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Azithromycin Pharmacodynamics Against Non-Typeable H. Influenzae

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Azithromycin Pharmacodynamics against Non-typeable *H. influenzae*

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Abstract

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is the third leading cause of death in the United States. Significant clinical disease in COPD patients has been tied to non-typeable *Haemophilus influenzae* (NTHI) infection. However, reasons why some NTHI strains persist despite antimicrobial therapy remain unknown.

Objective

The primary objective was to characterize the pharmacodynamics of azithromycin against a persistent pair of NTHI isolates.

Methods

A persistent pair of NTHI isolates (5P28H1 and 5P54H1) cultured from the sputum of an adult with COPD and determined to be the same strain by multilocus sequence typing was carried for 819 days. The Minimum Inhibitory Concentrations (MIC) were determined according to Clinical and Laboratory Standards Institute guidelines for 5P28H1 ($MIC_{azithromycin}$=2.0) and 5P54H1 ($MIC_{azithromycin}$=16.0). Time-kill experiments were performed using an array of azithromycin concentrations and samples were collected over 48h. The log reduction and integrated log ratio area over 48h were calculated and fit to a Hill-type model. A hollow-fiber infection model (HFIM) simulating azithromycin concentrations and pharmacokinetics in human serum and alveolar macrophages was performed over 240h for 5P28H1.

Results

Azithromycin displayed differential killing activity against 5P28H1 and 5P54H1. For 5P28H1, azithromycin concentrations >0.5 mg/L achieved complete killing by 48h. In

contrast, complete bacterial killing was observed by 48h for concentrations >4 mg/L in 5P54H1. Overall, azithromycin demonstrated dose-dependent bactericidal activity against both isolates. Pharmacodynamic analysis revealed a right shift in the comparative dose response curves. Model fits were excellent (R^2>0.99). Azithromycin serum concentrations simulated in HFIM did not appreciably reduce 5P28H1 viability by 240h, whereas simulated alveolar macrophage concentrations achieved complete killing by 26hrs.

Conclusion

Bactericidal activity was achieved for 5P28H1 at lower concentrations compared to 5P54H1, which demonstrated an attenuated killing profile. The differential pharmacodynamics of azithromycin suggests that antimicrobial pressure plays a role in counter selection of resistance for NTHI.

Introduction

Chronic Obstructive Pulmonary Disease (COPD) is a leading cause of morbidity and mortality worldwide (1, 2). COPD is characterized by irreversible airway obstruction, persistent inflammation, and a multitude of comorbidities (2). It is estimated that 24 million people are affected by COPD in the United States alone, with direct and indirect health care costs nearing $50 billion per year (3, 4). In contrast to other leading causes of death like cancer and cardiovascular disease, mortality due to COPD appears to be on a continuing upward trend (2). While smoking is the primary risk factor, air pollution and those exposed to dust, fumes, and chemical vapors comprise a large proportion of those developing COPD (2, 5). However, only about 25% of smokers actually develop this disease, indicating that other environmental or genetic factors may be involved in its pathogenesis (6).

Recently, it has become clear that bacteria may chronically colonize the lungs of COPD patients. Due to host immune responses, persistent bacterial colonization of damaged airways may lead to additional inflammation, promoting and advancing disease progression. This understanding led to the creation of the “Two-Hit” hypothesis, which posits that a combination of environmental and infection-related factors are crucial to COPD pathogenesis and progression (6). Apart from chronic bacterial colonization, bacterial infections are thought to play a major role in acute exacerbations of COPD. Treatment of exacerbations is believed to account for 45%-75% of direct COPD-related expenditures (7). These acute exacerbations not only incur significant financial costs, but additional social and indirect economic burdens including lost wages, absenteeism, and restricted activity (7).

It is estimated that approximately 50% of acute exacerbations have bacterial etiology (8). The most common species of bacteria isolated from COPD patients' lungs during an acute exacerbation is *Haemophilus influenzae* (1). *H. influenzae* exclusively infects humans and may cause pneumonia, bacteremia, or meningitis (9). While a vaccine exists for one of the six known types of *H. influenzae,* non-typeable (unencapsulated) strains predominate incident infections in the US (9). Thus, non-typeable *H. influenzae* (NTHI) is posited to play a significant role in progression of impaired pulmonary function observed in COPD patients (10).

