

EVALUATION OF DRUG COMBINATIONS AGAINST STATIONARY
PHASE AND PERSISTENT *BORRELIA IN VITRO*

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ABSTRACT

Lyme disease is the most commonly reported vector-borne disease in the United States. Although most patients respond well to antibiotic treatment, 10-20% suffer chronic symptoms of carditis, arthritis, and neurological impairment months after treatment. The cause of these symptoms is still undetermined, but there are many theories to explain this phenomena including autoimmune activation, the presence of antigenic debris, and treatment-resistant persistent bacteria. Evidence of treatment-resistant and non-culturable *Borrelia* has been seen in animals, but these results remain controversial.

Orally available, low toxicity drugs were tested in combination against *Borrelia burgdorferi in vitro* to determine if combined use would increase their activity. These combinations were tested against both stationary phase and persistent bacteria using a SYBR Green I/PI rapid viability assay, and were confirmed via epifluorescent microscopy. Several trends were seen among high activity combinations. Triple drug combinations including cefuroxime, a protein synthesis inhibitor [doxycycline, azithromycin, nitrofurantoin, etc.], and a free radical producing drug [methylene blue, artemisinin, etc.] had the highest activity against stationary phase cultures. However, drug combinations targeting DNA transcription [rifabutin] and either membrane permeability or homeostasis mechanisms [fluconazole, hydroxychloroquine, etc.] were highly effective against amoxicillin-treated persisters. These results suggest these pathways may be key targets for future *Borrelia* treatments.

PREFACE

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INTRODUCTION

Borrelia burgdorferi sensu stricto is the causative agent of Lyme disease, the most commonly reported vector-borne disease in the United States.² Lyme disease has an incidence rate of 8.6 confirmed cases per 100,000 people in the US, though recent reports have suggested that unreported cases may be as high as 300,000 a year.^{1,2} The incidence of reported Lyme cases in endemic regions has increased steadily from 1992 to 2006, and this trend is expected to continue.³ The number of infections in the United States is expected to increase in the coming years, causing this disease to be an emerging public health threat.

Lyme disease does not have a uniform geographic transmission, but instead is found in a scattered distribution of highly endemic foci.⁴ Disease transmission is seasonal, with the majority of reported cases occurring between June and August.³ This seasonality is likely the result of both an increase in tick foraging behavior and exposure to human hosts during these months. Various environmental factors such as the projected increase in temperature and humidity during winter and spring months is expected to result in a longer disease transmission window and the expansion of Lyme foci into new geographic regions.⁵ This disease expansion is projected to increase the amount of infections in the United States in the upcoming years.

Lyme disease is vectored by members of the *Ixodes* tick family and is transmitted to humans from rodents, birds, and various small mammals depending on the geographic region.⁶ As the *Ixodes* ticks undergo multiple developmental stages from nymph to adult, they are able to maintain infection with *Borrelia burgdorferi* transstadially.⁷ However, these ticks are unable to efficiently pass the bacteria transovarially to their offspring, so

the nymphal and larval ticks must acquire the bacteria through a blood meal taken from a previously infected host.⁸ The bacteria can then be transferred to human hosts during blood feeding by an infected tick.

Each Lyme endemic geographic foci has a slightly different enzootic disease cycle. The first discovered Lyme endemic foci is in the Northeast United States, where the transmission occurs between Maine and Maryland, as well as in Wisconsin and Minnesota.⁴ The primary vector in this area is the deer tick *Ixodes scapularis*. In this region, nymphal ticks transmit the infection primarily through white-footed mice and chipmunks, maintaining the disease enzootically.⁶ The adult *Ixodes* ticks feed primarily on larger mammals such as humans and deer, which are both incidental hosts and cannot further the disease transmission cycle.⁶ Despite the presence of incidental hosts in this cycle, this enzootic cycle is very efficient and results in the highest rates of *Borrelia* infection in ticks among the endemic foci in the US.

In the Pacific Northwest, the primary tick responsible for human infection is the *Ixodes pacificus*.⁴ In comparison to the *Ixodes scapularis*, this tick population tends to have lower infection rates, resulting in lower levels of Lyme disease in this region.⁴ The primary vectors and hosts for the Lyme disease life cycle in Europe and Asia are still uncertain, as the ticks in these regions tend to feed on a variety of hosts and show less species-specific feeding than the American tick species.⁴

Our current ability to accurately diagnose Lyme disease is suboptimal, resulting in confusion over diagnoses and a likely underreporting of case numbers. This diagnostic difficulty is partially due to the multi-stage nature of this disease, as each stage requires different diagnostic methods with variable rates of success. Only an early stage Lyme

disease infection can be diagnosed clinically, often through the presence of a characteristic rash known as an erythema migrans.⁹ Clinicians use a variety of methods to diagnose later stages of Lyme disease, but there is debate about the accuracy of these methods.

While most bacterial infections are traditionally diagnosed via bacterial culture, this method is not feasible for Lyme disease in many clinics due to the low yield from patient samples and long culture times.¹⁰ The best results from bacterial culture occur when the sample is collected directly from the erythema migrans, but even this yields highly variable results, with successful culturing occurring only 5-43% of the time.¹¹ Culturing the bacteria from patient blood or synovial fluid has been even less successful.¹¹ A clinician may also elect to perform a lumbar puncture if they suspect neurological involvement or to rule out other infections.¹² These samples are often tested using polyvalent ELISAs, however the interpretation of these results varies between laboratories. According to the guidelines set by the Infectious Disease Society of America (IDSA), patients experiencing symptoms for more than 4 weeks must have both IgM and IgG *Borrelia*-specific responses to be considered disease positive.¹³ However, this method is unable to determine if a positive result is due to a current or past infection. While researchers routinely use PCR to determine the presence of *Borrelia* DNA in patient samples, this method has not yet been approved for clinical diagnosis.¹³

Lyme disease is a multisystem disorder that can affect many parts of the body. In the early stages of infection, the bacteria are localized at the cutaneous site of the tick's blood meal where they form a characteristic target-shaped rash called the erythema migrans.⁹ This rash often appears 3-32 days after the tick bite and gradually expands as

the spirochetes disseminate through the skin, before fading 3-4 weeks later regardless of treatment.⁹ Despite the use of this rash for clinical diagnosis, only 50-75% of patients will exhibit this characteristic symptom, complicating their Lyme diagnosis.¹⁴ At this early stage of infection the rash is often accompanied by non-specific symptoms such as fever, malaise and regional swelling of the lymph nodes, which do not often aide the clinician in diagnosis.⁹

