efflux systems can simultaneously reduce the susceptibility of bacteria to multiple

antibiotics (Howden et al., 2011; Ziha-Zarifi et al., 1999). Multi-drug resistance may also be acquired in a single step by the horizontal transfer of genes or accessory genetic elements from the resident flora (Martinez and Baquero, 2002; Martinez-Suarez et al., 1987). Another noteworthy caveat is that for some infections, bacterial population sizes may well exceed the numbers we have considered here, thereby increasing the likelihood that mutants resistant to two antibiotics will be generated. As intriguing as they may be, a formal consideration of these realities is beyond the scope of this study.

3.5 APPENDIX

The following appendix contains supplementary information for the above investigation. All the figures, tables and protocols that follow have been pre-referenced in the text of the chapter.

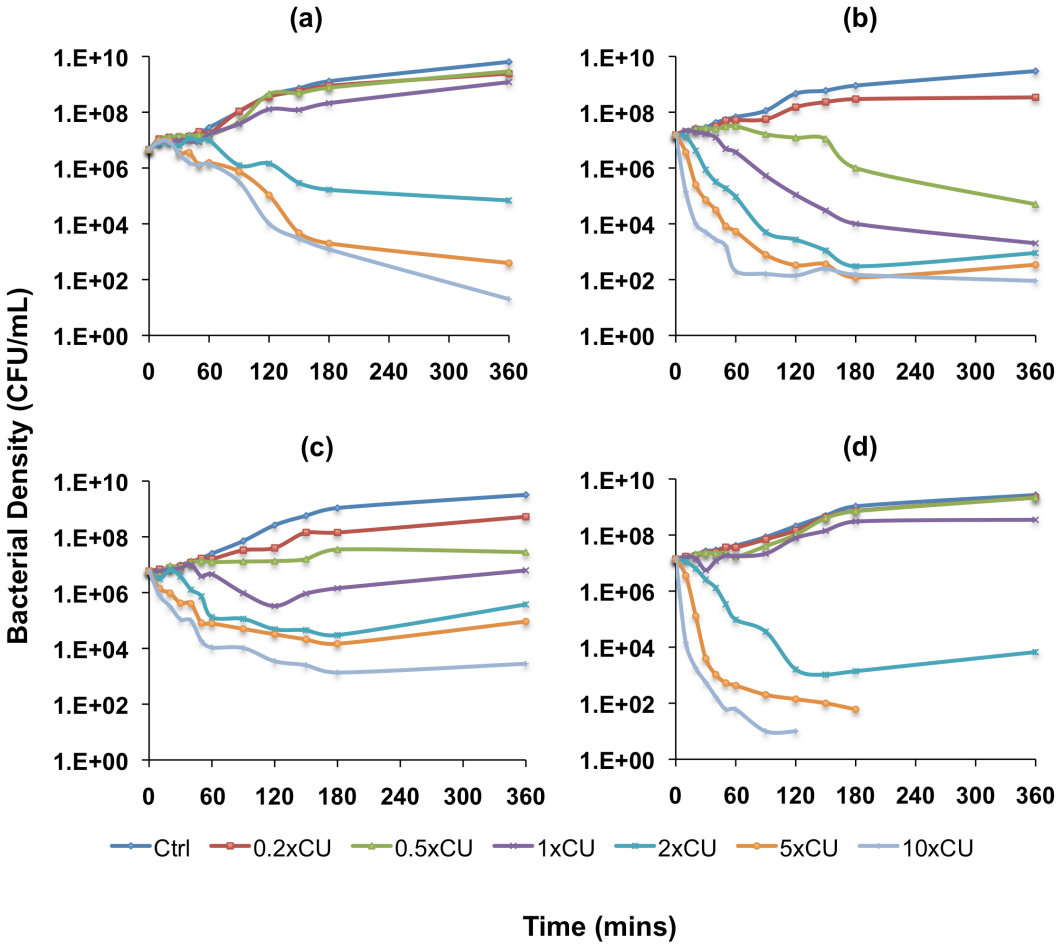

Figure 3A.1 Time-kill curves of *E. coli* CAB1 exposed to single antibiotics. Changes in viable cell density for cultures treated with varying concentrations (0.2xCU, 0.5xCU, 1xCU, 2xCU, 5xCU and 10xCU). Each multiple of cidal unit (xCU) is equivalent to the corresponding multiple of MIC (xMIC). (a) ampicillin (b) ciprofloxacin (c) tetracycline (d) tobramycin.

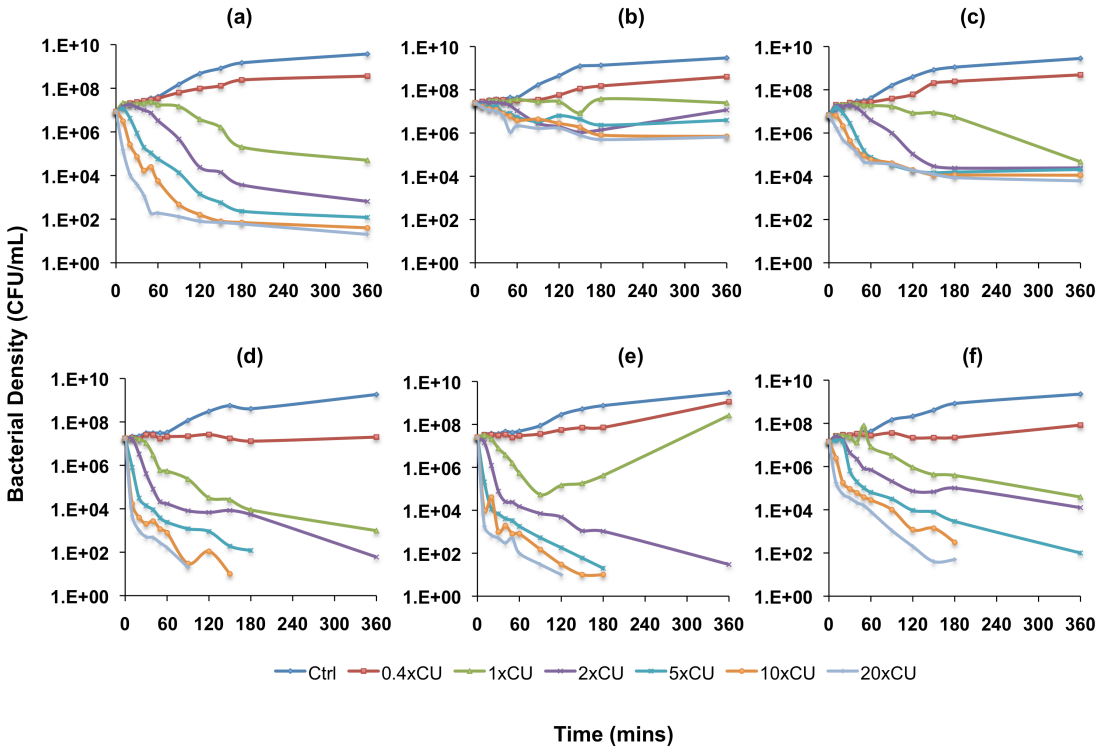

Figure 3A.2 Time-kill curves of *E. coli* CAB1 exposed to pairs of antibiotics. Changes in viable cell density for cultures treated with varying concentrations (0.4xCU, 1xCU, 2xCU, 5xCU, 10xCU and 20xCU) of each antibiotic pair. Each multiple of cidal unit (xCU) is equivalent to the sum of equal multiples of MIC (xMIC) of each drug, e.g. 1xCU is the combination of 0.5xMIC of each antibiotic. (a) ampicillin + ciprofloxacin (b) ampicillin + tetracycline (c) ciprofloxacin + tetracycline (d) ciprofloxacin + tobramycin (e) ampicillin + tobramycin (f) tetracycline + tobramycin.

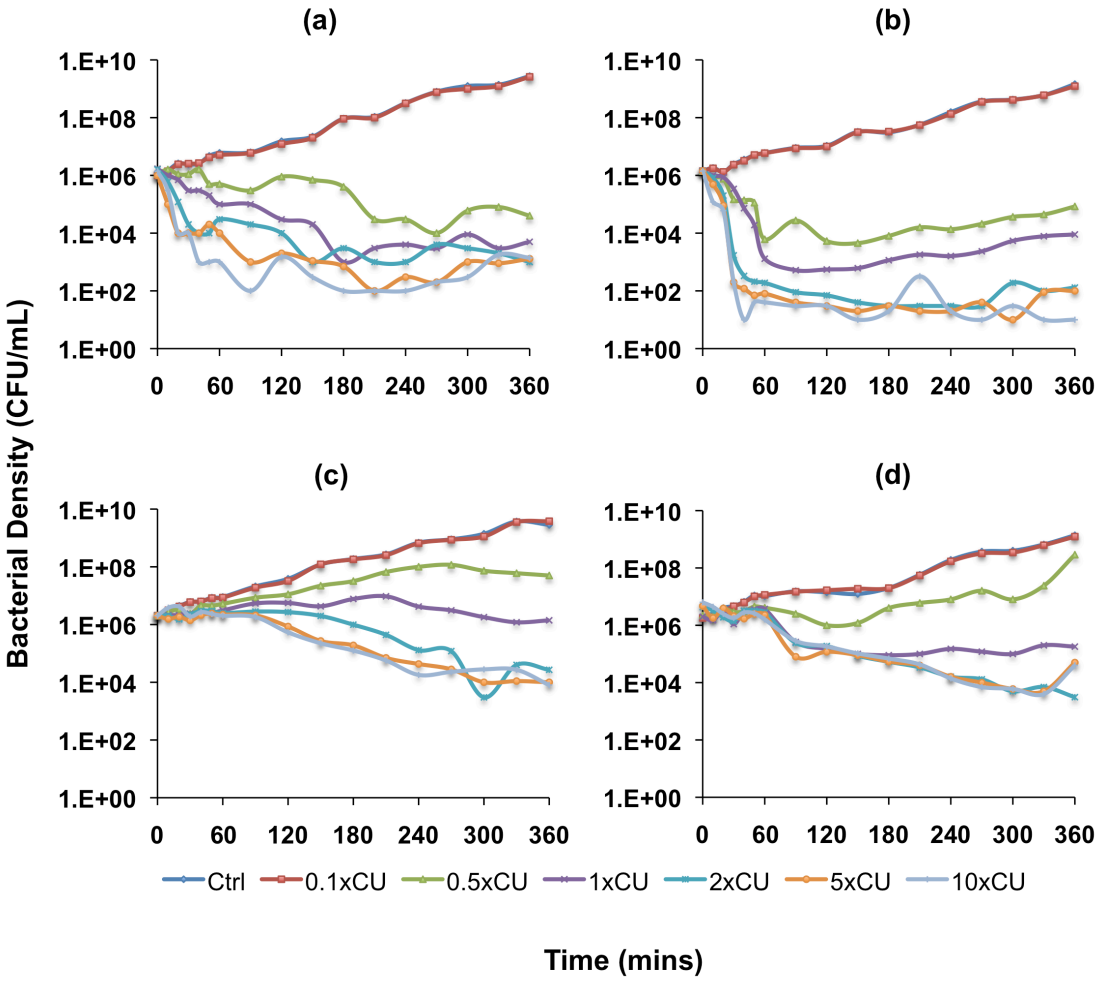

Figure 3A.3 Time-kill curves of *S. aureus* Newman exposed to single antibiotics. Changes in viable cell density for cultures treated with varying concentrations (0.1xCU, 0.5xCU, 1xCU, 2xCU, 5xCU and 10xCU) of each antibiotic. Each multiple of cidal unit (xCU) is equivalent to the corresponding multiple of MIC (xMIC). (a) ciprofloxacin (b) gentamicin (c) oxacillin (d) vancomycin.