Current guidelines for treating patients with acute COPD exacerbations include the prescription of an antibiotic and anti-inflammatory steroid agent (3). Azithromycin, a macrolide with both antibiotic and anti-inflammatory properties, is one of the most commonly prescribed antimicrobials for exacerbations and is being considered for prophylactic use in individuals with moderate-to-severe COPD (11, 12). However, treatment and long-term prophylaxis utilizing azithromycin is not a risk-free endeavor. Desai and colleagues (2010) noted a clinical association between antibiotic exposure and occurrence of resistant pneumococcal strains in a cohort of 127 adult COPD patients (8). In this study, it appeared that over 50% of pneumococcal strains obtained from patients exposed to macrolides, primarily azithromycin, at some point over a three and six month period displayed resistance (8). Additionally, individual and community azithromycin usage is known to increase population-level macrolide resistance in respiratory pathogens, garnering significant calls for judicious prescription practices (12).

Azithromycin exhibits antimicrobial activity by binding to the 50S ribosome and inhibiting protein translation. *H. influenzae* resistance mechanisms against macrolides include ribosomal methylase, intrinsic or acquired efflux pumps, and alterations in ribosomal proteins or RNA (13). While the clinical significance of efflux pumps remains controversial, strains with mutations in ribosomal proteins L4 or L22 and 23S rRNA tend to exhibit higher MICs for azithromycin than wild-type strains and may have significant clinical implication (13).

The pharmacokinetic and pharmacodynamic (PK/PD) profile of azithromycin against *H. influenzae* is crucial to the process of determining clinical susceptibility and improving patient outcomes (13). PK refers to the body's absorption, distribution, metabolism, and elimination of an agent (13, 14). Azithromycin is characterized by rapid absorption, poor bioavailability when taken orally (~37%), and extensive tissue distribution (14, 15). Peak serum concentrations (C_{max}) are estimated to be approximately 0.4 mg/L, with lung tissue and alveolar macrophage concentrations reaching up to 700 times that of serum (14, 15). The body does not metabolize azithromycin and excretion occurs through hepatic, transintestinal, and biliary routes (14). PD refers to the relationship between antimicrobial agent and pathogen (13). PD analysis reveals the effect of antimicrobial drug on killing and micro-organism growth dynamics (13). Azithromycin exhibits a concentration-dependent bactericidal effect on *H. influenzae*, in which activity is best determined by the serum concentration-time curve (AUC):MIC ratio (14). The AUC is the product of two PK parameters: C_{max} and the duration of exposure (14). It is important to note that the duration of exposure for

azithromycin can be quite long, with the terminal half-life estimated to be 68 hours and sub-inhibitory concentrations extending for up to 30 days (12, 14, 15).

The development of macrolide resistance in *H. influenzae* presents a major public health challenge. Since existing literature focuses primarily on pneumococcal resistance to macrolide antibiotics, there is a dearth of knowledge on the development of resistance in NTHI. Therefore, the underlying conditions allowing resistance to develop and optimal therapeutic regimens for treatment of NTHI in the context of COPD need delineation. In order to understand the driving factors of NTHI resistance to azithromycin, we characterized the pharmacodynamics of azithromycin against a pair of clinically persistent, serially obtained NTHI isolates. In addition, we evaluated the drug resistance profiles of the clinical isolates by simulating an in-vivo infection utilizing clinically relevant azithromycin concentrations and pharmacokinetic parameters.

Methods

Bacterial Strains

A unique collection of *H. influenzae* sputum isolates with corresponding whole genome sequence, epidemiologic, and clinical data were prospectively collected as part of a 20-year longitudinal study of COPD infection conducted in Buffalo, NY from 1994-2014 (16). A pair of clinically persistent, serially obtained non-typeable *H. influenzae* strains from an individual patient in this cohort was obtained. Strains 5P28H1 and 5P54H1 were isolated from the same patient at clinic visit 28 and 54, respectively, and were carried for 819 days (17). Over this time period, the patient experienced 4 courses of azithromycin therapy. These strains were of the same multilocus sequence type, with 5P54H1 harboring a mutation in the L22 ribosomal protein (17). MICs were determined according to CLSI guidelines. 5P28H1 exhibited $MIC_{azithromycin}$ of 2 mg/L and 5P54H1 exhibited $MIC_{azithromycin}$ of 16 mg/L (17).