After early localized infection the patient develops an early stage disseminated infection, at which stage the spirochetes have disseminated further from the cutaneous injection site. During this stage approximately half of patients will experience multiple secondary erythema migrans on the skin, which are usually smaller and have less gradual size increase compared to the primary rash.⁹ It has been shown that *Borrelia* are also able to penetrate the central nervous system at this stage of infection.¹⁴ In one study *Borrelia* DNA was found in the cerebrospinal fluid of 2/3 of patients at the early disseminated phase.¹⁴ As with the central nervous system, *Borrelia* are able to begin dissemination throughout the body at this stage and can infect multiple organs. This results in a variety of recurrent symptoms including arthritis, meningitis, carditis, encephalopathy and neurological impairment.¹⁵ These symptoms often clinically manifest through complaints of general malaise, headaches, sore neck and joint pain.¹⁵

Late stage Lyme disease, also called the disseminated stage of infection, occurs months to years after the initial infection in patients that were either untreated or treatment-resistant.¹⁵ Patients with late stage Lyme disease often experience similar symptoms to patients with early Lyme disease, however the time between these episodes of these recurrent symptoms is increased in late stage patients.⁹ Chronic arthritis normally

develops in patients with late stage infection, with most of the inflammation occurring around major joints, such as the knees.⁹

For patients with early stage Lyme disease, antibiotic treatment is typically very effective. The treatment for early stage Lyme disease is an antibiotic regimen consisting of doxycycline, amoxicillin, or cefuroxime axetil for 10-21 days depending on the severity of symptoms.¹³ In areas with greater than 20% infection rate of *Borrelia* in the tick vectors, doxycycline is also approved for use as a prophylaxis treatment after a confirmed tick bite before a positive diagnosis.¹³ If the patient is unable to take these drugs, macrolide antibiotics are generally recommended for use as second line drugs only.¹³ However, Lyme disease patients with cardiac or neurological complications are treated ceftriaxone intravenously for 14 days instead of taking the oral regime.¹³

Late stage Lyme patients are treated with the same antibiotic regimen as early stage patients, for a longer length of 28 days. However, if these patients have neurological involvement, the use of an intravenous beta-lactam antibiotic is recommended rather than the oral regime.¹³ While intravenous antibiotic therapy is more expensive and has a higher risk of complications than an oral regime, patients treated with intravenous ceftriaxone or penicillin G have been found to be less likely to develop neuroborreliosis.¹³ Due to this finding, patients with serious complications such as carditis or neurological impairment are recommended intravenous therapy rather than the commonly used oral antibiotics.

Despite the majority of patients seeing success with the previously outlined antibiotic regimens, 10-20% of patients experience symptoms such as fatigue, muscular

pain, and neurologic impairment for longer than 6 months after treatment. These patients are diagnosed with Post-Treatment Lyme Disease Syndrome (PTLDS), sometimes called Chronic Lyme disease, though this diagnosis is still controversial.^{1,16} Patients who have been diagnosed with PTLDS tend to have significantly lower quality of life and decreased functional abilities when compared to non-PTLDS Lyme patients.¹⁷

The evidence for Post-Treatment Lyme disease in both humans and animals is varied and highly controversial. Laboratory mice have been used as a model for PTLDS, as they are a natural host for *Borrelia* in the wild.¹⁸ However, mice do not exhibit the same cardiac or arthritic symptoms as humans with chronic Lyme infections, making them a suboptimal model for PTLDS.¹⁹ Despite dissimilar symptoms, the presence of *Borrelia* DNA was confirmed in the tissue of chronically infected mice up to 9 months after treatment via both PCR and xenodiagnoses.^{19,20} Despite the presence of DNA in these mice after treatment, live spirochetes were unable to be cultured from these samples.^{19,20} These results have come under criticism, however, from other groups over a lack of standardization of inoculum size, insufficient antibiotic treatment, and other methodological concerns.²¹

Further studies in ceftriaxone-treated infected mice have shown that *Borrelia* infections in mice can have a resurgent pattern.²² The treated mice tested consistently negative for *Borrelia* through microscopy, culture, xenodiagnoses and PCR from 1 to 8 months after infection.²² However, the presence of non-culturable *Borrelia* was discovered in treated mice 12 months after infection through PCR.²² At month 12, the levels of *Borrelia* flaB DNA in the treated mice were similar to the levels found in the saline-treated infected control group.²² These results suggest that *Borrelia* actually

replicated after antibiotic removal in a form that is not culturable and that mice may be used as a viable model for long-term treatment study of PTLDS.

Evidence of PTLDS in humans has been similarly controversial. One study showed culturable *Borrelia* could be recovered from the blood of 43 out of 47 patients presenting with PTLDS symptoms after treatment with a third generation cephalosporin.¹⁰ Another group were able to culture live *Borrelia* from patient skin and blood samples months after antibiotic treatment.²³ However, these results were deemed by some to be unrepeatable and no cultures have been reliably grown from patient plasma or cerebrospinal fluid samples.^{10,24} *Borrelia* DNA has been found to positively correlate to active infections, and is cleared from the body quickly following infection, making it a positive indicator of active infection.²⁵ Using this marker, *Borrelia* DNA was found to be excreted in patient urine samples previously treated for Lyme disease, indicating the possibility of chronic infection.¹⁵

Due to the inconclusive nature of results concerning the existence of PTLDS in humans, the IDSA does not officially recognize PTLDS and does not recommend any long-term use of antibiotics for treatment.¹⁴ The basis for this recommendation comes from four studies examining the use of long-term antibiotics to treat patients with PTLDS symptoms. Krupp *et al.* studied the effect of long-term treatment with one month of intravenous ceftriaxone on fatigue, mental acuity and clearance of bacterial antigens. This study found significant improvement in patient fatigue, but not in the two other criteria studied.²⁶ A study by Fallon *et al.* also examined the effect of intravenous ceftriaxone on memory and cognitive function in PTLDS patients with a longer treatment period of 10 weeks. An improvement in mental acuity was seen at the end of the treatment period, but

this improvement was not seen upon testing at 14 weeks after treatment. However, this study contained only 32 patients, and should be repeated with more participants before definitive conclusions can be drawn.^{27,28} Klempner *et al.* studied the effect of adding 2 months of doxycycline treatment to a one month of intravenous ceftriaxone regimen for both IgG seropositive and seronegative PTLDS patients. After 180 days, the patients were shown to have no significant benefits from the doxycycline treatment.²⁴ However, these trials have been criticized for being underpowered and using criteria that exceeded the minimum clinically important differences, which could obscure any treatment effects.²⁸ All of these studies concluded that the benefits of long-term antibiotic treatments for Lyme did not outweigh the risks associated with the treatments.^{24,26,27} A review of all four trials by Delong *et al.* has deemed these statements to be unsubstantiated by these trials, and warns against using these trials to rule out treatment possibilities in the clinic.²⁸