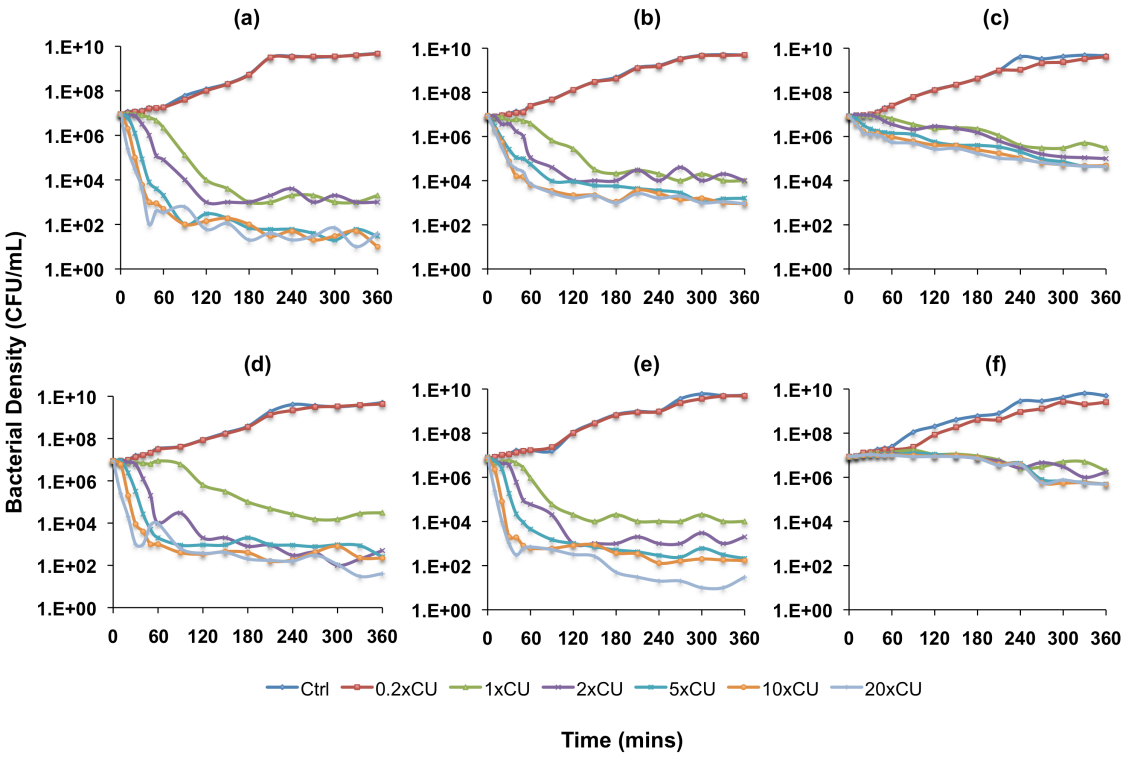

Figure 3A.4 Time-kill curves of *S. aureus* Newman exposed to pairs of antibiotics. Changes in viable cell density for cultures treated with varying concentrations (0.2xCU, 1xCU, 2xCU, 5xCU, 10xCU and 20xCU) of each antibiotic pair. Each multiple of cidal unit (xCU) is equivalent to the sum of equal multiples of MIC (xMIC) of each drug, e.g. 1xCU is the combination of 0.5xMIC of each antibiotic. (a) gentamicin + ciprofloxacin (b) ciprofloxacin + oxacillin (c) ciprofloxacin + vancomycin (d) gentamicin + oxacillin (e) gentamicin + vancomycin (f) oxacillin + vancomycin.

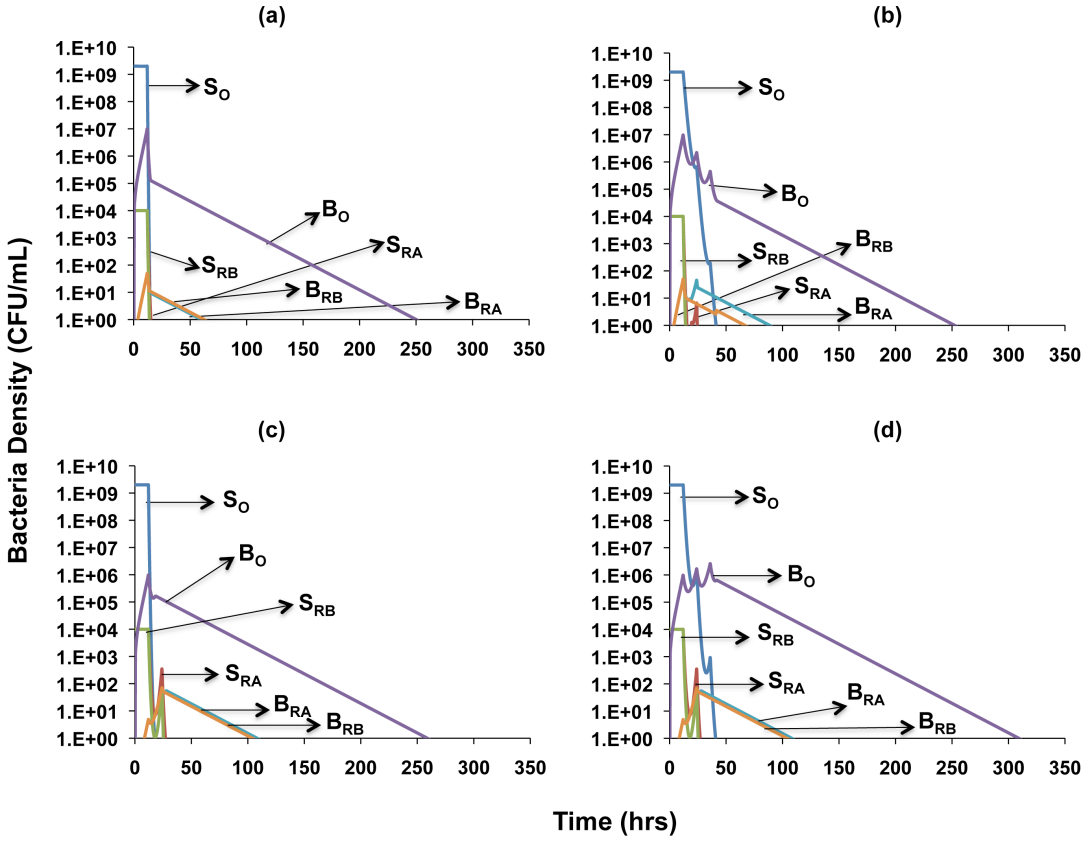

Figure 3A.5 Effects of increasing dose and decreasing rates of migration into spatial refuge on clearance dynamics. Unless otherwise noted, parameter values are the same as those used for corresponding simulations shown in Figure 5. (a) Clearance dynamics with a higher dose of antibiotics, assuming synergistic interactions (Amax=10, Bmax=10) (b) Clearance dynamics with a higher dose of antibiotics, assuming suppressive interactions (A_{max}=10, B_{max}=10 (c) Clearance dynamics with a lower rate of migration of cells into the spatial refuge assuming synergistic interactions (f_b=10^{-6}, g_b=10^{-7}) (d) Clearance dynamics with a lower rate of migration of cells into the spatial refuge assuming suppressive interactions (f_b=10^{-6}, g_b=10^{-7})

Table 3A.1 Pharmacodynamic function parameter estimates and standard errors for *E. coli* experiments.

Antibiotic(s)	ψ_{max} (h^{-1})	ψ_{min} (h^{-1})	κ	MIC or rMIC
Ciprofloxacin	1.59±0.20	-15.7±1.06	1.42±0.14	0.70±0.06
Ampicillin	1.57±0.15	-1.16±0.30	4.53±1.99	3.47±0.45
Tetracycline	1.30±0.15	-8.32±0.76	1.46±0.19	0.92±0.08
Tobramycin	1.08±0.35	-16.6±1.08	2.67±0.48	1.20±0.19
Ciprofloxacin + Ampicillin	1.98±0.48	-18.0±4.21	1.29±0.30	1.68±0.30
Ciprofloxacin + Tetracycline	1.91±0.15	-5.69±0.22	2.56±0.29	2.22±0.13
Ciprofloxacin + Tobramycin	1.43±0.69	-15.2±1.12	1.30±0.25	0.34±0.10
Ampicillin + Tetracycline	1.24±0.23	-5.96±4.72	0.61±0.24	1.15±0.25
Ampicillin + Tobramycin	1.41±0.85	-15.1±0.99	1.58±0.37	0.38±0.12
Tetracycline + Tobramycin	1.48±0.55	-9.68±1.17	1.45±0.38	0.92±0.21

Table 3A.2 Pharmacodynamic function parameter estimates and standard errors for *S. aureus* experiments.

Antibiotic(s)	ψ_{max} (h^{-1})	ψ_{min} (h^{-1})	κ	MIC or rMIC
Ciprofloxacin	1.53±0.27	-3.01±0.26	1.55±0.48	0.34±0.07
Gentamicin	1.28±0.26	-4.06±0.23	1.62±0.41	0.23±0.05
Oxacillin	1.51±0.04	-1.04±0.08	1.42±0.14	1.56±0.08
Vancomycin	1.07±0.02	-1.53±0.01	2.43±0.08	0.56±0.007
Ciprofloxacin + Gentamicin	1.66±0.47	-4.26±0.33	1.74±0.63	0.42±0.13
Ciprofloxacin + Oxacillin	1.50±0.27	-2.88±0.18	2.05±0.65	0.49±0.11
Ciprofloxacin + Vancomycin	1.46±0.21	-1.23±0.14	1.82±0.59	0.70±0.13
Gentamicin + Oxacillin	1.41±0.19	-3.67±0.15	2.33±0.61	0.62±0.10
Gentamicin + Vancomycin	1.50±0.34	-3.85±0.25	1.86±0.62	0.47±0.12
Oxacillin + Vancomycin	1.41±0.03	-0.095±0.02	3.00±0.50	0.81±0.17

Text 3A.1 Differential equations used for simulation of the two-compartment mathematical model.

$$\frac{dS_O}{dt} = S_O(gr_{AB} - w - f_b - f - mu_{SA} - mu_{SB}) + gP_O + g_bB_O$$

$$\frac{dS_{RA}}{dt} = S_{RA}(gr_B - w - f_b - f - mu_{SB}) + gP_{RA} + g_bB_{RA} + mu_{SA}S_O$$

$$\frac{dS_{RB}}{dt} = S_{RB}(gr_A - w - f_b - f - mu_{SA}) + gP_{RB} + g_bB_{RB} + mu_{SB}S_O$$

$$\frac{dS_{RAB}}{dt} = S_{RAB}(\psi - w - f_b) + g_bB_{RAB} + mu_{SB}S_{RA} + mu_{SA}S_{RB}$$

$$\frac{dP_O}{dt} = P_O(-w - f_b - g) + fS_O$$

$$\frac{dP_{RA}}{dt} = P_{RA}(-w - f_b - g) + fS_{RA}$$

$$\frac{dP_{RB}}{dt} = P_{RB}(-w - f_b - g) + fS_{RB}$$

$$\frac{dB_O}{dt} = B_O(bgr_{AB} - w_b - g_b - mu_{BA} - mu_{BB}) + f_b(P_O + S_O)$$

$$\frac{dB_{RA}}{dt} = B_{RA}(bgr_B - w_b - g_b - mu_{BB}) + f_b(P_{RA} + S_{RA}) + mu_{BA}B_O$$

$$\frac{dB_{RB}}{dt} = B_{RB}(bgr_A - w_b - g_b - mu_{BA}) + f_b(P_{RB} + S_{RB}) + mu_{BB}B_O$$

$$\frac{dB_{RAB}}{dt} = B_{RAB}(\psi_b - w_b - g_b) + f_bS_{RAB} + mu_{BA}B_{RB} + mu_{BB}B_{RA}$$

Growth Rates:

$$\psi = \psi_{\max}\left(\frac{R}{R+k_m}\right)$$

$$gr_{AB} = \psi - H_{AB}$$

$$gr_A = \psi - H_A$$

$$gr_B = \psi - H_B$$

$$\psi_b = \psi_{\max b}\left(\frac{R_f}{R_f+k_m}\right)$$

$$bgr_{AB} = \psi_b - bH_{AB}$$

$$bgr_A = \psi_b - bH_A$$

$$bgr_B = \psi_b - bH_B$$

$$H_{AB} = \left[\frac{(\psi_{\max} - \psi_{\min AB}) * \left(\frac{A+B}{MIC_{AB}}\right)^{\kappa_{AB}}}{\left(\frac{A+B}{MIC_{AB}}\right)^{\kappa_{AB}} - \left(\frac{\psi_{\min AB}}{\psi_{\max}}\right)}\right]$$

$$H_A = \left[\frac{(\psi_{\max} - \psi_{\min A}) * \left(\frac{A}{MIC_A}\right)^{\kappa_A}}{\left(\frac{A}{MIC_A}\right)^{\kappa_A} - \left(\frac{\psi_{\min A}}{\psi_{\max}}\right)}\right]$$

$$H_B = \left[\frac{(\psi_{\max} - \psi_{\min B}) * \left(\frac{B}{MIC_B}\right)^{\kappa_B}}{\left(\frac{B}{MIC_B}\right)^{\kappa_B} - \left(\frac{\psi_{\min B}}{\psi_{\max}}\right)}\right]$$

$$bH_{AB} = \left[\frac{(\psi_{\max b} - \psi_{\min AB}) * \left(\frac{A+B}{bMIC_{AB}}\right)^{\kappa_{AB}}}{\left(\frac{A+B}{bMIC_{AB}}\right)^{\kappa_{AB}} - \left(\frac{\psi_{\min AB}}{\psi_{\max b}}\right)}\right]$$

$$bH_A = \left[\frac{(\psi_{\max b} - \psi_{\min A}) * \left(\frac{A}{bMIC_A}\right)^{\kappa_A}}{\left(\frac{A}{bMIC_A}\right)^{\kappa_A} - \left(\frac{\psi_{\min A}}{\psi_{\max b}}\right)}\right]$$

$$bH_B = \left[\frac{(\psi_{\max b} - \psi_{\min B}) * \left(\frac{B}{bMIC_B}\right)^{\kappa_B}}{\left(\frac{B}{bMIC_B}\right)^{\kappa_B} - \left(\frac{\psi_{\min B}}{\psi_{\max b}}\right)}\right]$$