Antibiotic and media

Azithromycin analytical grade powder was commercially purchased (Sigma Chemical Company, St. Louis, MO). Stock solutions of azithromycin in 10% DMSO were prepared at the start of each experiment. MIC values for 5P28H1 and 5P54H1 were previously determined by broth microdilution in Mueller-Hinton Broth according to CLSI guidelines (17). Brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) supplemented with 10 μg/mL NAD and 10 μg/mL Hemin was used for all static time kill experiments. Colonies were enumerated on chocolate agar (Thermo Fisher Scientific, Waltham, MA).

Static Time Kill Experiments

Static time kill experiments were performed over 48 hours as previously described, using a starting inoculum of 10^8 CFU/mL (18). In short, fresh bacterial colonies from overnight growth were added to BHI broth to create a concentrated suspension. From this suspension, 2 mL were removed and diluted with BHI broth and standard azithromycin stock solution to achieve a 10^8 inoculum in a total reaction volume of 20 mL. Each 20 mL culture was incubated in a 37°C water bath with constant shaking for 48 hours. At 0, 1, 2, 4, 8, 24, 26, 28, 32, and 48 hours, .1 mL samples were withdrawn and serially diluted in sterile saline for CFU/mL enumeration. Colony counts were determined by plating 50 µL of each diluted sample onto BHI chocolate agar with an automated spiral dispenser (WASP; Don Whitely Scientific Limited, West Yorkshire, England) and incubating plates for 24 hours at 35°C with 5% CO_2. Time kill experiments were performed at azithromycin concentrations chosen as a function of the isolates' MIC: 64, 32, 16, 8, 4, 2, and .5 mg/L. Two growth controls (10% DMSO in BHI and BHI only) were run in parallel.

Pharmacodynamic Modeling

An integrated PK/PD approach was used to quantify the effect of azithromycin against the clinical isolates. The area under the CFU curve (AUCFU) for each azithromycin concentration was normalized by the AUCFU in the absence of drug to obtain the log ratio area **(Equation 1).**

Equation 1:

$$Log\ ratio\ area = \log_{10} \frac{AUCFU_{drug}}{AUCFU_{control}}$$

Plots of the log ratio area vs. azithromycin concentration were then constructed, and azithromycin's activity (E) was modeled by the Hill function **(Equation 2)**, where E_{max} is the maximal drug effect, EC_{50} is the concentration required to obtain half the maximal effect, C is the concentration, E_0 is the effect in the absence of drug, and H is the sigmoidicity constant (version 12, Systat Software Inc., San Jose, CA). Overall model fits were analyzed based on coefficients of determination (*R^2).*

Equation 2:

$$E = E_0 - \frac{E_{max}\, x\, (C)^H}{(EC_{50})^H + (C)^H}$$

Hollow Fiber Infection Model (HFIM)

HFIM was used to evaluate how clinically relevant azithromycin regimens affect bacterial burden of lung infection over 240 hours (19, 20). The HFIM utilized cellulosic cartridge C3008 (FiberCell Systems, Frederick, MD). Essentially, bacteria colonize the extracapillary space of the cartridge while nutrients and antibiotic are exchanged through hollow fibers. Apart from growth controls for both clinical isolates, azithromycin dosing regimens were administered in a two-tiered fashion for 5P28H1 to achieve identical areas under concentration-time curve (AUC) as those deemed physiologically relevant. We simulated azithromycin pharmacokinetics in both serum and alveolar macrophages. A high-burden lung infection was simulated in this model, using a starting inoculum of 10^8 CFU/mL.

Serum drug concentrations for hollow fiber modeling were determined by digitizing data from a 5-day Z-pack pharmacokinetics study in human subjects (21). Azithromycin protein binding was estimated to be 51% at .02 mg/L and 7% at 2 mg/L via the FDA package insert. We assumed a linear correlation between these two values

and determined the free amount of azithromycin in plasma accordingly. Azithromycin concentrations in alveolar macrophages have been previously determined (15). Using Sigma Plot (Systat Software Inc., San Jose, CA), these estimations were used to calculate the AUC in order to determine the effective drug concentration for use in HFIM over 10 days (shown in **Equation 3**).