Those who believe in the possibility of PTLDS have developed many theories to explain the lack of cultivable cells from these patient samples. It has been suggested that the similarity between the *Borrelia* membrane antigen OspA and the human adhesion molecule LFA-1 could result in the activation of the immune system against this self LFA-1 antigen and trigger an autoimmune reaction. This theory would explain the chronic arthritis often seen in PTLDS, but has been largely discredited as the primary reason for PTLDS.²⁹ Others believe that PTLDS symptoms are the result of coinfection with other untreated parasitic or bacterial infections such as *Babesia*, *Bartonella henselae*, and *Anaplasma*, all of which have been shown to be able to coinfect and be co-transmitted with *Borrelia burgdorferi* by ticks in laboratory conditions.³⁰

Another theory for PTLDS is the continued presence of antigens in immune protected sites in the body. It has been suggested that *Borrelia* may be able to evade the immune system long-term by migrating to immune protected sites such as the central nervous system, which would explain the neurologic impairment often associated with the disorder.³¹ Long-term immune evasion is also thought to occur due to altered *Borrelia* morphology *in vivo* that would alter the ability of the immune system to recognize the bacterial antigens through the formation of biofilms or blebbing, both of which morphologies can occur *in vitro*.³¹

The most studied theory for the existence of PTLDS symptoms in Lyme patients is the role of persistent bacteria that have survived previous antibiotic treatment. Persisters are a term used to define a heterogeneous subgroup within a bacterial population with phenotypic variants that allow for survival in the presence of antibiotics and other stressors while retaining their genetic susceptibility.³² These persistent bacteria are thought to be generally dormant in their resistant form, though they can often revert back to their original phenotype and resume growth.³² The presence of persistent *Borrelia* in PTLDS patients would explain the presence of bacterial DNA and immune activation without the presence of culturable cells, as has been indicated in many studies.^{15,19,20} One study claimed that they were able to grow persistent atypical forms of *Borrelia* from 60-80% of PTLDS patient samples using special growth conditions.³³ These atypical forms of *Borrelia* were found to be less motile than traditional spirochetes and exhibited increased levels of blebbing *in vitro*.³³

Further studies have linked the morphology of *Borrelia* to its ability to become persistent. Three morphologies of *Borrelia* have been identified *in vitro*; spirochetal

forms, spheroplast or biofilm-like forms, and round bodies or coccoid forms.^{34,35} *In vitro*, *Borrelia* are predominantly spirochetal in growing log phase cultures.³⁴ Once the culture reaches stationary phase, the coccoid and biofilm forms became more abundant.³⁴ These coccoid forms have also been shown to form *in vivo* after exposure to Lyme antibiotics.³⁶ These atypical forms of *Borrelia* have also been shown to have altered drug susceptibilities *in vitro*, with the coccoid and biofilm-like microcolony forms being the least susceptible to antibiotic treatment.^{34,37} These persistent atypical forms were shown to be able to withstand exposure to commonly used Lyme antibiotics at higher concentrations than can be achieved clinically.^{34, 38}

The commonly used Lyme antibiotics have been found to be highly effective against actively growing log phase *Borrelia* cultures, but have little activity against stationary phase populations.³⁴ As stationary phase cultures contain higher amounts of atypical persistent forms of *Borrelia in vitro*, these cultures have been used as a model for persistent and late stage Lyme infections. However, it is important to note that the age of the culture is important in its viability as a model for PTLDS and late stage Lyme disease, as persister development in a culture has been shown to be age-dependent.³⁴

Previous drug screens of a FDA-approved drug library (Johns Hopkins Clinical Compound Library version 1.3) and the National Cancer Institute Compound Library have been used to identify drugs with higher activity against stationary phase *Borrelia* cultures than the commonly used Lyme antibiotics.^{34,39} These previous library screens identified hits with high activity against stationary phase cultures including daptomycin, clofazimine, cefoperazone, sulfa drugs, daunorubicin, mitomycin c, and doxorubicin.^{34,39} However, despite these drugs' high activity against stationary phase *Borrelia*, these drugs

also have high toxicity and must be administered parentally or intravenously, making them both inconvenient and potentially dangerous for use in the currently controversial field of PTLDS patient treatment.³⁹

In order to address this issue, I chose to examine 13 drugs that were orally bioavailable, had low toxicity, and were previously shown to have high activity against stationary phase *Borrelia* populations (<60% viable cells remaining after antibiotic exposure).⁴⁰ These drugs were tested both in double and triple drug combinations in order to study the effects of a combinatorial approach to *Borrelia* antibiotic treatment. The drug combinations were tested against both stationary phase *Borrelia* and persistent *Borrelia* populations, the latter of which was created through previous treatment with amoxicillin. Using this method, I was able to examine the activity of these drugs against bacterial populations that were partially and fully composed of persistent bacteria in order to gain better understanding of which bacterial subpopulation each drug was the most active against.

The use of combination therapy to treat disease is currently being employed in many areas, most notably to combat the HIV epidemic, cancer, malaria, and drug-resistant tuberculosis.⁴¹ Along with increased activity against these pathogens, the use of multiple drugs is thought to slow the development of genetic drug resistance within a population.⁴¹ Despite the use of drug combinations in other fields, the use of multiple antibiotics against Lyme disease has still not been fully examined, with current IDSA guidelines only recommending single drug therapy.¹³ However, the recommendation against the use of drug combinations for Lyme disease is based primarily on lack of proven efficacy and toxicity concerns, which I have addressed in this study through the

use of low toxicity drugs that are frequently used effectively in combination for other diseases.¹³

The International Lyme and Associated Diseases Society (ILADS) has recently published their own guidelines for treatment against Lyme disease that differs from the IDSA's.⁴² This society's guidelines suggest longer term treatment for early Lyme patients, with 4-6 weeks of first line antibiotic treatment rather than the IDSA's recommended 10-21 days.⁴² This society's guidelines also suggest continuation of treatment until patients are non-symptomatic, and continued long-term antibiotic treatment of relapsed Lyme patients.⁴² Importantly, the ILADS guidelines give clinicians more freedom in selecting treatment options, and mention combination antibiotic treatments as a possibility for patients not sufficiently treated by long-term single drug therapy.⁴² However, despite this allowance for doctors to prescribe drug combinations to Lyme patients, the ILADS does state that there is currently only very low quality evidence regarding the efficacy of drug combinations against this disease.⁴² There is a clear need for studies examining the effect of antibiotic drug combinations against *Borrelia* both *in vitro* and *in vivo*, a need this study will attempt to fill.