Mutation Rates:

$$mu_{SA} = \mu_A \left(\frac{\psi}{\psi_{\max}} \right)$$

$$mu_{SB} = \mu_B \left(\frac{\psi}{\psi_{\max}} \right)$$

$$mu_{BA} = \mu_A \left(\frac{\psi_b}{\psi_{\max}} \right)$$

$$mu_{BB} = \mu_B \left(\frac{\psi_b}{\psi_{\max}} \right)$$

Resources:

$$\frac{dR}{dt} = w(C - R) - e(gr_{AB}S_O + gr_A S_{RB} + gr_B S_{RA} + \psi S_{RAB})$$

$$\frac{dR_f}{dt} = w_b(C - R_f) - e(bgr_{AB}B_O + bgr_A B_{RB} + bgr_B B_{RA} + \psi_b B_{RAB})$$

Antibiotics:

$$\frac{dA}{dt} = A(-w - d_A)$$

$$\frac{dB}{dt} = B(-w - d_B)$$

CHAPTER 4

Pharmacokinetics and Pharmacodynamics meets Population Dynamics meets Immunology: Predictions and Hypotheses for the Design and Evaluation of Antibiotic Treatment Regimens

Peter Ankomah and Bruce R. Levin

In preparation; to be submitted to PLoS Pathogens

4.1 INTRODUCTION

The goals of antibiotic treatment of bacterial infections are straightforward and interrelated: to maximize the likelihood and rate of cure, to minimize the toxic and other deleterious side-effects of treatment, and to minimize the chance and/or extent of resistance emerging during the course of treatment. In addition to the advantage to the individual patient, controlling acquired resistance reduces the contribution of treatment to the spread of resistance in hospitals and open communities. How does one choose the most effective antibiotic(s) for a given infection and determine its optimum dose, frequency and term of administration to achieve these goals?

One answer has been to combine *in vitro* studies of the pharmacodynamics (PD) of the antibiotics and bacteria and the *in vivo* pharmacokinetics (PK) of the antibiotics in treated patients or model organisms (Craig, 1998; Drusano, 2004). Central to this "rational" (as opposed to purely empiric) approach to antibiotic treatment are three PK/PD indices: (i) the ratio of the peak antibiotic concentration achieved *in vivo* to the lowest level required to prevent the *in vitro* growth of the bacteria (the Minimum Inhibitory Concentration), C_{MAX}/MIC, (ii) the ratio of the area under the concentration-

time curve to the MIC, AUC/MIC and (iii) the amount of time the antibiotic concentration exceeds the MIC, T>MIC. The therapeutic efficacies of different classes of antibiotics are considered to be best described by one of these indices. Thus, antibiotic regimens that are based on the same index can be empirically evaluated and compared using *in vitro* experimental systems (e.g. hollow fiber models) or with laboratory organisms, typically neutropenic or other immunocompromised mice (Mouton et al., 2011).

PK/PD indices have the virtue of reductionism; save for host variation in PK (Drusano, 2007), the treatment regimen is founded on a single index that can be estimated in a standardized way. Although there is evidence that antibiotic use protocols based on these indices are correlated with treatment success, (Ambrose et al., 2001; Forrest et al., 1993; Kashuba et al., 1999; Preston et al., 1998), it is not at all clear whether these protocols are optimal (McKinnon and Davis, 2004; Udekwu et al., 2009). Treatment fails and resistance emerges even when PK/PD-based protocols are used and adhered to (Forrest et al., 1993; Tapsall et al., 1998). Are there ways to develop antibiotic treatment regimens that would lead to lower rates of treatment failure and emergence of resistance than those based on PK/PD indices alone?

Mathematical and computer simulation models could provide a framework to facilitate the development of optimal antibiotic treatment protocols. They have been successfully used to design and evaluate antibiotic use regimens for hospitals and to evaluate the relationship between antibiotic use and epidemiology of resistance in open communities, e.g (Bergstrom et al., 2004; Bonhoeffer et al., 1997; D'Agata et al., 2007; Webb et al., 2005). To a lesser extent, mathematical models have also been used to

explore and evaluate protocols for the treatment of individual patients with single and multiple antibiotics (Ankomah and Levin, 2012; Bonhoeffer et al., 1997; D'Agata et al., 2008; Lipsitch and Levin, 1998). However, while it is well known that the clearance of bacterial infection can be attributed to a collaboration of the host's immune defenses and antibiotics, with few exceptions, e.g. (D'Agata et al., 2008; Geli et al., 2012; Handel et al., 2009), models of antibiotic treatment do not consider the host's contribution. And, the few models that do allow for a generalized host contribution to the clearance of the infection also fail to consider other significant realities bacterial infection. Included among these are the physiological variation in susceptibility of the infecting bacteria, like the phenomenon of persistence (Lewis, 2010), and tissue and other within-host heterogeneities that contribute to dynamics of bacterial infections and their clearance. Finally, most existing models of the within-host dynamics of antimicrobial chemotherapy treat inherited resistance as a discrete state, rather than the reality of a continuum of declining susceptibility to these drugs.

In this report, we use a mathematical model and computer simulations to explore the efficacy of different antibiotic dosing and term-of-use regimens on the rate of clearance (cure) and emergence of resistance in self-limiting as well as potentially lethal acute bacterial infections. Our model combines the pharmacokinetics of periodic antibiotic dosing with multi-parameter functions for the pharmacodynamics of the antibiotics and bacteria and the innate and adaptive host immune response. The model also considers the effects of phenotypic resistance (persistence), inherited variation in the susceptibility of the bacteria to the treating drugs, and within-host variation in the efficacy of antibiotic action. In our analysis, we give primary consideration to the

relationship between the dose, frequency and term of administration of the antibiotic and the rate of clearance of the infection as well as the emergence of resistance. Our assessments of the properties of this model provide a number of predictions (hypotheses) about the consequences of different antibiotic treatment regimens on the course of bacterial infections. Contrary to recent arguments against "orthodox" high dose therapy (Read et al., 2011), our results suggest that under most conditions, high dose therapy is more effective than more moderate dosing as measured by both the rate of clearance and the likelihood of emergence of resistance. We discuss the current status of data in support of the predictions of this study, briefly consider those elements that require testing and suggest how they can be tested.

4.2 METHODS

4.2.1 The Model: The model we develop here is an extension of that we employed in (Levin and Udekwu, 2010) that incorporates innate and adaptive host immune responses and the emergence of resistance (Figure 4.1). In the following, we outline the different elements of this model.

4.2.2 Population growth and maintenance: In the absence of antibiotics, the maximum growth rate of the bacteria of population B_i (ϕ_{iMAX}) is proportional to the concentration of a limiting resource, R μg/ml:

$$\phi_{iMAX}(R) = V_i\left(\frac{R}{k+R}\right)$$

where k is the concentration of the resource at which the population is growing at half its maximum rate, and V_i is the maximum resource-independent growth rate (Monod, 1949). Resources continually infuse into the site of the infection out of a resource reservoir C at a rate w μg/mL per day and are consumed by the bacteria at a rate proportional to their

maximum growth rate and a conversion efficiency parameter, e µg per cell (Stewart and Levin, 1973). The latter is the amount of resource required to produce a single new cell. With these definitions and assumptions, the rate of growth of bacterial population B_i and the rate of change in the resource concentration are given by:

$$\frac{dB_i}{dt} = \phi_{iMAX}(R)B_i$$

$$\frac{dR}{dt} = w(C-R) - e\sum_{i=1}^{n} B_i\phi_{iMAX}(R)$$

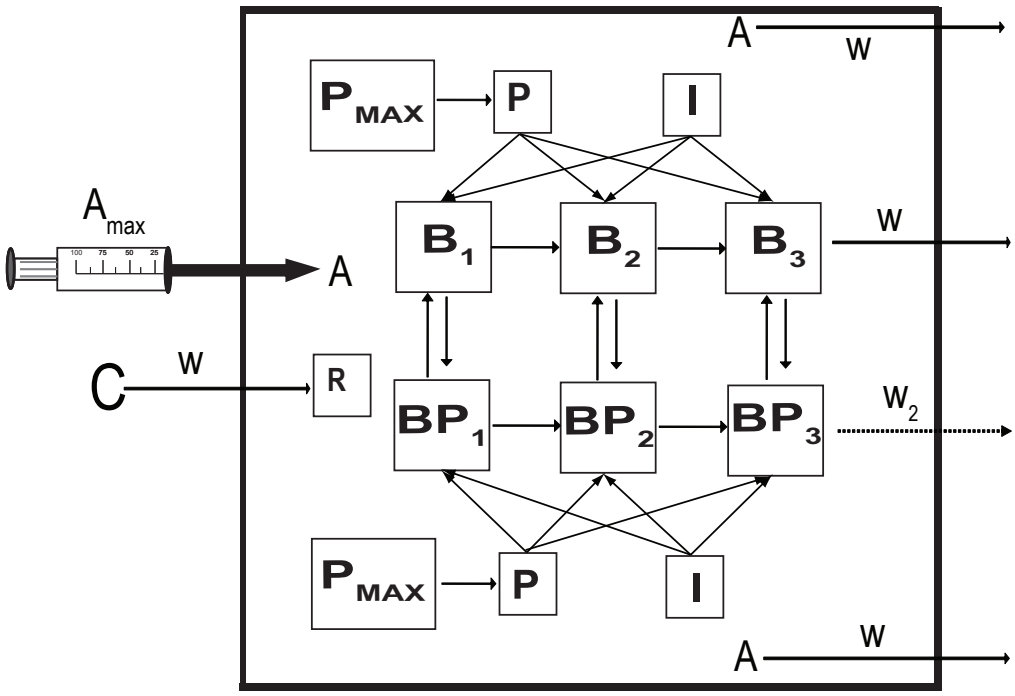

Figure 4.1. Schematic diagram showing the mathematical model of the population and evolutionary dynamics of bacteria with host immune responses and antibiotic treatment. B_i, rapidly-growing bacteria; BP_i, slowly-growing persister bacteria; i = 1, susceptible to antibiotic action; i=2, intermediate-resistance; i=3, high-level resistance. P_{MAX}, reservoir of inactive innate immune cells; P, activated innate immune cells; I, adaptive immune cells. C, resource reservoir; R, concentration of resource in infection site; A_{max}, dose of antibiotic added periodically; A, concentration of antibiotic in infection site; w, rate of flow of resources into and out of the infection site; w_2, rate of washout of persisters.

4.2.3 The Bacterial Populations: There are three populations of bacteria, with densities and designations B_1, B_2 and B_3. These bacteria can vary in their MIC_i, with the higher index number bacteria being less susceptible to the antibiotic than the lower index populations. The bacteria can also exhibit different maximum rates of growth, V_i, due to fitness costs of resistance. Bacteria of more susceptible states can generate those of lower susceptibility by mutation: $B_1 \rightarrow B_2$ at a rate μ_1 per cell per generation and $B_2 \rightarrow B_3$ at a rate μ_2 per cell per generation. For convenience and also because the effect is negligible, we do not consider reverse mutation. The cells of each of these bacterial populations can be in one of two states: (i) rapidly replicating and phenotypically susceptible to the antibiotics, or (ii) replicating slowly and phenotypically refractory to the antibiotics. The latter subpopulations, BP_1, BP_2 and BP_3, represent a refuge from the antibiotics as would be expected for persisters as well as cells in biofilms and other sub-habitats where the efficacy of the antibiotics is reduced. We assume that these populations divide at a low rate, $\psi_{pi}(R)$, where $\psi_{pi}(R) << \phi_{iMAX}(R)$, and that bacteria change from the susceptible to the refractory state at rate f_{SP} per cell per hour and return to the susceptible state at a rate f_{PS} per cell per hour, $f_{SP} < f_{PS}$.