Equation 3

$$Azithromycin\ Concentration\ for\ HFIM = F_{unbound}\ x \left(\frac{AUC_{0-240hours} mgL/hour}{240\ hours}\right)$$

Four models were analyzed: **A.** 5P28H1 growth control **B.** 5P54H1 growth control **C.** 5P28H1 + .063 mg/L Azithromycin (AUC-matched serum concentration) **D.** 5P28H1 + 80.6 mg/L Azithromycin (AUC-matched alveolar macrophage concentration). Samples were serially drawn over 240 hours and plated on chocolate agar. To determine shifts in population dynamics, population analyses were performed on all models over 240 hours by plating samples on chocolate agar containing 1, 2, 4, 8, and 16 mg/L azithromycin.

Results

Static time kill results for 5P28H1 ($MIC_{azithromycin}$: 2 mg/L) are shown in **Figure 1**. By 48 hours, all concentrations of azithromycin above .5 mg/L achieved complete killing for isolate 5P28H1. The three highest concentrations of azithromycin (64, 32, and 16 mg/L) exhibited the most rapid bactericidal effect, completely killing 5P28H1 by 24 hours. At the only azithromycin concentration tested beneath the MIC of 5P28H1 (.5 mg/L), minimal killing was observed. The growth controls for 5P28H1 displayed an approximate 1-log decrease in CFU/mL by 48 hours. A concentration-dependent effect was visible, as decreases in azithromycin concentration led to increased time-to-complete killing. The bactericidal activity of azithromycin against 5P54H1 ($MIC_{azithromycin}$: 16 mg/L) is shown in **Figure 2**. Concentrations of azithromycin at or above the MIC of 5P54H1 exhibited complete killing by 24 hours. At a concentration of 8 mg/L (half the MIC of 5P54H1), azithromycin appeared to effectively eliminate viability by 28 hours. At 4, 2, and .5 mg/L of azithromycin, initial reductions in viability were essentially nullified after 8 hours.

To analyze the pharmacodynamic interaction between azithromycin and our two strains, we fit a Hill-type function to our time-kill data. The Hill-type function and model parameters are displayed in **Figure 3.** The Hill-model displayed excellent model fits, as the R^2 values in both sets were greater than .99. The values for E_{max} and EC_{50} were higher in 5P54H1 (2.38 and 1.47, respectively) than in 5P28H1 (2.25 and .858, respectively), indicating azithromycin to be both less efficacious and less potent against 5P54H1.

In order to simulate human infection, we performed HFIM on 5P28H1 and 5P54H1. The results of the HFIM are displayed in **Figure 4.** It is evident that both

5P28H1 and 5P54H1 in the absence of antibiotic were able to grow in the HFIM, achieving bacterial densities greater than 10^9 CFU/mL. While 5P28H1 was previously shown to survive in the conditions of the Hollow Fiber cartridge, this was the first test of 5P54H1 viability in the HFIM. The AUC-matched serum concentration (.063 mg/L) of azithromycin was virtually unable to produce any killing effect against 5P28H1. However, at a concentration mimicking that within alveolar macrophages (80.6 mg/L), 5P28H1 exhibited complete loss of viability by 26 hours. A population analysis of 5P28H1 performed throughout the HFIM did not reveal any significant phenotypic changes in antibiotic susceptibility over 10 days (data not shown).

Discussion

To our knowledge, this is one of the first studies examining azithromycin pharmacodynamics against resistant NTHI in COPD patients. In this study, we elucidated the pharmacodynamics of azithromycin against a pair of clinically persistent NTHI isolates. We determined that azithromycin is effective in killing 5P28H1 and 5P54H1 in a dose-dependent fashion. As the viability of 5P54H1 had not been tested in the HFIM previously, we provided proof-of-principle for this isolate to be further examined in the model.

The threat of antibiotic resistance is particularly alarming in the context of COPD. It has been shown that alveolar macrophage activity and other bacterial lung defense mechanisms are severely inhibited in COPD patients (22-24). Considering that chronic bacterial infection may propel lung damage in COPD and that lowered lung defenses of COPD patients may foster persistent bacterial colonization, a formidable cycle of disease progression exists. COPD patients often rely on both antibiotic and corticosteroid treatment to alleviate symptoms. When antibiotic treatments fail, COPD patients may experience rapid acceleration of disease and significant social, physical, and financial costs. Understanding the pharmacodynamics of azithromycin against clinical strains of NTHI may help tailor future treatment regimens and prevent the development of macrolide resistance.