In order to more closely mirror the effects of these drugs in the human body, the chosen drugs were tested as concentrations close to the maximum concentration in patient plasma (C_{max}). However, it is common practice in drug testing to use a drug concentration of at least 2 µg/mL, so any drug that had a lower C_{max} value than this standard was tested at 2 µg/mL.⁴³ The only drugs that were used at concentrations that greatly differed from their C_{max} value were daptomycin and the triple combination of daptomycin + cefoperazone + doxycycline, due to the use of these drugs as a positive

control. These drugs were previously found to be among the most highly active drugs against stationary phase *Borrelia* populations at the concentration of 10µg/mL.³⁵

Therefore, the inclusion of these drugs as positive controls in this study allowed for comparison between the antibiotic killing of these drug combinations against previously published data.

MATERIALS AND METHODS

Bacterial strains and culture methods

Borrelia burgdorferi strain B31 (ATCC35210) was obtained from the American Type Tissue Collection (Manassas, VA, USA). All bacteria used in this study were passaged no more than 7 times. The cultures were grown in BSK-H media (HiMedia Laboratories, Mumbai, India) supplemented with 6% rabbit serum (Sigma Aldrich, St. Louis, MO, USA), that was filter-sterilized using a 0.2 µm filter. The *Borrelia* cultures were incubated in a capped 50 mL conical tube (BD Biosciences, CA, USA) for at least 7 days at 33°C without shaking, until the culture reached stationary phase. If the culture was slow-growing, the transition to stationary phase was determined both visually and through the use of microscopic counting. After the culture reached stationary phase, half of the 50 mL culture was aliquoted into 96 well plates for drug testing, with 100 µL of culture in each desired well. The remaining culture was treated with 6 µg/mL of amoxicillin for 6 days to create a culture of persistent *Borrelia*. After amoxicillin treatment the persistent bacterial cell suspension was aliquoted into 96 well plates with 100µL of cells in each desired well for drug testing, without washing of the bacterial culture to remove the amoxicillin.

Drug Testing against Stationary phase and Persistent *Borrelia* populations

The drugs used in the study were diluted from filter-sterilized individual stock concentrations. The drugs were diluted so that 2 μ L of the diluted drug solution added to 100 μ L of the bacterial culture would result in the desired drug concentration per well shown in Table 1. For double and triple drug combinations, 2 μ L of each of the diluted drugs were added to 100 μ L of the bacterial culture in the 96 well plate. After the drugs were added to the bacterial cultures in the 96 well plates, the plates were sealed and stored in a high humidity environment at 33°C for 7 days without shaking. After 7 days, the seal was removed and the culture underwent viability testing.

SYBR Green I/PI Viability Testing

The treated bacterial cultures underwent viability testing using a combination of a SYBR Green I/PI rapid viability assay along with epifluorescent microscopy, as previously described.⁴⁴ The SYBR Green I/PI assay works as follows: SYBR Green I is a green permeant dye that stains all cellular DNA, whereas propidium iodide (PI) is an orange-red impermeant dye that stains only the DNA of dead or damaged cells with a compromised cell membrane. Thus live or viable cells with intact membranes will be stained green by SYBR Green I, while damaged or dead cells with a compromised membrane will be stained orange-red by PI. The SYBR Green I/PI assay can be used to measure the viability of bacteria in the sample well of 96-well plates. A microplate reader is used to measure the green: red fluorescence ratio, which determines the ratio between live and dead cells, respectively, in the sample. The viability counts of wells with a similar or lower green: red fluorescence ratio than that of the positive control triple drug combination daptomycin + cefoperazone + doxycycline were confirmed using

epifluorescent microscopy. Wells that had media discoloration were also confirmed via microscopy, as the discoloration was seen to effect the plate-reader determined fluorescence ratio.

Table

1. Pharmacokinetics of the drugs screened against stationary phase and persistent *Borrelia burgdorferi* populations.

	C _{max} * (µg/mL)	Oral Bioavailability* (%)	Drug screen concentration (µg/mL)
Artemisinin	0.6	30	2
Methylene Blue	3.9	72.3	4
Nitrofurantoin	0.9	90	2
Azithromycin	0.6	38	2
Doxycycline	3.17	80	3
Rifaximin	0.004	0.4	2
Ciprofloxacin	2.9	79	3
Rifabutin	1.03	53	2
Cefuroxime	2.8	68	3
Pyrimethamine	1.2	90	2
Clofazimine	1.41	62	2
Hydroxychloroquine	0.004	74	2
Fluconazole	6.72	90	6
Daptomycin	6	NA	10

*All values were taken from current literature

Microscopy

The bacterial viability counts were confirmed using 10 μ L aliquots of treated culture from the indicated wells and examined using a Zeiss AxioImager M2 microscope and a SPOT slider color camera. Images of three random fields of view were captured for each sample to ensure an accurate viability count. These images were quantitatively analyzed using Image J software, and the average of the replicate viability counts were determined for each sample. All combinations were tested using two different *Borrelia* cultures as replicates. Both cultures were used as soon as they reached stationary phase, although differences in growing times meant that the cultures were of different ages when tested against the selected drugs. These replicates were both tested against the selected drugs both at stationary phase and after a 6-day treatment with 6 μ g/mL amoxicillin without washing to create a persistent population.

Fluorescence Data Analysis

The SYBR Green I/PI green: red fluorescence ratios were transformed to cell viability counts using the previously published linear regression method.⁴⁴ In order to determine the equation used to transform the raw fluorescence data to residual viable cell counts, the fluorescence values of *Borrelia* cultures with fixed amounts of live: dead cells were tested. An aliquot of 7 day old stationary phase *Borrelia* culture was autoclaved for at 121°C for 15 minutes to make a ‘dead’ culture. Samples of this autoclaved culture were added to samples taken from the same stock culture at the fixed live: dead ratios of 0:100, 20:80, 50:50, 80:20 and 0:100 μ L of live: dead culture. These samples then underwent viability testing using the SYBR Green I/PI method and the subsequent green: red ratios were determined.