4.2.4 Pharmacodynamics and pharmacokinetics: Central to the PD of this model is a Hill function, for which the net rate of growth or death of a bacterial population, ψ_i, is a function of the concentration of the antibiotic, A μg/mL, and the limiting resource, R μg/mL:

$$\psi(A,R)_i = \phi_{iMAX}(R) - \frac{\left[(\phi_{iMAX}(R) - \phi_{iMIN}(R)) * \left(\frac{A}{MIC_i}\right)^{\kappa}\right]}{\left[\left(\frac{A}{MIC_i}\right)^{\kappa} - \frac{\phi_{iMIN}(R)}{\phi_{iMAX}(R)}\right]}$$

where $\phi_{iMAX}(R)$ is the maximum resource-limited growth rate of the bacteria, $\phi_{iMIN}(R)$ is the minimum bacterial growth rate (maximum antibiotic kill rate), κ, the Hill coefficient is a shape parameter that describes the sensitivity of the bacterial growth rate to changes in antibiotic concentration, and MIC_i is the minimum inhibitory concentration of the antibiotic. The level of bacterial susceptibility to antibiotics in this model is directly proportional to the MIC (CLSI, 2005; Kahlmeter et al., 2003). We assume that intermediate (B_2) and high-level (B_3) resistant populations have MICs, respectively two- and ten-fold greater than that of the susceptible population (B_1). For the pharmacokinetics, we assume that in the absence of input the concentration of the antibiotic declines exponentially at rate d per hour, and is also lost due to flow from the site of infection at rate w.

$$\frac{dA}{dt} = -(d + w)A$$

4.2.5 The innate and adaptive immune responses: Our model incorporates two components of mammalian immune defenses, a rapid innate response and a slowly developing adaptive response.

4.2.5.1 The innate Immune Response: Our model of the innate immune response is similar to that developed by (Kochin et al., 2010). Activated effector cells (phagocytes) are recruited into the site of the infection at a rate proportional to the density of cells in an inactive reservoir and a rate parameter η per hour. The total density of cells in the reservoir is $P_{MAX,}$ and P represents the density of activated phagocytes, with the latter becoming inactive at a rate γ per hour. The rate of recruitment is proportional to the total

density of the infecting bacterial population, $N = B_1+B_2+B_3+ BP_1 + BP_2 + BP_3$ via a Monod-like hyperbolic function.

$$\frac{dP}{dt} = \eta(P_{MAX} - P)\left(\frac{N}{N+\sigma_P}\right) - \gamma P$$

where $\sigma_P > 0$ is a saturation constant employed to reflect the relationship between the rate of recruitment of phagocytes and the density of bacteria in the site of the infection.

4.2.5.2 Adaptive immune response: The adaptive immune response proceeds via a clonal expansion of effector cells, *I*, that are specific for the collective of antigens borne on the infecting bacteria. The intensity of the adaptive response increases at a rate governed by the maximum rate at which the *I* population increases, α per hour, and the density of the target population of bacteria. The constant σ_I is the density of bacteria at which the adaptive immune response increases at half its maximum rate (Antia et al., 1994). Since we are modeling short-term infections, we assume that there is no waning of the adaptive immune response over the course of the infection.

$$\frac{dI}{dt} = \alpha I\left(\frac{N}{N+\sigma_I}\right)$$

4.2.6 Bacterial Population dynamics under immune action: We assume the sensitivity to inhibition (killing) by the innate and adaptive immune response is the same for the all replicating populations of bacteria and proportional to the product of their densities, *P* and *I* and the mass action constants k_p and k_i (per immune cell per hour), respectively. We also assume that the three refuge populations of bacteria are killed at a lower rate than the more rapidly replication subpopulations. The mass action constants for the innate and adaptive immune responses for these refuge populations are, respectively, j_p and j_i, where $j_p < k_p$ and $j_i < k_i$.

With the above definitions and assumptions, the rates of change in the densities of the bacterial populations are given by:

$$\frac{dB_i}{dt} = \psi_i(A,R)B_i - k_P B_i P - k_i B_i I + f_{PS} BP_i - f_{SP} B_i$$

$$\frac{dBP_i}{dt} = \psi_{Pi}(R)BP_i - j_P PBP_i - j_i BP_i I - f_{PS} BP_i + f_{SP} B_i$$

4.2.7 Computer simulations: We use a semi-stochastic algorithm to solve the above array of coupled differential equations. The changes in the densities of the bacteria, immune cells and concentrations of the resource and antibiotic are deterministic. The corresponding differential equations are solved by the Euler method with a finite step size Δt. The generation and loss of persisters and mutation to antibiotic resistance are stochastic. We incorporate these stochastic elements into the model via a Monte Carlo protocol. To illustrate this algorithm we consider that used for the generation of persisters. At each finite time interval Δt, the probability that a single persister cell BP_1 will be produced by the B_1 population is $f_{SP}B_1\Delta t$, where Δt is chosen so that this product is less 1. If a random number x ($0<x<1$) is less than or equal to $f_{SP}B_1\Delta t$, a single B_1 is removed from that population and enters the persister BP_1 population. Mutations that change the resistance state of the bacteria are generated using a similar protocol, at rates proportional to bacterial growth rates and the product of the number of individuals of the ancestral state and the mutation rate, μ_1 or μ_2. In addition, since stochastic extinction processes are important at lower population densities (Handel et al., 2007; zur Wiesch et al., 2011), we assume that when the density of a bacterial population is less than 5 cells/mL, there is a 50% chance of extinction of that population with each iteration of the simulation. In Table 4A.1, we list the variables and parameters of the model and the

ranges and/or standard values of the parameters employed in our simulations. Whenever possible, we use parameter values in the ranges of those estimated experimentally for *S. aureus* and *E. coli* (Johnson and Levin, 2013; Regoes et al., 2004; Udekwu et al., 2009; Wiuff et al., 2005). For more justifications of the parameter values used, see the footnotes to Table 4A.1.

We initiate the simulations with a single bacterium of state B_1, a single phagocyte P=1 and a single adaptive immune cell I =1. We choose parameter values to address the reality that a bacterial population increases exponentially and reaches substantial densities before the host response begins to control the population. We also assume that treatment is not initiated until the bacterial population is at a level where the infection is symptomatic. We consider infections of two major types: (i) a self-limiting infection for which combined innate and adaptive immune responses will, in the absence of antibiotics clear the infection over a clinically realistic term (Gwaltney et al., 2004; Ternhag et al., 2007), and (ii) a non-self-limiting and therefore potentially lethal infection for which clearance does not occur in the absence of antimicrobial agents. Antibiotic treatment commences when infecting bacteria attain their resource-limited density at which time antibiotics at a concentration A_{MAX} are pulsed into the site of infection every T hours. The simulation used for this model was programmed in Berkeley Madonna™. Copies of the program are available at www.eclf.net/programs.

4.3 RESULTS:

4.3.1 Self-limited infection: We open with a consideration of the null case, the dynamics of an infecting bacterial population in the absence of an immune response and antibiotic treatment. Under these conditions, the infecting bacteria (B_1) grow to high densities but

are limited by the availability of resources for growth (Figure 4.2a). The susceptible bacteria grow to densities that are high enough that they generate a concomitantly high level of persister bacteria (BP_1). Intermediate-resistance bacteria (B_2) are generated but do not ascend due to resource restriction. Figure 4.2b illustrates infection dynamics when the innate immune defenses operate, in the absence of antibiotics or the adaptive immune response. Here, the maximum density of bacteria is lower than that in the null case (Figure 4.2a) but the infection is not cleared. If however, we allow for an adaptive immune response, the infection is cleared well before the end of the 20-day simulation (Figure 4.2c). The addition of a small dose of antibiotics in combination with the immune response leads to a somewhat earlier clearance of the infection (Figure 4.2d). The cidal activity of the antibiotics also reduces the density of the infection population earlier than immune action alone. Of note though, at this treatment level the resources that are freed up with the decrease in density of the B_1 population enable the temporary ascent of intermediate-resistance bacteria, but these too are eventually cleared by the immune response.

In the subsequent section, we examine the efficacy of various treatment regimens on the time to clearance of the bacteria and the rate of evolution of intermediate and high-level resistance. As our measure of clearance, we consider the average number of days required for the density of the total bacterial population to be less than 1 CFU/mL over ten independent simulations. For the emergence of resistance, we consider the number out of 100 simulations in which B_2 or B_3 bacteria are produced before day 20.

In Figure 4.3a and 4.3b we examine the effects of dose on these two measures of treatment efficacy. The average time to clearance and the fraction of runs in which B_2

bacteria emerge decline with increasing concentrations of the antibiotic. This decline, however, is not monotonic. After a point, increasing the dose of the drug has little or no effect on either of these measures of antibiotic efficacy.

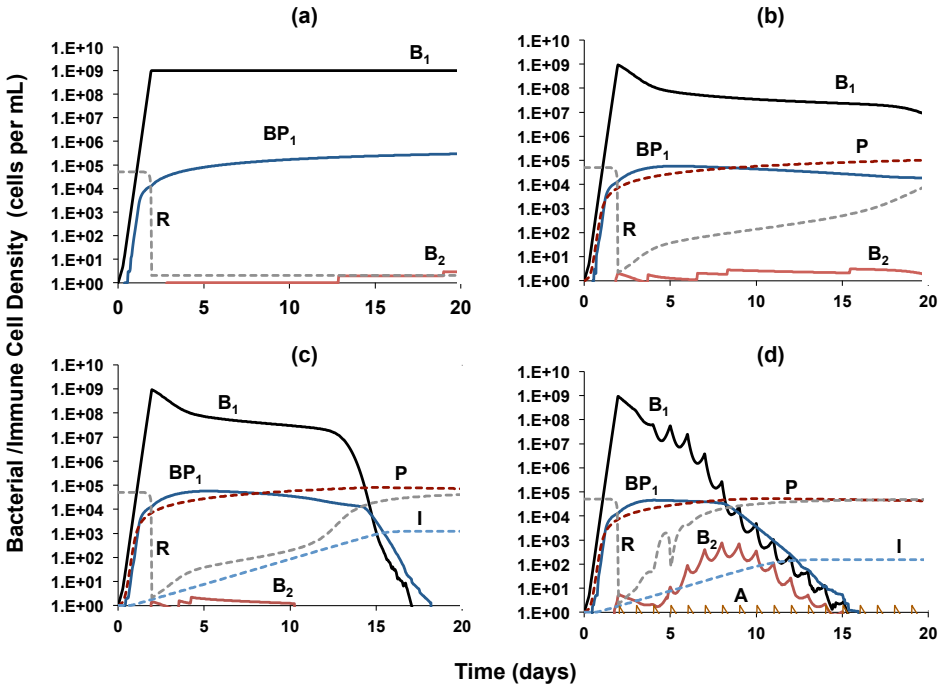

Figure 4.2 Bacterial population dynamics of a self-limited infection with immune action and antibiotic treatment. Changes in the densities of the bacteria and immune cells under the following conditions: (a) No immune action, no antibiotic treatment (b) Innate immune action (c) Innate and adaptive immune action (d) Innate and adaptive immune action with antibiotic treatment, dose = 2 μg/mL. Standard parameter values used for the simulations are listed in Table 4A.1.

This is a reflection of the Hill function pharmacodynamics. For example, with the Hill function parameters used for the susceptible strain, increasing the concentration of the antibiotic from 1.5xMIC to 2.5xMIC decreases the net growth rate from -0.125 to –0.2815 or by a factor of 1.25. The corresponding factor decreases that accompany

increasing the dose from 2.5xMIC to 5xMIC and from 10xMIC to 20xMIC are, respectively, 0.64 and 0.13.