Multiple challenges exist in correlating antimicrobial susceptibilities with clinical therapeutic efficacy. Antimicrobial susceptibilities for NTHI, including those for azithromycin, are derived utilizing microbiological breakpoints (13). These breakpoints are determined based on drug-effect differences between an azithromycin-naïve

population of *H. influenzae* (wild-type) and a population exhibiting decreased azithromycin activity (13, 25). However, microbiological breakpoints may not correlate with clinical outcome and may not be useful for individual case management (13). Clinically relevant breakpoints incorporate a number of factors, including: results of clinical studies, PK information regarding tissues and fluids, dose-effect relationships, and mathematical modeling (13). The possible disconnect between microbiological susceptibility breakpoints and clinically relevant breakpoints was evident in our study. Isolate 5P28H1, though deemed susceptible to azithromycin through microbiological breakpoints, was not cleared by the patient after four courses of azithromycin therapy. This disparity may have led to counter-selection and the genetic and phenotypic development of azithromycin resistance observed in isolate 5P54H1. Thus, our results highlight the need for clinically relevant breakpoints in *H. influenzae* susceptibility testing.

Our observation regarding the inability of simulated serum concentration exposures in the HFIM to kill 5P28H1 yields insight into a less-defined topic in azithromycin treatment: utility of serum AUC versus site-specific AUC metrics. It has been posited that site-specific azithromycin concentration for lower respiratory tract infections is better correlated with antimicrobial effect than serum or systemic measures (26, 27). Despite this, site-specific concentrations within the lungs and on epithelial surfaces remain poorly understood. Lucchi *et al.* determined the concentration of immediate and extended release azithromycin in epithelial lining fluid (ELF) in patients with lung cancer and found values ranging from 0 to 6.81 mg/L (15). In a separate study, healthy individuals were administered a single 500 mg dose (comparable to the

first dose in a Z-pak regimen) and the amount of azithromycin in ELF was undetectable (28). Thus, it appears that underlying health conditions as well as individual physiologic characteristics may affect the achievable azithromycin concentrations in ELF. To our knowledge, no studies have examined the concentration of azithromycin in ELF of COPD patients. Additionally, our population analysis of 5P28H1 during the HFIM revealed no significant changes in antibiotic susceptibility by day 10. Ultimately, this may implicate that multiple regimens of azithromycin were necessary to initiate the changes in 5P28H1 to increase its MIC 8-fold.

Utilizing azithromycin for COPD prophylaxis and treatment of a multitude of respiratory diseases could have far reaching consequences. Since commensal bacteria are also exposed to azithromycin during treatment, macrolide resistance may develop in the microbiota. This process may already be occurring, as multiple studies have found macrolide resistance in nasopharyngeal and oropharyngeal isolates after azithromycin regimens (12). This may facilitate the transfer of resistance elements between commensal and pathogenic bacteria, increasing the population burden of macrolide resistance. Azithromycin prescription has also been linked to the development of penicillin and multi-drug resistance (12). A Spanish study performed in 2002 found that macrolide consumption was more important in driving penicillin resistance than consumption of β-lactams (29). Therefore widespread use of azithromycin could drive an increase in resistance across drug classes, posing a significant threat to antimicrobial stewardship efforts and public health. Thus, broad scale azithromycin prescription practices may need reevaluation.

While this study sheds light on important issues facing the treatment and management of COPD exacerbations using azithromycin, we recognize a few limitations to our study. The first limitation is generalizability. The findings from a pair of isolates obtained from a single patient in Buffalo, NY may not be generalizable to all individuals chronically infected with NTHI. Additionally, we recognize that a broader array of physiologically relevant concentrations of azithromycin for time kill experiments and the hollow-fiber infection model could have been used. Therefore in future studies we hope to utilize a broader array of concentrations with physiological relevance.

Figures

Figure 1. Static Time Kill results for isolate 5P28H1 ($MIC_{azithromycin}$: 2 mg/L) evaluating the bactericidal activity of an array of azithromycin concentrations.