To further ensure accuracy of this cell viability equation, 10 μ L aliquots from each of the live: dead mixed cultures were analyzed using the previously described method for epifluorescent microscopy cell viability counting. The raw fluorescence values for each live: dead ratio sample were then graphed against the microscopically determined cell viability number, and analyzed via linear regression (Figure 1). The equation of that linear regression line was then used to transform the rest of the green: red fluorescence data into an approximation of their cell viability count without requiring microscopic evaluation for each sample. As the drug combinations with the best activity against either stationary phase or persister populations were confirmed using microscopic analysis, which has been determined to be the most accurate and thorough method for cell viability determination, only the microscopic values will be presented in this study for the analysis of drug activity against the bacterial populations.

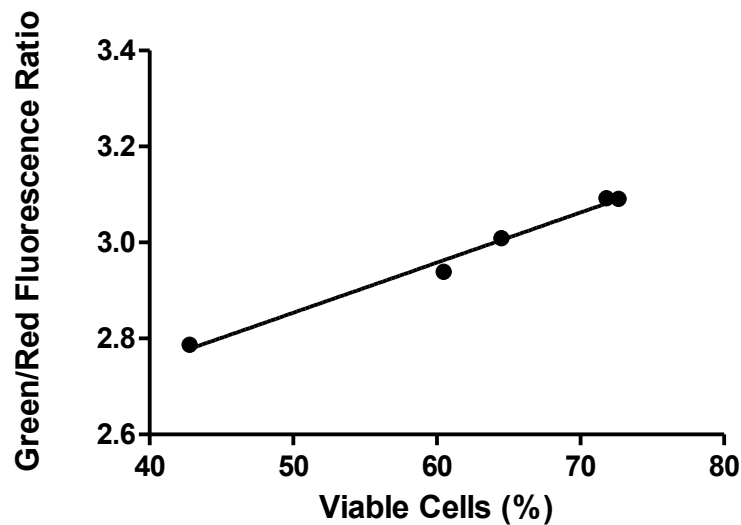


Figure 1. Linear relationship between the percent viable *Borrelia burgdorferi* cells and the green: red fluorescence. Fluorescence ratios of live: dead *Borrelia* suspensions measured via the SYBR Green I/PI rapid viability assay, and the viability counting performed via epifluorescent microscopy. The fluorescence cell viability equation was determined by linear regression

performed between the fluorescence ratio and the viability cell count. $R^2 = 0.9879$.

RESULTS

The drug combinations were first screened using the SYBR Green I/PI rapid viability assay, the data from which was transformed using linear regression analysis to the percentages of residual viable and killed cells remaining after drug treatment for both bacterial populations. The combinations that had activity similar to the positive control drug combination of daptomycin + cefoperazone + doxycycline were confirmed via microscopy.³⁵ Confirmation of a combination's activity was determined through comparison between the cell viability data for two replicates using different bacterial cultures of the same culture phase. Only the drug combinations with high enough activity to be confirmed via microscopy for both biological replicates against either population are presented as a validated hits. In order to account for inter-plate and inter-replicate variation, all post-treatment cell viability data was standardized against the cell viability data from the no drug control sample for the same plate.

Once the drug combinations were confirmed for both replicates, the drugs that had a proportion of less than 0.90 residual viable cells remaining when compared to the no drug control when analyzed via microscopy, were deemed to be active. Drugs found in multiple highly active combinations against a *Borrelia* population were deemed to be highly active against that population. These drugs were then broken down into three groups for mechanistic analysis: drugs with high activity against stationary phase populations, drugs with high activity against amoxicillin-treated persistent populations, and those with high activity against both populations.

Highly Effective Drugs against Stationary Phase Bacteria When Used in Combination

Artemisinin

Artemisinin is a sesquiterpene lactone whose antimalarial ability results from its endoperoxide bridge.⁴¹ While artemisinin's activation mechanism is still unclear, it is thought that artemisinin is primarily triggered by the presence of heme. *Borrelia* have been shown by some to accumulate iron intracellularly when grown in a high iron environment.^{45,46} However, recent studies have suggested that *Borrelia* intracellularly aggregate manganese at higher rates than iron. It has also been shown that while these bacteria can grow normally in iron-deficient media, the presence of manganese is essential for normal bacterial growth and motility. Gene homology also suggests that key genes in *Borrelia* such as SodA and Fur have over 50% homology to manganese-dependent enzymes in other bacterial species, further suggesting that manganese plays a larger role in the function of *Borrelia* than iron.⁴⁷ Studies using manganese-containing tetraphenylporphyrins as a synthetic heme model have shown that these compounds are able to activate artemisinin as effectively as heme.^{48,49} This suggests that the intracellular manganese in *Borrelia* may be able to activate artemisinin in lieu of endogenous heme.

Once activated, artemisinin's endoperoxide bridge causes the creation of reactive oxygen species which can be rearranged into carbon-centered radicals.⁵⁰ Both species of free radicals accumulate in neutrally charged lipids and thiols resulting in lipid peroxidation. The free radicals can also cause damage to DNA and metabolic enzymes resulting in disruption of DNA transcription and cellular metabolism.⁵¹ Artemisinin has also been suggested to be involved in protein alkylation, resulting in inhibition of cysteine proteases.^{41,52}

Artemisinin is highly effective against stationary phase *Borrelia* when used in triple drug combinations including cefuroxime (Table 2). Cefuroxime, a commonly prescribed antibiotic for early Lyme disease, is a second generation cephalosporin that functions through the inhibition of cell wall biosynthesis.^{53,54} Cefuroxime specifically disrupts cell wall repair mechanisms by inhibiting the pathways needed for the transportation and insertion of peptidoglycan into the cell wall.^{53,54} Cefuroxime appears to complement artemisinin activity for stationary phase *Borrelia*, likely due to cefuroxime's ability to disrupt the cell wall and allow for increased intracellular penetration of artemisinin or through production of reactive oxygen species.

The triple drug combination of cefuroxime + artemisinin + azithromycin had the highest activity against stationary phase *Borrelia* in this study. Azithromycin is a macrolide antibiotic that inhibits bacterial protein synthesis.⁵⁵ This combination's high antibacterial activity could result from the cell's inability to repair proteins damaged by the free radicals produced by artemisinin. The inhibition of protein synthesis by azithromycin could also result in defects in peptidoglycan production, resulting in increased penetration of artemisinin into the cells. Artemisinin also showed high activity in combination with cefuroxime and nitrofurantoin, a drug that creates electrophiles and alters protein function.⁵⁶ The damage caused by these electrophiles along with an inhibition of protein function could also affect mechanisms needed to repair proteins, resulting in accumulation of damaged and possibly toxic proteins, leading to cell death.