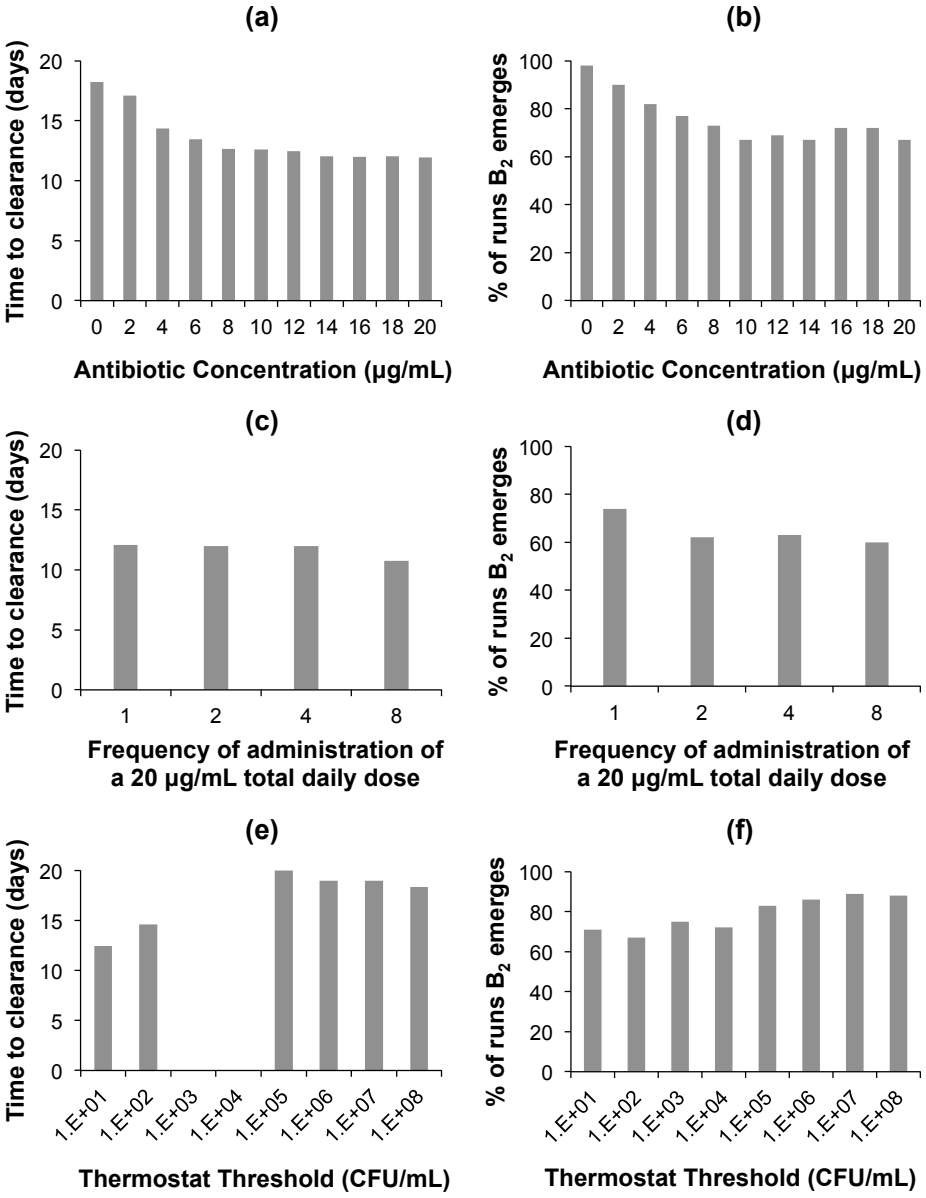

Figure 4.3. The effects of different treatment regimens on the average time to clearance of the bacteria (left column) and fraction of simulations in which bacteria with intermediate levels of resistance emerge (right column). (a and b) Single daily doses of different concentrations of the antibiotic (c and d) 20 µg/mL of the antibiotic administered at different frequencies ranging from one dose of 20 µg/mL to 8 doses of 2.5 µg/mL per day (e and f) Different density thresholds for the cessation of antibiotic dosing in thermostat regimens, standard treatment of 10 µg/mL per day.

Bacteria exhibiting high-level antibiotic resistance (B_3) were not produced in these or any other simulations for this self-limited infection because the immune response cleared the infection before B_2 could ascend to densities high enough to allow for the generation of B_3 bacteria by mutation.

In Figures 4.3c and 4.3d we illustrate the effect of the frequency of administration on clearance and resistance. On first consideration, it may seem surprising that dosing rates have little effect on these measures of the efficacy of treatment. Why this is the case can be seen in Figure 4A.1 (in Appendix) where we follow the dynamics of the changes in density of bacteria for dosing either once (Figure 4A.1a) or eight (Figure 4A.1b) times daily while keeping the total administered dose constant. High concentrations of the drug dosed at a low frequency rapidly reduce the density of bacteria, but without frequent replenishment, antibiotic concentrations wane to low levels due to the decay and washout. At these lower concentrations of the bactericidal effects of treatment are modest (Figure 4A.1a). By partitioning the total amount of drug employed for treatment into more frequently administered doses, the amplitude of the oscillations in concentration is damped and the rate of antibiotic-mediated killing more constant (Figure 4A.1b). Although this leads to a somewhat higher rate of clearance of the susceptible population, the frequency of administration of the drug has little effect on the rate of clearance of the persister sub-population and thus the infection at large. The net effect is that both the rate of clearance and intensity of selection for resistance are relatively insensitive to frequency of administration of the drug.

In Figures 4.3e and 4.3f, we consider the effects of a ‘thermostat’ treatment regimen, whereby drugs are only administered when the density of bacteria exceed a

threshold. The assumption here is that this threshold represents the density below which symptoms would be abrogated. We illustrate the effects of varying these thresholds on the rates of clearance (Figure 4.3e) and emergence of resistance (Figure 4.3f). At lower threshold densities, clearance occurs more rapidly than at higher thresholds and the rate of emergence of resistance is lower. There is, however, an intriguing exception to this result; with threshold densities of 10^3 and 10^4, clearance did not occur in any of the runs. The reason for this is the interplay between the density of the bacteria and the immune response. At the very low threshold densities (10^1 and 10^2), the hiatuses in treatment are relatively rare and thereby the antibiotics are effective in reducing the density of the bacterial population. Since the intensity of the immune response is directly proportional to the density of the infecting bacteria, at high threshold densities (10^5 and above) immune-mediated killing plays a major role in clearance. On the other hand, because the numbers of bacteria are relatively large, mutants of intermediate resistance are more likely to be generated (see Figure 4.3f and Figure 4A.2b, in Appendix). At intermediate threshold densities the joint action of the antibiotics and the immune system are least effective in clearing the bacteria (Figure 4A.2a, in Appendix). Hiatuses in antibiotic dosing are relatively frequent and because the densities of bacteria are low, the adaptive immune response is only marginally stimulated and thereby plays only a modest role in killing the bacteria.

In Figure 4.4a we consider the treatment dynamics of an infection for which there is already a minority population of B_3 cells prior to the initiation of therapy. The density of susceptible bacteria is rapidly reduced whilst the resistant minority ascend to high densities. In this self-limiting infection however, they are eventually cleared by the

immune response. In Figure 4.4b, we show that the rate of ascent and the density of these resistant bacteria can be reduced by treating with a higher antibiotic concentration. If a thermostat treatment regimen is employed, even at the higher antibiotic concentrations used in 4b the dosing hiatuses result in a rapid ascent of resistant bacteria to high densities and increase the time to clearance of the infection (Figure 4.4c).

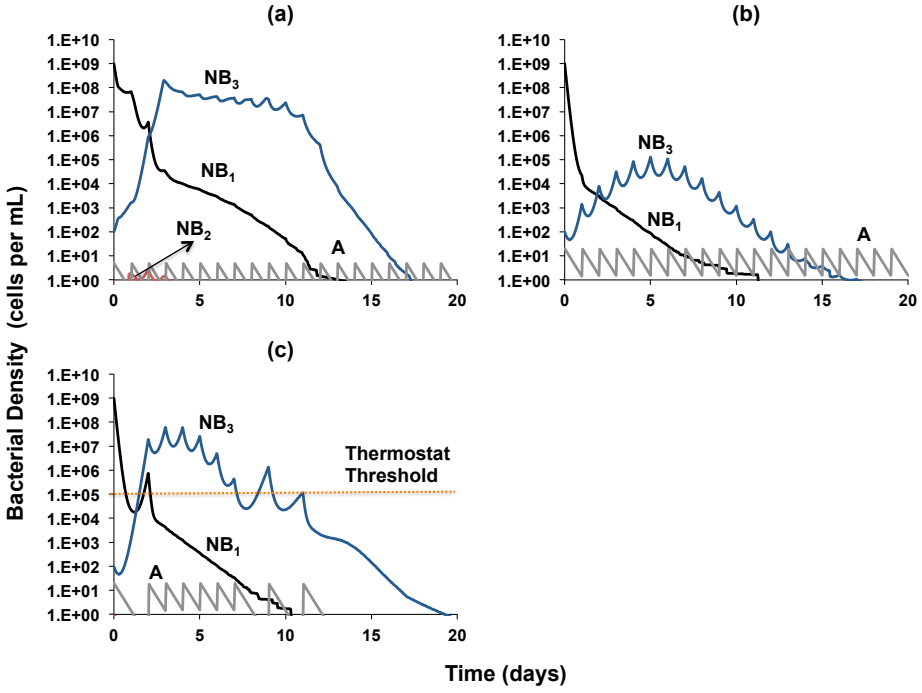

Figure 4.4 Bacterial population dynamics of a self-limited infection with pre-existing high-level resistant bacteria. Changes in the densities of the bacteria ($NB_1=B_1+BP_1$, $NB_2=B_2+BP_2$, $NB_3=B_3+BP_3$) under the following conditions: (a) Dose = 10 µg/mL (b) Dose = 20 µg/mL (c) Dose = 20 µg/mL, thermostat threshold = 10^5 bacteria. Standard parameter values used for the simulations are listed in Table 4A.1.

4.3.2 Non-self-limited infection that would be lethal in the absence of antimicrobial intervention: For this scenario, we assume an infection that occurs under conditions of relative immunodepression as would be for the elderly (Gardner, 1980), patients who have undergone major medical/surgical procedures (Angele and Faist, 2002), etc. To

explore this situation, we use the same model as for the self-limiting infections but modify the immune response parameters so that the infection is not cleared in the absence of treatment and the bacteria maintain a level determined by availability of resources.

Under these conditions, the combination of the innate and adaptive immune action only marginally reduces the density of bacteria (Figure 4.5a). An immunological boost, such as by infusing bacteria-specific antibodies, as in serum therapy (Casadevall and Scharff, 1994) can lead to clearance (Figure 4.5b). Antibiotic treatment can also promote clearance (Figure 4.5c). It worth noting that, at least in this example, the antibiotics more rapidly reduce the density of bacteria than immune serum.

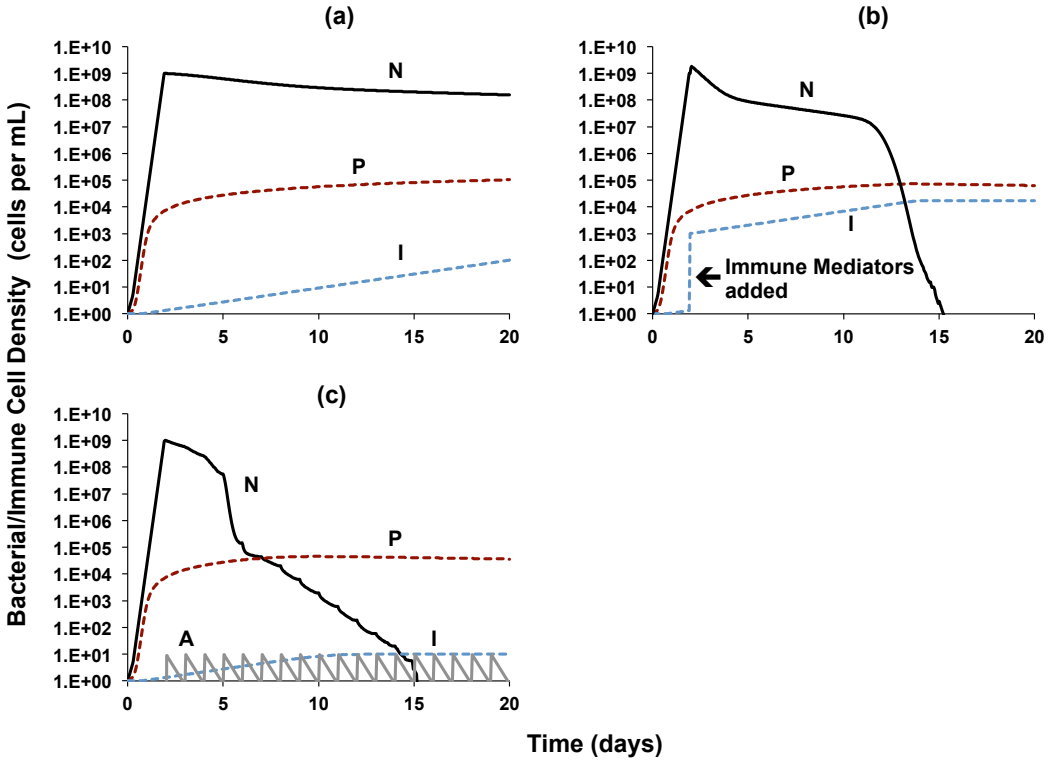

Figure 4.5 Bacterial population dynamics of a non-self-limited infection with immune action and antibiotic treatment. Changes in the densities of the bacteria ($NB_1=B_1+BP_1$, $NB_2=B_2+BP_2$, $NB_3=B_3+BP_3$) under the following conditions: (a) Innate and adaptive immune action (b) infusion of exogenous adaptive immune mediators (c) Innate and adaptive immune action with antibiotic treatment, dose = 10 µg/mL. Standard parameter values used for the simulations are listed in Table 4A.1.