5P28H1

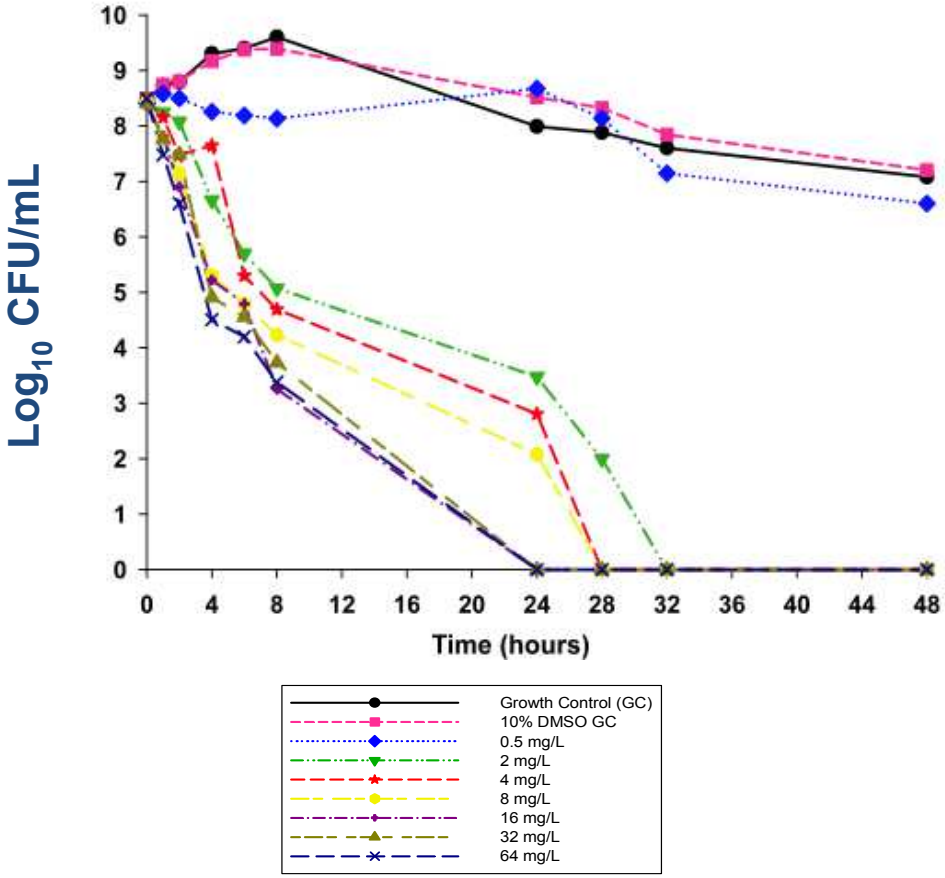

Log10 CFU/mL
10
9
8
7
6
5
4
3
2
1
0
0
4
8
12
16
20
24
28
32
36
40
44
48
Time (hours)
Growth Control (GC)
10% DMSO GC
0.5 mg/L
2 mg/L
4 mg/L
8 mg/L
16 mg/L
32 mg/L
64 mg/L

Figure 2. Static Time Kill results for 5P54H1 ($MIC_{azithromycin}$: 16 mg/L) evaluating the bactericidal activity of an array of azithromycin concentrations.