Table 2. Proportion of viable and killed cells after 7-day treatment with high activity artemisinin-containing combinations against stationary phase *Borrelia burgdorferi* when compared to no drug control

Controls		Viable Cells	Killed Cells	Std Error			
Drug Treatment	Conc. (µg/mL)						
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.97	0.03	0.05			
Daptomycin	10	0.88	0.12	0.08			
Daptomycin + Cefoperazone + Doxycycline	10	0.64	0.36	---			
Artemisinin + Drug treatment					Cefuroxime + Artemisinin + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std Error	Viable Cells	Killed cells	Std Error
Azithromycin	2	>0.9	<0.1	---	0.59	0.41	0.00
Nitrofurantoin	2	>0.9	<0.1	---	0.73	0.27	0.05
Fluconazole	6	>0.9	<0.1	---	0.82	0.18	0.00
Ciprofloxacin	3	>0.9	<0.1	---	0.83	0.17	0.05
Doxycycline	3	>0.9	<0.1	---	0.85	0.15	0.20
Clofazimine	2	>0.9	<0.1	---	0.87	0.13	0.01
Hydroxychloroquine	2	>0.9	<0.1	---	0.88	0.12	0.04
Pyrimethamine	2	>0.9	<0.1	---	0.89	0.11	0.07

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

Despite artemisinin's high activity against stationary phase populations in multiple combinations, it was not widely effective against amoxicillin-treated persistent populations. However, the triple drug combination of artemisinin + cefuroxime + rifabutin did have high activity against persistent *Borrelia* populations (Table 3). Rifabutin, an RNA synthesis inhibitor, functions by blocking the production of RNA. By preventing RNA synthesis, the bacteria are no longer able to produce proteins or regulate its gene expression to adapt to changing environments. Without the ability to alter gene expression in response to cellular damage, the cell would likely be less able to repair the damage caused by artemisinin.

Table 3. Proportion of viable and killed cells after 7-day treatment with high activity artemisinin-containing combinations against amoxicillin-treated persistent *Borrelia burgdorferi* when compared to no drug control

Controls							
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.78	0.22	0.27			
Daptomycin	10	0.70	0.30	0.11			
Daptomycin + Cefoperazone + Doxycycline	10	0.42	0.58	---			
Artemisinin + Drug treatment					Cefuroxime + Artemisinin + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed cells	Std. Error	Viable Cells	Killed cells	Std. Error
Rifabutin	2	>0.9	<0.1	---	0.69	0.31	---
Fluconazole	6	>0.9	<0.1	---	0.77	0.23	0.07
Methylene Blue	4	>0.9	<0.1	---	0.84	0.16	0.25
Hydroxychloroquine	2	>0.9	<0.1	---	0.85	0.15	0.30
Clofazimine	2	>0.9	<0.1	---	0.85	0.15	0.25
Doxycycline	3	>0.9	<0.1	---	0.86	0.14	0.15

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

The other artemisinin-containing drug combination with high activity against persisters was the combination of artemisinin + cefuroxime + fluconazole. Fluconazole is an antifungal drug that inhibits cytochrome p-450-dependent 14 α -sterol demethylase, resulting in an inhibition of ergosterol.⁵⁸ While normally exclusive to fungal membranes, *Borrelia* utilize ergosterol in their membranes to maintain fluidity and membrane integrity.⁵⁹ The membrane dysfunction caused by the addition of fluconazole could allow for increased cellular penetration of the other drugs, but it could also function through disruption of nutrition uptake. A previous study showed 5 genes encoding ion transporters were upregulated in persistent *Borrelia*, suggesting the importance of nutrient uptake in this population.⁶⁰ The inhibition of ergosterol synthesis could result in membrane dysfunction, altering the ability of the persistent cells to uptake nutrients necessary for survival in their dormant state.

Nitrofurantoin

Nitrofurantoin is a nitrofuran antibiotic commonly prescribed for urinary tract infections that functions through the creation of free radicals and protein dysfunction.⁶¹ Nitrofurantoin must be activated through intracellular reduction, which results in the

creation of electrophiles. These reactive species result in the inhibition of components of the citric acid cycle, along with causing damage to DNA, RNA and protein synthesis mechanisms.⁵⁵ Nitrofurantoin also functions as a diamide and causes the creation of non-native disulfide bonds in bacterial proteins, resulting in protein dysfunction. At sufficiently high concentrations, nitrofurantoin was also shown in *E. coli* to completely inhibit protein synthesis.⁵⁵

Nitrofurantoin is widely effective against stationary phase *Borrelia* populations when used in triple drug combination including cefuroxime (Table 4). The added effect of cefuroxime is likely due to an increased ability of the drugs to penetrate the cells, especially due to nitrofurantoin's required intracellular activation. The most effective nitrofurantoin combination against stationary phase *Borrelia* involves artemisinin and methylene blue, both drugs implicated in the production of free radicals. As described with cefuroxime + artemisinin + azithromycin, the addition of a free radical producing drug to a protein disrupting drug appears to be highly effective, likely by preventing the repair of damaged cellular proteins.

Drug combinations including both methylene blue and nitrofurantoin have high activity against both stationary phase and persistent *Borrelia* populations (Table 4) (Table 5). The addition of cefuroxime to this double drug combination increases the combinations' antibacterial activity against stationary phase populations, but has the opposite effect against persisters. Cefuroxime has been shown to have decreased activity against dormant cells when compared to growing log phase cells.³⁴ It has been suggested that both methylene blue and nitrofurantoin require cellular penetration for full antimicrobial activity.⁶² It is possible that cefuroxime's decreased activity prevents

methylene blue and nitrofurantoin from entering persisters as readily as stationary phase bacteria, which would result in reduced levels of oxidative damage within these cells.

The triple drug combination of cefuroxime + nitrofurantoin + rifabutin was highly effective against amoxicillin-treated persistent populations, while showing little activity against stationary phase populations (Table 5). Rifabutin functions through the inhibition of DNA transcription, which can result in the inhibition of protein synthesis.⁶³ The highly penetrative nature of rifabutin in combination with cefuroxime may allow for the increase in penetration necessary for nitrofurantoin to be intracellularly activated in the persistent *Borrelia* populations.⁶³ Once the drugs are activated, the combination of free radicals and protein synthesis inhibition likely inhibits the bacteria from repairing the oxidative damage, resulting in cell death.