Relative to the self-limited infection described previously, larger antibiotic doses are required to clear this type of infection. In Figure 4.6a we show that clearance only occurs at antibiotic concentrations of 6 μg/mL or greater. Here as well, there is a saturation effect such that clearance rate changes relatively little at higher antibiotic doses. Lower doses of antibiotics are also more likely to lead to the emergence of resistance than higher doses (Figure 4.6b). At doses of 2 and 4 μg/mL, highly resistant bacteria (B_3) emerged in 16% and 91% of simulations respectively. On first consideration, this relationship between dose and the frequency of resistance emerging may seem counter-intuitive. The reason for this result is illustrated in Figure 4A.3 (in Appendix). At the lower dose of antibiotics, the susceptible population maintains densities near that imposed by resource limitation (Figure 4A.3a). The effect is to competitively suppress the rate of ascent of B_2, the population with intermediate levels of resistance and thereby postpone or reduce the likelihood of the emergence of the B_3 population (compare Figures 4A.3a and 4A.3b).

As noted in our consideration of a self-limiting infection, the frequency of administration of fractions of a total daily dose has little effect on the rate of clearance or emergence of resistance (Figure 4.6c and 4.6d). For this type of potentially lethal infection, however, the hiatuses in treatment that occur in thermostat regimens preclude clearance. Moreover, there is an increasing rate of emergence of B_2 and B_3 bacteria with increasing thermostat threshold densities (Figure 4.6e). If high-level resistant bacteria exist prior to the initiation of therapy even greater doses of the antibiotic are needed for these drugs to promote clearance than would be for self-limiting infections (Figure 4A.4, in Appendix).

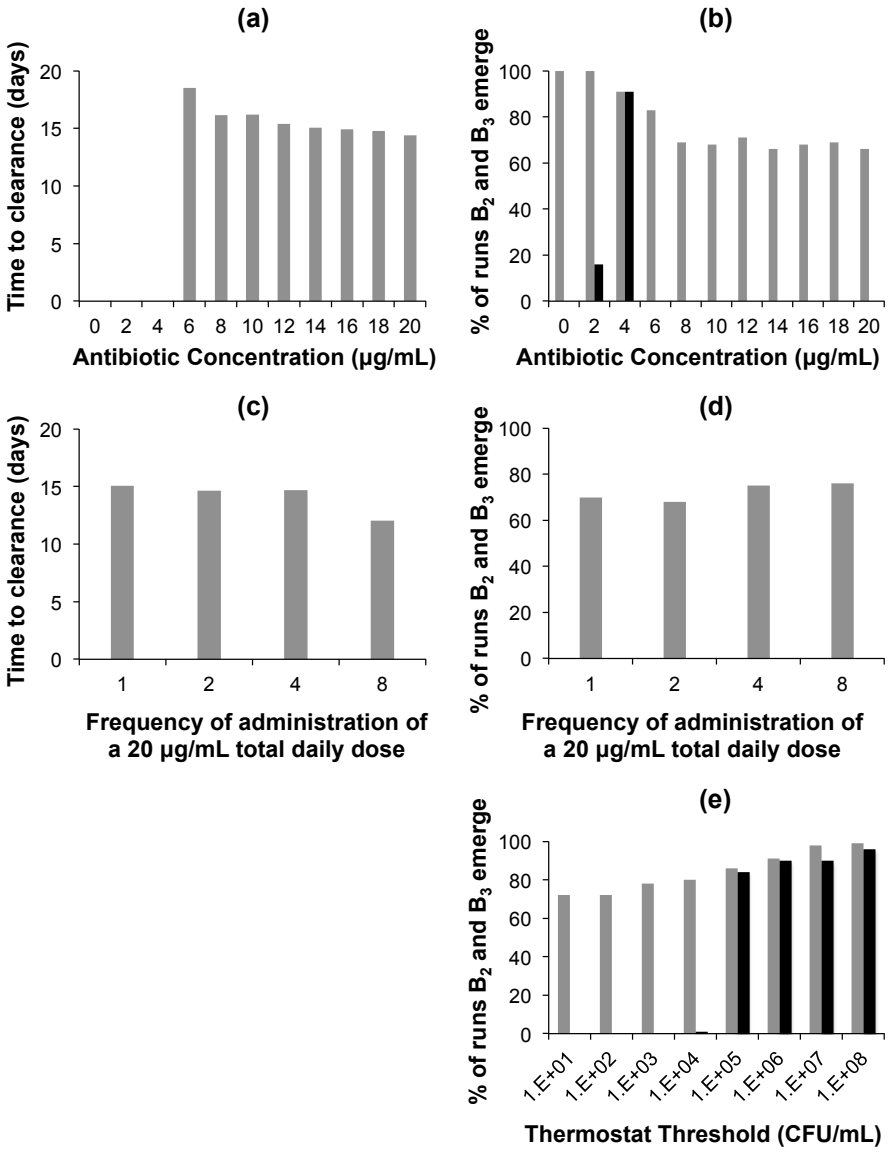

Figure 4.6 The effects of different treatment regimens on the average time to clearance of the bacteria (left column) and fraction of simulations in which bacteria with intermediate (grey bars) and high (black bars) levels of resistance emerge (right column). (a and b) Single daily doses of different concentrations of the antibiotic (c and d) 20 µg/mL of the antibiotic administered at different frequencies ranging from one dose of 20 µg/mL to 8 doses of 2.5 µg/mL per day (e) Different density thresholds for the cessation of antibiotic dosing in thermostat regimens, standard treatment of 10 µg/mL per day.

Of course, in this model we are not directly considering the morbidity or mortality of the treated patient. It is, however, reasonable to assume that the intensity of symptoms

and the likelihood of mortality would be proportional to the density of the bacterial population and the time it is maintained at these densities. Both of these consequences of infection also depend in a negative way on the immune response, immunopathology. The latter would be proportional to the density of activated phagocytes and the rate of increase in the intensity of the adaptive immune response. As shown in Figures 4.7a and 4.7b, increasing the dose of the antibiotic reduces both of these elements of immunopathology. Here too, there is a saturation effect, i.e. diminishing returns with increasing doses.

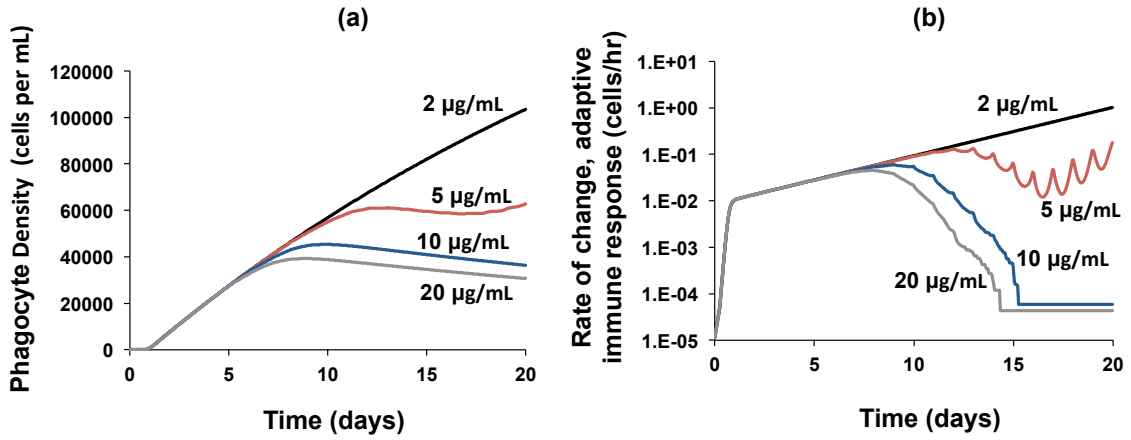

Figure 7. Levels of immune-cell activity associated with different doses. (a) Density of activated innate immune cells and (b) Rate of change of the adaptive immune response corresponding to 2, 5, 10, and 20 µg/mL doses. Standard parameter values used for the simulations are listed in Table 4A.1.

4.4 DISCUSSION:

Mathematical models are necessarily simplistic caricatures; they capture only a limited subset of the complex array of interconnected elements that contribute to biological processes. This is certainly the case for the models we develop and employ for this

consideration of the pharmacodynamic, pharmacokinetic, innate and adaptive immune responses that govern the population and evolutionary dynamics of antibiotic treatment of bacterial infections. Although for our numerical analyses we used parameters in realistic ranges for *S. aureus* and *E. coli,* we do not consider these models to be quantitatively precise analogues of the dynamics of antibiotic treatment of specific infections with these or other bacteria. The purpose of the models is to provide a framework for exploring and evaluating different antibiotic treatment regimens and generating qualitative rather than quantitative predictions about the microbiological and evolutionary consequences of these regimens. Ultimately the utility of the models and our analysis lies in the validity of the predictions made, how these can account for existing observations and be used to generate hypotheses for additional empirical studies. Some of the predictions made in this study have been supported experimentally *in vitro*, in laboratory animals and in treated patients, while others have yet to be evaluated. In the following, we discuss the major predictions, the evidence in their support and opposition, the limitation of this evidence and the implications of this theoretical study for the optimal design of antibiotic treatment regimens.

Our analysis of the properties of the mathematical model indicates that for both acute self- and non-self-limiting infections, the term of the infection will be inversely proportional to the dose of the antibiotic. This prediction has been corroborated in a number of *in vitro* studies (Gumbo et al., 2004; Tam et al., 2005a; Tam et al., 2005b), animal model experiments (Daikos et al., 1990; Knudsen et al., 2000; Knudsen et al., 2003) and in patients (Dunbar et al., 2003; Forrest et al., 1993; Moise-Broder et al., 2004; Moore et al., 1987; Preston et al., 1998). In line with clinical reality (Markou et al., 2003;

Pea et al., 2005), we find that the need for high doses of antibiotics in potentially lethal infections is even greater than for self-limiting infections. The magnitude of the immunopathology responsible for the symptoms and the mortality of these non-self-limited infections is proportional to the density of the target population of bacteria and the term of the infection (Chuang et al., 2012; Ho et al., 2009). Our study indicates that these densities and terms of the infection will be inversely proportional to the concentration of the antibiotics used for treatment.

The results of this study predict that if at the onset of treatment all of the infecting bacteria are susceptible to the antibiotic, the likelihood of *de novo* resistance evolving will decline with the dose of the antibiotic. By rapidly reducing the density of the infecting bacteria, higher doses of antibiotics supplement the action of immune defenses and bring the numbers of bacteria down to levels where resistant mutants are not likely to be generated. Moreover, if as in the case of fluoroquinolones, the generation of clinically significant levels of resistance is a multi-step process (Marcusson et al., 2009) and first step mutants are already present at the onset of or evolve during treatment, the likelihood of high-level clinical resistance emerging also declines with the dose. These predictions are well supported by *in vitro* experiments (Blaser et al., 1987; Firsov et al., 2003; Gumbo et al., 2004; Olofsson et al., 2005; Olofsson et al., 2006; Tam et al., 2007; Tam et al., 2005a; Tam et al., 2005b; Thorburn and Edwards, 2001), animal models (Fantin et al., 1994; Knudsen et al., 2003; Stearne et al., 2007; Wiuff et al., 2003) and in patients (Guillemot et al., 1998; Hansen et al., 2009; Thomas et al., 1998).

Although it is convenient to consider susceptibility and resistance as qualitatively distinct states, in reality the susceptibility of bacteria to antibiotics is a quantitative rather

than a qualitative phenomenon (CLSI, 2005; Kahlmeter et al., 2003). As long as the antibiotic dose is high enough, even if there are preexisting populations of cells of reduced antibiotic susceptibility, higher doses of antibiotics can retard their rate of ascent and thereby the likelihood that they will be transmitted or, in the case of a potentially lethal infection, lead to treatment failure. For *Streptococcus pneumoniae* infections, for instance, there is both *in vitro* and *in vivo* evidence to show that by employing higher doses, beta lactam antibiotics can be used to treat some infections containing populations of bacteria that are officially 'non-susceptible' to the drug (Klugman et al., 1995; Odenholt et al., 2003; Viladrich et al., 1996). Of course, because of toxicity and other side effects, there are limits to the concentrations at which most antibiotics can be employed. Nevertheless, there is evidence that the doses at which some antibiotics are employed could be increased to enhance bactericidal responses in patients with little or no toxic side effects (Diacon et al., 2007; Roberts et al., 2008; van Ingen et al., 2011). At this juncture, for most antibiotic-bacteria combinations, there is no general way to predict the overlap between concentrations of the drug that will limit the rate of ascent of pre-existing resistant mutants and those that will be toxic to the treated patient. These concentrations will have to be determined empirically, with experimental animals and clinical trials.