5P54H1

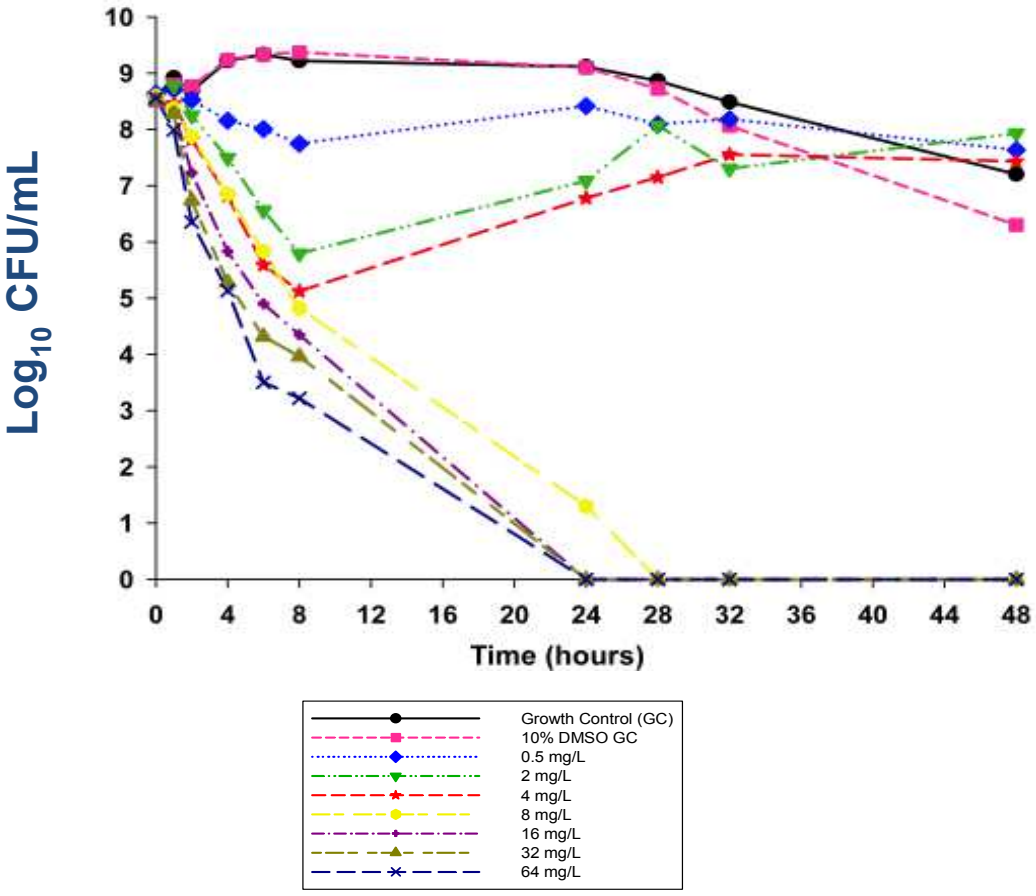
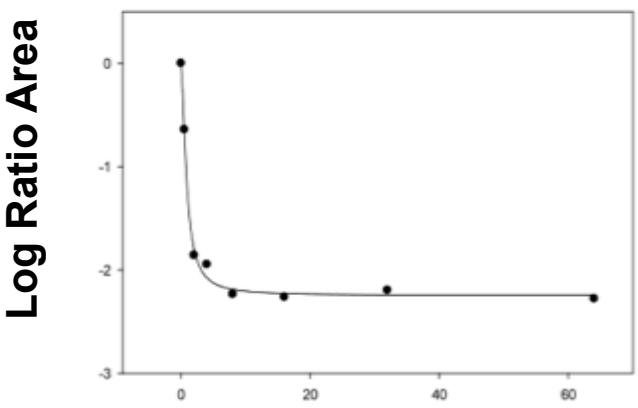

Log10 CFU/mL
10
9
8
7
6
5
4
3
2
1
0
0
4
8
12
16
20
24
28
32
36
40
44
48
Time (hours)
Growth Control (GC)
10% DMSO GC
0.5 mg/L
2 mg/L
4 mg/L
8 mg/L
16 mg/L
32 mg/L
64 mg/L

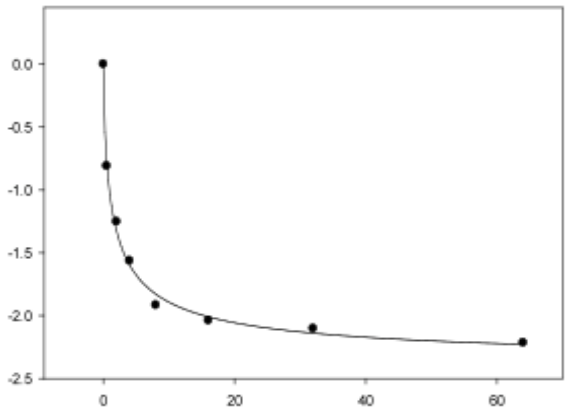
Figure 3. The Hill-type model fit for azithromycin concentrations versus log-ratio area for 5P28H1 and 5P54H1. Model parameters, including E_{max} and EC_{50} are included with percent standard error.