Table 4. Proportion of viable and killed cells after 7-day treatment with high activity nitrofurantoin-containing combinations against stationary phase *Borrelia burgdorferi* when compared to no drug control

Controls							
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.97	0.03	0.05			
Daptomycin	10	0.88	0.12	0.08			
Daptomycin + Cefoperazone + Doxycycline	10	0.64	0.36	---			
		Nitrofurantoin + Drug treatment			Cefuroxime + Nitrofurantoin + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Methylene Blue	4	0.85	0.15	0.01	0.72	0.28	0.09
Artemisinin	2	>0.9	<0.1	---	0.73	0.27	0.05
Rifabutin	2	>0.9	<0.1	---	0.75	0.25	---
Clofazimine	2	>0.9	<0.1	---	0.86	0.14	0.03
Hydroxychloroquine	2	>0.9	<0.1	---	0.90	0.10	0.03

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

Table 5. Proportion of viable and killed cells after 7-day treatment with high activity nitrofurantoin-containing combinations against amoxicillin-treated persistent *Borrelia burgdorferi* when compared to no drug control

Drug Treatment	Controls						
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.78	0.22	0.27			
Daptomycin	10	0.70	0.30	0.11			
Daptomycin + Cefoperazone + Doxycycline	10	0.42	0.58	---			
Nitrofurantoin + Drug treatment					Cefuroxime + Nitrofurantoin + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Rifabutin	2	>0.9	<0.1	---	0.57	0.43	---
Methylene Blue	4	0.74	0.26	0.12	0.78	0.22	0.09

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by “---”.

Azithromycin

Azithromycin is a second-generation macrolide antibiotic derivative of erythromycin.⁵⁴ Azithromycin differs from other macrolides through the presence of methyl-substituted nitrogen in its macrolide ring, allowing for increased potency against gram negative bacteria.⁶⁴ The drug binds to the large bacterial ribosomal subunit,

inhibiting the synthesis of fully formed proteins.^{64,65} Azithromycin has also been implicated in biofilm prevention in *Pseudomonas aeruginosa*, a morphology associated with increased levels of persistence in *Borrelia* populations.⁶⁶

The two drug combinations with the highest activity against stationary phase populations include azithromycin and cefuroxime in combination with free radical producing drugs methylene blue and artemisinin (Table 6). As azithromycin appears to have high activity only when combined with cefuroxime, it is likely that the cefuroxime is required for cell wall disruption and increased drug penetration into the cells. The high activity against stationary phase populations further suggests the importance of the ability of stationary phase *Borrelia* populations to correct oxidative damage to both DNA and proteins intracellularly. Oxidative damage to DNA can result in the accumulation of toxic and misfolded proteins, and prevention of necessary cellular functions. The cells also require proteins for both maintenance of the cell wall as well as protection of other proteins from stress-related damage. Without these proteins, the cellular membrane and vital cell functions may be compromised.

Despite azithromycin's high activity against stationary phase populations, the drug has less activity against persistent *Borrelia* populations. Azithromycin only shows activity against persistent populations when in combination with hydroxychloroquine or methylene blue (Table 7). Methylene blue and azithromycin drug combinations likely functions through inhibition of repair mechanisms for damaged proteins. Genes involved in maintenance of protein integrity and repair have been found to be upregulated in doxycycline-treated persisters, including the genes coding for clpP and HSP proteins.⁶⁰

This gene upregulation suggests that protein management is important in persister maintenance.

Table 6. Proportion of viable and killed cells after 7-day treatment with high activity azithromycin-containing combinations against stationary phase *Borrelia burgdorferi* when compared to no drug control

	Controls						
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.97	0.03	0.05			
Daptomycin	10	0.88	0.12	0.08			
Daptomycin + Cefoperazone + Doxycycline	10	0.64	0.36	---			
	Azithromycin + Drug treatment				Cefuroxime + Azithromycin + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Artemisinin	2	>0.9	<0.1	---	0.59	0.41	0.00
Methylene Blue	4	0.88	0.12	0.11	0.64	0.36	0.13
Rifabutin	2	>0.9	<0.1	---	0.78	0.22	---
Clofazimine	2	>0.9	<0.1	---	0.78	0.22	0.02
Hydroxychloroquine	2	>0.9	<0.1	---	0.80	0.20	0.01

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by “---”.

Table 7. Proportion of viable and killed cells after 7-day treatment with high activity azithromycin-containing combinations against amoxicillin-treated persistent *Borrelia burgdorferi* when compared to no drug control

Controls							
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.78	0.22	0.27			
Daptomycin	10	0.70	0.30	0.11			
Daptomycin + Cefoperazone + Doxycycline	10	0.42	0.58	---			
		Azithromycin + Drug treatment			Cefuroxime + Azithromycin + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std Error	Viable Cells	Killed Cells	Std Error
Hydroxychloroquine	2	>0.9	<0.1	---	0.80	0.20	0.28
Methylene Blue	4	0.80	0.20	0.23	0.80	0.20	0.15

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

The addition of cefuroxime to this drug combination does not appear to alter the antibacterial activity, suggesting that the cefuroxime is neither required for intracellular penetration nor does it inhibit the function of methylene blue or azithromycin. It is possible that methylene blue's ability to cause lipid peroxidation provides the necessary increase in cell permeability, as was likely seen with the addition of fluconazole to

artemisinin.⁶⁷ This suggests that membrane penetration may be more important than cell wall penetration for antibacterial activity against persistent populations.

Hydroxychloroquine is a highly permeant drug that functions through alteration of the intracellular pH, which can have deleterious effects on cellular metabolism, DNA and proteins.⁶⁸ While hydroxychloroquine does not create free radicals like methylene blue, the rapid alteration of the intracellular pH likely results in the induction of similar stress, the repair of which is likely blocked by azithromycin. Stress response mechanisms have been found to be vital to persister formation in many different species.⁶⁹ This suggests that the intracellular damage created by these drugs in combination with inhibition of possible repair mechanisms may be affecting pathways vital for persister cell maintenance and survival.

Highly Effective Drugs against Both Stationary Phase and Persistent *Borrelia* When Used in Combination

Methylene Blue

Methylene blue is the only drug that in the screen with high activity against both stationary phase and persistent populations. Methylene blue is a photosensitive dye once used as an antimalarial that produces free radicals and hydroxides when exposed to light.⁶² These free radicals cause lipid peroxidation, resulting in the loss of membrane integrity, and possibly damage to the peptides in the bacterial cell wall.⁶⁷ If methylene blue is able to penetrate the cell wall, the drug can bind to and modify guanine residues, though the effect of this on inhibition of DNA replication has not been determined.⁷⁰

Methylene blue was found to be highly active against stationary phase *Borrelia* populations, being found in 4 out of the 5 most active drug combinations against

stationary phase populations (Table 8). Methylene blue showed significantly higher activity against stationary phase populations when used in triple drug combination including cefuroxime ($p < 0.05$), though the addition of cefuroxime does not significantly increase the combination's antibacterial activity against persistent populations ($p > 0.05$). It has been shown that the addition of amoxicillin to *Borrelia* populations inhibits cell wall synthesis, indicating that the cell walls of persisters may be inherently weaker than their stationary phase counterparts.⁶⁰ This further suggests that cellular wall penetration is more important for antibacterial ability against stationary phase than persistent populations.