On first consideration, the results of this study seem inconsistent with the argument presented by Andrew Read and colleagues against the "orthodoxy" of high dose antibiotic treatment protocols (Read et al., 2011). They are also inconsistent with the results of rodent malaria model experiments, which provide the empirical support for Read and colleagues' arguments (Huijben et al., 2010): for mixed infections of

susceptible and resistant *Plasmodium chabaudi* in mice, they found low-dose chemotherapy to be more effective in reducing the rate of ascent of the resistant parasites than high doses. This result is what would be anticipated by classical population genetic theory; that the rate of ascent of a character will be proportional to the intensity of selection for that character (Crow and Kimura, 1970). In this case, the "character" is resistance to the chemotherapeutic agent, and the dose of that agent is proportional to the intensity of selection for resistance. If, as Read and colleagues assume, resistance engenders a fitness cost on the pathogen, at lower doses of the antimicrobial, the advantage gained by resistance may not exceed its cost and its ascent can be "competitively suppressed" by the intrinsically more fit co-infecting susceptible pathogens.

How general this "competitive suppression" effect is remains to be seen. Even when resistance engenders a fitness cost, compensatory mutations may ameliorate these costs (Bjorkman et al., 1998; Comas et al., 2012; Levin et al., 2000; Nagaev et al., 2001; Schrag and Perrot, 1996) and thereby minimize these competitive suppression effects on the dynamics of chemotherapy-mediated selection for resistance. Also, in reality, the population dynamics of resistance in bacteria are not uniquely determined by the concentration of the antibiotic at a particular point in time, but as suggested by our model, depends on a complex interplay between multiple processes including pharmacokinetics, the pharmacodynamics of susceptible and resistant bacteria and the contribution of the innate and adaptive immune system to the clearance of infections.

While our analysis supports the use of higher doses of antibiotics for treatment, it also suggests that there are diminishing returns to increasing antibiotic concentrations. In

addition to the potential deleterious side effects, after a point, the gain in antibiotic-mediated killing and the capacity to limit the *de novo* evolution of resistance declines as the concentration of drug increases. In our models this can be attributed to the saturation effect associated with Hill function pharmacodynamics. It worth noting that a number of *in vitro* studies (Corvaisier et al., 1998; Delacher et al., 2000; Hyatt et al., 1995; Madaras-Kelly et al., 1996; Regoes et al., 2004) and experiments using animal models (Bonapace et al., 2002; Boylan et al., 2003; Kim et al., 2002; Louie et al., 2001) have demonstrated that the pharmacodynamics of antibiotics are consistent with saturating functions like those employed in our model. We are unaware of clinical studies that have a directly explored the saturation effect, but there have been studies that support the proposition that after a point increasing doses of antibiotics have diminishing effects on clinical outcome (Diacon et al., 2010; Wallis et al., 2011). It would seem particularly useful for the optimal use of existing antibiotics to have more studies determining the doses of antibiotics beyond which there is little or no effect on clinical outcome.

In the preceding discussion we have focused on the concentration of the antibiotic administered but not the frequency of administration. Clearly they are related and there is a play-off between these two elements of antibiotic treatment. The results of our analysis suggest that there is only a modest effect of dividing the high concentrations of antibiotics into more frequently administered lower doses. This is suggestive of a practical advantage of administering drugs at higher doses. This strategy may allow for less frequent dosing and thereby ease the logistics of treatment and improve adherence.

What about the term, the length of therapy? It has been suggested that using lower doses for short amounts of time would be an effective way to reduce the rate of ascent of

resistance and thereby constitute a prudent use of antimicrobials (Read et al., 2011). The assumption is that by reducing the density, rather than clearing the bacteria, such 'light touch therapy' would promote clearance by immune responses whilst reducing the intensity of selection for resistance. To explore this "light touch" approach, we used a 'thermostat' model of antibiotic treatment where drugs are only administered when the density of bacteria is above some minimum threshold. We assume that the thresholds correlate with bacterial densities that elicit symptoms in a patient. Because of the hiatuses in treatment, the total amount of drugs employed and the amount of time a patient is under therapy are less than they would be for a treatment regimen with a pre-defined term. The results of our analysis suggest that the downside of this form of light touch therapy will offset the virtues of prudence. It can increase the term of treatment of self-limiting infections and preclude clearance of potentially lethal infections. For both types of infections, it also increases the likelihood of resistance emerging and ascending during therapy.

While our theoretical results question the generality of more moderate therapeutic regimens, it is difficult to predict how our thermostat model will hold up for the treatment of a broad array of real infections. It bears noting, however, that our thermostat regimen is analogous to non-adherence and there are several lines of evidence indicating that non-adherence is a major risk factor for both treatment failure and the ascent of resistance (Alexiou et al., 2007; Burman et al., 1997; Ringdahl, 2000). Be this as it may, our model points to questions that should be addressed to evaluate moderate treatment regimens based on the manifestation of symptoms: (i) What are the densities of bacteria at which patients can cease taking antibiotics without affecting the rates of microbiological cure?

(ii) What is the relationship between these bacteriological loads and patient symptoms? To obtain answers to these questions, it will be critical to monitor the densities of the infecting populations of bacteria and determine the relationship between these densities and the symptoms of the infection during the course of treatment. When this information is available, the term of therapy may then be modulated by the manifestation of symptoms rather than a pre-prescribed term.

In reference to clearing infections, Paul Ehrlich recommended that drugs be used as early in the infection as possible and at high doses (Ehrlich, 1913). The results of this computer-assisted theoretical study support this century old recommendation, but raise a number of questions about the details of this "hit them hard" protocol with respect to the microbiological, immunological and evolutionary components of the rational design of antibiotic treatment regimes. We believe these questions can and should be answered empirically and look forward to the results of these studies.

4.5 APPENDIX:

The following appendix contains supplementary information for the above investigation. All the figures, tables and protocols that follow have been pre-referenced in the text of the chapter.

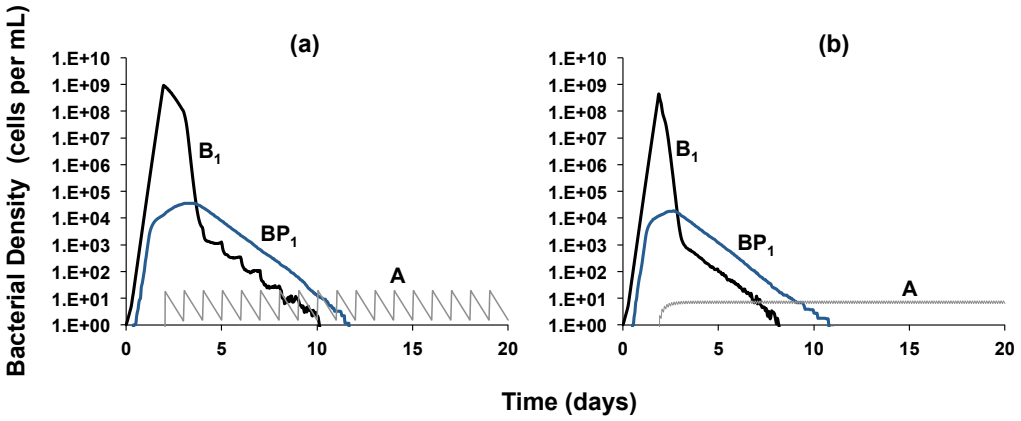

Figure 4A.1. Bacterial population dynamics of a self-limited infection with different frequencies of administration of a constant total daily dose. Changes in the densities of the bacteria under the following conditions: (a) One dose of 20 µg/mL per day (b) Eight doses of 2.5 µg/mL per day. Standard parameter values used for the simulations are listed in Table 4A.1.

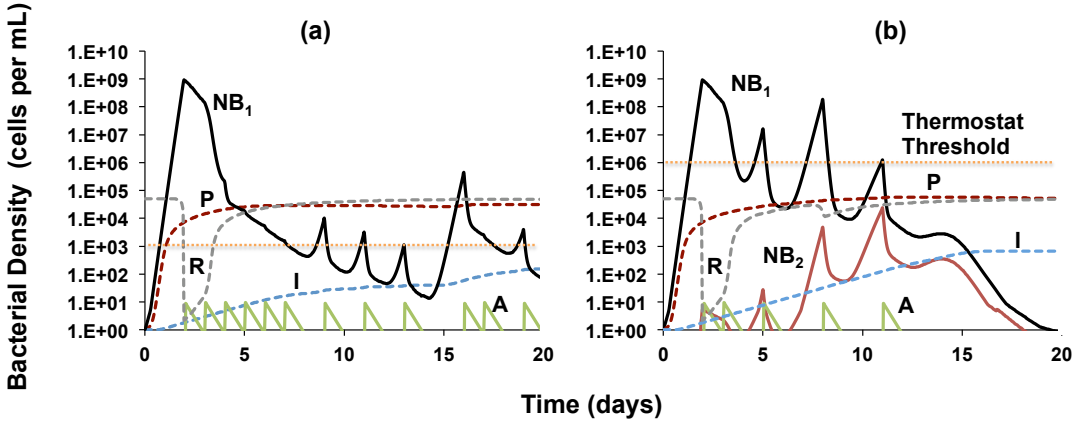

Figure 4A.2. Bacterial population dynamics of a self-limited infection with thermostat treatment regimens. Changes in the densities of the bacteria (NB_1=B_1+BP_1, NB_2=B_2+BP_2) under the following conditions: (a) Thermostat threshold = 10^3 bacteria (b) Thermostat threshold = 10^6 bacteria. Standard parameter values used for the simulations are listed in Table 4A.1.

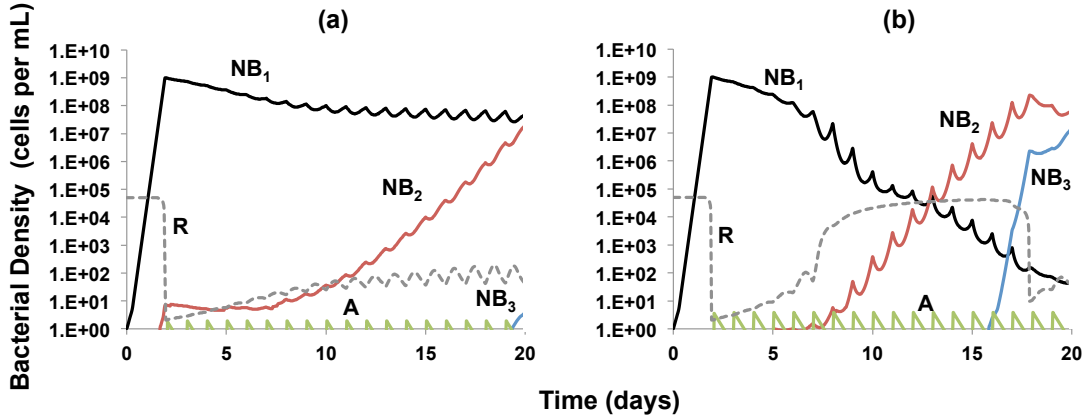

Figure 4A.3. Bacterial population dynamics of a non-self-limited infection with low dose treatment. Changes in the densities of the bacteria (NB_1=B_1+BP_1, NB_2=B_2+BP_2, NB_3=B_3+BP_3) under the following conditions: (a) Dose = 2 µg/mL and (b) Dose = 4 µg/mL. Standard parameter values used for the simulations are listed in Table 4A.1.

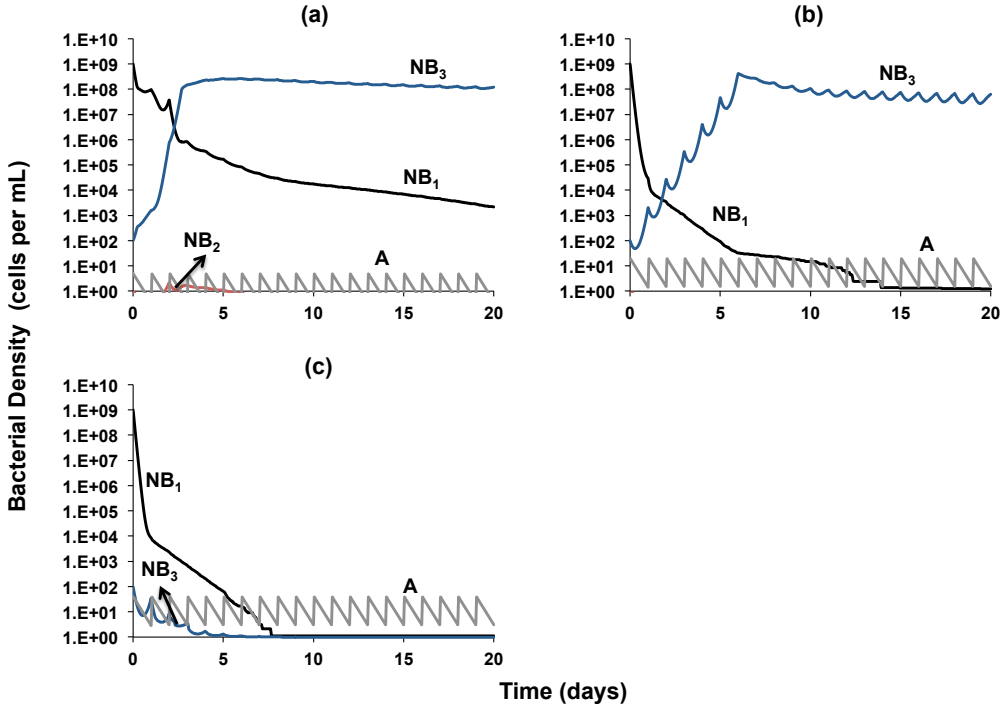

Figure 4A.4. Bacterial population dynamics of a non-self-limited infection with pre-existing high-level resistant bacteria. Changes in the densities of the bacteria (NB_1=B_1+BP_1, NB_2=B_2+BP_2, NB_3=B_3+BP_3) under the following conditions: (a) Dose = 5 µg/mL (b) Dose = 20 µg/mL (c) Dose = 40 µg/mL. Standard parameter values used for the simulations are listed in Table 4A.1.