5P28H1



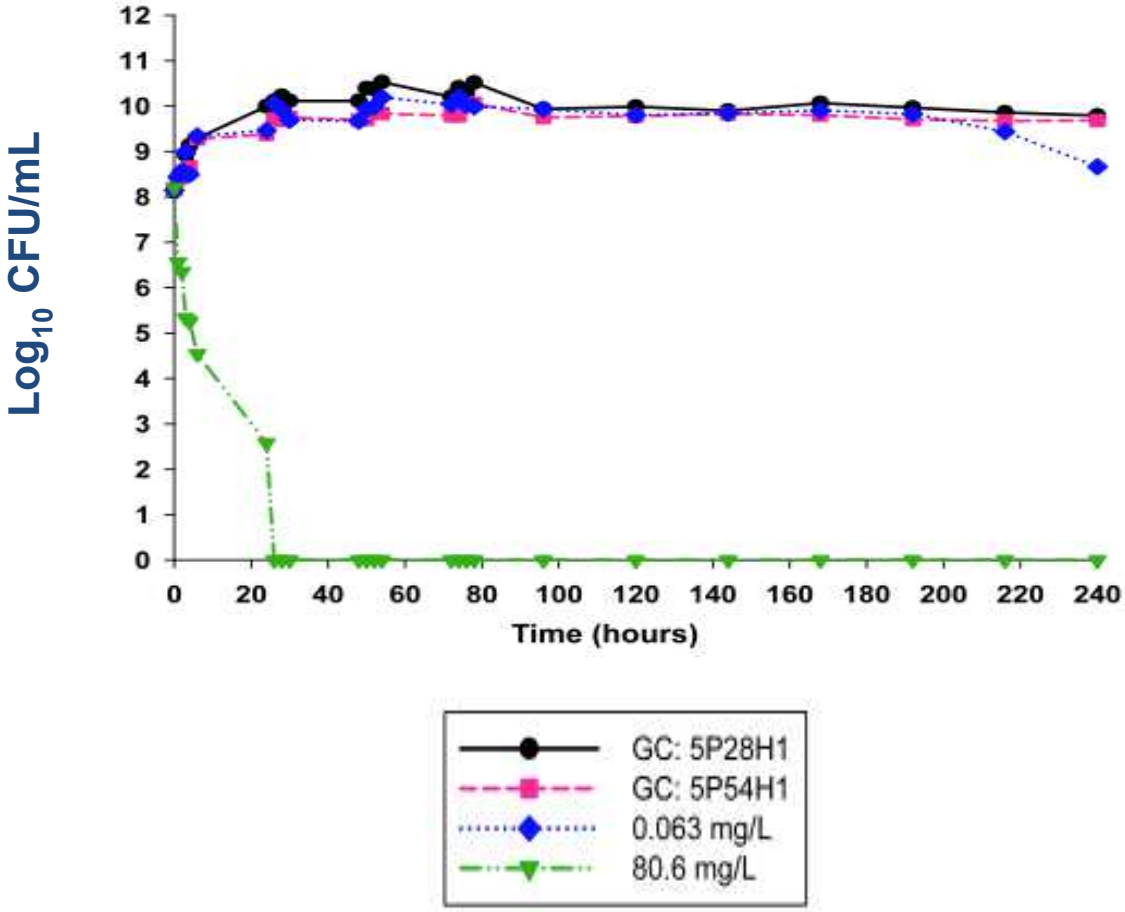
R^2	0.998
E_{max} (% SE)	2.25 (0.09)
EC_{50} (% SE)	0.858 (0.09)

5P54H1



R^2	0.998
E_{max} (% SE)	2.38 (0.14)
EC_{50} (% SE)	1.47 (0.28)

Figure 4. HFIM model results over 10-days (240 hours). Viable bacteria as determined by CFU/mL are plotted against time. Simulated concentrations of azithromycin in serum (.063 mg/L) and alveolar macrophages (80.6 mg/L) were tested against 5P28H1.


Log10 CFU/mL
12
11
10
9
8
7
6
5
4
3
2
1
0
0
20
40
60
80
100
120
140
160
180
200
220
240
Time (hours)
GC: 5P28H1
GC: 5P54H1
0.063 mg/L
80.6 mg/L

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