Table 4A.1. Values and ranges for variables and parameters used for generating numerical solutions to the model.

Variable/Parameter	Description	[a]Value or range considered
Variables		
A	Antibiotic concentration (µg/mL)	0 – 40 (10)
B_i	Density of bacteria (cells per mL); population wholly susceptible to antibiotic action, i=1; intermediate-resistance, i=2; high-level resistance, i=3.	$1\text{-}10^{10}$
BP_i	Density of persisters (cells per mL); population wholly susceptible to antibiotic action, i=1; intermediate-resistance, i=2; high-level resistance, i=3.	$1\text{-}10^{10}$
R	Concentration of the limiting resource (µg/mL)	0-500
Parameters		
V_i	Maximum hourly growth rate of replicating bacteria	(0.5)
s_2, s_3	Fitness costs of resistance; assessed as decreases in maximum hourly growth rate for B_2 and B_3 populations	0 – 0.025
VP_i	Maximum hourly growth rate of persisters	0.001
ψ_{min}	Maximum hourly death rate generated by the antibiotic	(-0.75)
MIC_i	Minimum Inhibitory Concentration of antibiotic A for population B_i (µg/mL)	1, 2, 10
κ	Hill coefficient	(1)
w	Hourly washout rate, rapidly-replicating bacteria	(0.2)
w_2	Hourly washout rate, persisters	(0.001)
f_{SP}	Hourly rate at which B_i is converted into BP_i	(0.005)
f_{PS}	Hourly rate at which BP_i is converted into B_i	(0.05)
C	Reservoir resource concentration (µg/mL)	(500)
e	Efficiency of resource conversion into cells (µg/cell)	$(5\text{x}10^{-7})$
k	Concentration of resource at half maximal growth (µg/mL)	(1)
A_{max}	Antibiotic concentration added at each dosing period (µg/mL)	0 – 40 (10)
d	Antibiotic decay rate (h^{-1})	(0.1)

T	Time between doses (h)	3 – 24 (24)
μ_1, μ_2	Mutation rate (mutations per cell division)	10^{-8}, 10^{-9}
k_p	Rate constant for phagocyte clearance of replicating populations	[c] $5x10^{-6}$, [d] $5x10^{-7}$
k_i	Rate constant for lymphocyte clearance of replicating populations	[b,c] $5x10^{-4}$, [d] $5x10^{-5}$
j_p	Rate constant for phagocyte clearance of persister populations	[c] $5x10^{-8}$, [d] $5x10^{-9}$
j_i	Rate constant for lymphocyte clearance of persister populations	[b,c] $5x10^{-5}$, [d] $5x10^{-6}$
η	Rate of phagocyte recruitment	($3x10^{-4}$)
P_{MAX}	Phagocyte reservoir (cells per mL)	(10^6)
γ	Rate of phagocyte inactivation	(10^{-3})
α	Rate of increase, lymphocytic cells	0.01, [d] 0.02
σ_p	Saturation constant that reflects the relationship between rate of phagocyte recruitment and bacterial density (cells per mL)	[c] (10^4)
σ_I	Bacterial density at which adaptive immune response is at half-maximum activity (cells per mL)	[c] (10^3)

[a] Values in parentheses are the standard values used for numerical analysis of the model. Save for simulations in which parameters are varied, unless otherwise stated, the standard parameter values are employed for all simulations.

[b] As in (Antia et al., 1994), we assume that as adaptive immune cells need to be at high densities to control the infection, the clearance rate constant, k_i, is less than the initial density of adaptive immune cells. In addition, we assume that the bacterial density at which the specific immune response grows at half its maximum rate will be intermediate between the initial bacterial density and the saturation density.
i.e. $k_i, j_i << 1 << \sigma_i <<$ bacterial saturation density.

[c] We also assume that antigens that elicit specific immune responses are present at higher densities on bacterial surfaces than those that generate non-specific (innate) responses (Antia et al., 1996), and that the adaptive immune response exhibits more effective bactericidal activity than the innate immune response.
i.e. $\sigma_I < \sigma_p$, and $k_i > k_p$, $j_i > j_p$.

[d] In modeling potentially lethal infections, we assume that relative to a self-limited infection, the immune mass action parameters are an order of magnitude lower, and also halve the rate of recruitment of adaptive immune mediators.

CHAPTER 5: SUMMARY AND OUTLOOK

In this thesis, I have described a number of theoretical and experimental studies designed to explore the pharmaco-, population and evolutionary dynamics of single- and multi-drug therapy. We utilized novel Hill function-based pharmacodynamic studies to assess the impact of single antibiotics and antibiotic combinations on the *in vitro* rate of exponential-phase bacterial killing as well as the effect on genotypically-susceptible but phenotypically-resistant subpopulations of bacteria, persisters. To explore the potential clinical implications of our PD studies, quantitative insights from those analyses were incorporated into mathematical models of antibiotic treatment. The models combined the population and evolutionary dynamics of bacteria, the pharmacodynamics of antibiotics and bacteria, the pharmacokinetics of antibiotics, phenotypically-resistant subpopulations such as persisters and bacteria in spatial refuges as well as innate and adaptive host immune responses. We used computer simulations to explore the properties of these models, giving primary consideration to: (i) the effects of drug-drug interactions, and (ii) the effects of varying different components of a treatment regimen (dose, dosing frequency, term of therapy) on time to clearance of an infection and the emergence and/or ascent of single- or multi-drug resistance.

The results of our studies have demonstrated that the types of interactions between antibiotics can substantially affect clearance and resistance dynamics during treatment. Antagonistic and/or suppressive interactions between drugs decrease rates of clearance and increase the likelihood that single drug resistant bacteria will emerge and ascend. Even though combining drugs generally helps prevent the evolution of multi-drug resistance regardless of the type of drug interactions between the component drugs of the

combination, we find that non-adherence to therapy can thwart this evolutionary virtue of multi-drug therapy. Under various scenarios where patients do not take their drugs as prescribed, the type of interactions between drugs becomes important; more synergistic combinations are better able to prevent the evolution of multi-drug resistance and are also less sensitive to the effects of hiatuses in dosing.

Our results indicate that *a priori* assumptions about the type of interactions that will exist between antibiotics made on the basis of mechanisms of action or the pharmacodynamics of individual drugs are likely to be incorrect. Moreover, we also show that drug interactions cannot be assumed to stay constant (both quantitatively and qualitatively) when conditions change. Antibiotics may, for instance, interact additively over one concentration range but synergistically over another. Our work therefore suggests that with our current, limited, understanding of the mechanisms of antibiotic cidal action, drug interactions should be determined empirically over clinically relevant concentration ranges.

While only theoretical, our study examining the relationship between the dose, dosing frequency and term of a single-drug regimen and treatment success yielded interesting and potentially useful insights and predictions. Our results demonstrate the utility of high-dose therapy in limiting the term and likelihood of mortality of infections, preventing the emergence and/or ascent of resistance and minimizing immunopathology. We also show that the effects of high doses saturate such that, after a point, there are minimal gains to be made by further increases in dose. We only found modest effects of dose fractionation at higher doses, suggesting a practical advantage for high dose therapy; antibiotics may be administered less frequently without diminishing their efficacy. Our

results also indicate that more moderate regimens that make treatment coincident with patient symptoms can adversely affect the likelihood of successful therapy.

As would be expected from any worthwhile thesis, the studies contained herein have generated more questions than answers and provided new directions for further research. For one, we have not elucidated the biochemical, cellular or physiological reasons for the interactions that we observed between drugs from different classes. Of particular import, a mechanistic explanation remains to be determined for our results showing sub-MIC antagonism between all drugs assessed in our multi-drug PD *M. marinum* experiments. Why, also, did we find limited correlation between the efficacy of drug combinations in the exponential phase of bacterial killing and in the reduction of persister populations? While our analyses indicate that there may be no simple general rules to explain these observations, the reasons for this lack of generality need to be explored.

Research that sheds further light on the mechanisms of antibiotic cidal activity will help generate answers to some of these questions. Studies like those of Kohanski *et al.* (Kohanski et al., 2007) directed at determining whether there are generalizable mechanisms of antibiotic action for different classes of antibiotics are laudable, and these lines of enquiry should be continued. Such studies should also be extended into model organisms to determine how *in vivo* conditions affect the mechanisms of antibiotic activity. It should be recognized that our experimental pharmacodynamic assessments were conducted using classical *in vitro* culture methods to examine cidal dynamics for large bacterial population sizes. Complementary studies using microfluidic culture assays

will provide increased resolution at single-cell and small population levels and generate insights that are not readily evident with conventional culture methods.

It bears noting that conventional antibiotics are not the only viable way to treat bacterial infections; a number of novel approaches are under consideration as potential effectors of antibacterial therapy. Endolysins produced by bacteriophage are one example, having been used to control pathogenic Gram-positive bacteria in a number of animal models (Loeffler et al., 2001; Nelson et al., 2001). Anti-virulence strategies involving the use of inhibitors to target: (i) bacterial toxins (Karginov et al., 2005; Scobie et al., 2005), (ii) adhesive structures/molecules (Svensson et al., 2001a; Svensson et al., 2001b), (iii) specialized secretory systems (Bailey et al., 2007; Muschiol et al., 2006), (iv) virulence gene expression (Hung et al., 2005; Shakhnovich et al., 2007) and (v) cell-to-cell signaling (Hentzer et al., 2003; Manefield et al., 2002; Wright et al., 2005) are showing promise as antibacterial therapies. Agents that destabilize biofilm matrices such as norspermidine (Kolodkin-Gal et al., 2012) and D-amino acids (Kolodkin-Gal et al., 2010) as well as those that interrupt signaling pathways which promote biofilm formation and maintenance (Hentzer et al., 2003; Janssens et al., 2008) could be used clinically to enhance treatment in biofilm-associated infections.

Host-targeting approaches to anti-bacterial therapy are now also starting to gain some traction. One of these is a return to the old idea of passive antibody (serum) therapy as a means of boosting the hosts' immunological response (Casadevall et al., 2004). Advances in recombinant DNA and cloning technologies have meant that pure and highly specific antibodies can be produced in a relatively facile manner for treating various bacterial diseases (Casadevall et al., 2004). Another host-based antimicrobial approach

involves denying the pathogen access to host factors it requires for pathogenicity. This has been investigated mostly for mycobacteria, and it has been demonstrated that inhibiting some host kinases can reduce bacterial burdens in animal models (Napier et al., 2011; Wu et al., 2012a). It is expected that most of these new-generation agents will be used in conjunction with existing antibiotics (Smith and Romesberg, 2007). Our work would suggest that assessing the pharmacodynamics of these new types of antimicrobial combinations should be a crucial component of their evaluation.

The within-host mathematical models we have developed here would also benefit from additional refinement. In particular, our pharmacokinetic considerations were limited by our assumption of first-order kinetics for all drugs. Subsequent models should incorporate realistic pharmacokinetics for different drugs since this is an important factor affecting the concentrations of individual drugs that will interact during therapy. Non-cidal effects of antibiotics on bacterial population dynamics such as antibiotic-associated increases in mutation rate (Gillespie et al., 2005; Henderson-Begg et al., 2006; Kohanski et al., 2010) and elevation of persister densities (Johnson and Levin, 2013) should also be factored into the models.

In conclusion, we hope that the work described in this thesis stimulates further thought (and action) to help improve success rates of antibiotic treatment regimens and minimize the contribution of bacterial infections to human mortality. The prospect of a post-antibiotic era is too dark to consider, and all stakeholders should join hands and work stringently to prevent this from occurring.

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