Drug combinations including methylene blue had less than 0.90 residual viable cells remaining compared to the no drug control in all combinations except those involving artemisinin, hydroxychloroquine and fluconazole. It is possible that the similarity between the antibacterial mechanisms of these drugs for both membrane and intracellular damage resulted in competition for the drug targets rather than additive benefit. As with previously discussed combinations, free radical producing methylene blue has high activity in combination with cell wall disruptors and protein synthesis inhibitors such as doxycycline. However, methylene blue also appears to work well in combination with transcription inhibitors such as rifabutin, rifaximin and ciprofloxacin. However, these DNA transcription inhibitors did not have high antibacterial activity when used with other free radical producing drugs. This suggests that methylene blue may cause more oxidative damage to non-protein targets than either artemisinin or nitrofurantoin, as DNA transcription will have cellular effects beyond protein synthesis inhibition including mitochondrial damage or epigenetic changes in the bacteria. It is

possible that methylene blue's exclusive ability to modify guanine residues is the cause of this extra damage.

Table 8. Proportion of viable and killed cells after 7-day treatment with high activity azithromycin-containing combinations against stationary phase *Borrelia burgdorferi* when compared to no drug control

	Controls						
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.97	0.03	0.05			
Daptomycin	10	0.88	0.12	0.08			
Daptomycin + Cefoperazone + Doxycycline	10	0.64	0.36	---			
	Methylene Blue + Drug treatment				Cefuroxime + Methylene Blue + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Azithromycin	2	0.88	0.12	0.11	0.64	0.36	0.13
Pyrimethamine	2	0.81	0.19	0.06	0.69	0.31	0.08
Rifaximin	2	0.86	0.14	0.04	0.69	0.31	0.22
Nitrofurantoin	2	0.85	0.15	0.01	0.72	0.28	0.09
Rifabutin	2	>0.9	<0.1	---	0.75	0.25	---
Ciprofloxacin	3	0.84	0.16	0.07	0.77	0.23	0.20
Doxycycline	3	0.85	0.15	0.11	0.82	0.18	0.00
Clofazimine	2	0.88	0.12	0.13	>0.9	<0.1	---

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control

standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by “---”.

Many drug combinations containing methylene blue had high activity against both stationary phase and persistent populations. Interestingly, while the addition of cefuroxime increases the anti-persister activity of most of the combinations, it appears to lessen the activity of combinations including rifabutin (Table 9). Rifabutin inhibits the DNA transcription mechanism.⁶³ The highly lipophilic nature of these drugs may allow for efficient cell permeability without the need for cell wall disruption in the persistent population. Cefuroxime's intracellular presence may also block rifabutin's ability to effectively bind to and inhibit DNA transcription mechanisms.⁶³ While rifabutin anti-persister function does not require cell wall disruption, less lipophilic drugs may require the increased permeability in the bacterial cell wall for efficient penetration.⁶³

Table 9. Proportion of viable and killed cells after 7-day treatment with high activity methylene blue-containing combinations against amoxicillin-treated persistent *Borrelia burgdorferi* when compared to no drug control

Drug Treatment	Controls						
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.78	0.22	0.27			
Daptomycin	10	0.70	0.30	0.11			
Daptomycin + Cefoperazone + Doxycycline	10	0.42	0.58	---			
Drug Treatment	Methylene Blue + Drug treatment				Cefuroxime + Methylene Blue + Drug treatment		
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Rifabutin	2	0.59	0.41	---	0.76	0.24	---
Ciprofloxacin	3	0.90	0.10	0.22	0.76	0.24	0.14
Nitrofurantoin	2	0.74	0.26	0.12	0.78	0.22	0.09
Rifaximin	2	0.86	0.14	0.27	0.79	0.21	0.25
Azithromycin	2	0.80	0.20	0.23	0.80	0.20	0.15
Doxycycline	3	0.85	0.15	0.26	0.83	0.17	0.15
Artemisinin	2	>0.9	<0.1	---	0.84	0.16	0.25
Hydroxychloroquine	2	0.87	0.23	0.31	0.88	0.12	0.21
Clofazimine	2	0.88	0.12	0.19	0.88	0.12	0.20

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

Highly Effective Drugs against Persistent *Borrelia* When Used in Combination

Doxycycline

Doxycycline is a tetracycline derivative protein synthesis inhibitor with widespread activity against both gram positive and gram negative bacteria.⁷¹ Doxycycline binds to the bacterial ribosome and inhibits the ribosomal binding of aminoacyl t-RNA, a necessary step in the initiation of protein synthesis.⁷² Doxycycline is a commonly used antibiotic against early stage Lyme disease, but has not been found to be effective against stationary phase *Borrelia* when used individually.³⁴ However, when added in combination with methylene blue, doxycycline has high activity against stationary phase populations, as has been seen with other free radical producing and protein synthesis inhibiting drug combinations (Table 10).

Doxycycline does, however, has high activity against persistent populations in triple drug combinations including cefuroxime and either rifabutin or ciprofloxacin, both drugs involved in inhibition of RNA synthesis (Table 11). Ciprofloxacin binds directly to DNA, preventing binding of DNA gyrase and not allowing the DNA to form a negative superhelix.^{73,74} RNA synthesis inhibitors affect the ability of the cell to regulate gene expression and also prevent protein synthesis. It is likely that the combination of doxycycline and a RNA synthesis inhibitor are able to synergistically inhibit protein synthesis by targeting protein synthesis at different stages of the pathway.

Table 10. Proportion of viable and killed cells after 7-day treatment with high activity doxycycline-containing combinations against stationary phase *Borrelia burgdorferi* when compared to no drug control

Drug Treatment	Controls						
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.97	0.03	0.05			
Daptomycin	10	0.88	0.12	0.08			
Daptomycin + Cefoperazone + Doxycycline	10	0.64	0.36	---			
Drug Treatment	Doxycycline + Drug treatment				Cefuroxime + Doxycycline + Drug treatment		
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Methylene Blue	4	>0.9	<0.1	0.11	0.82	0.18	0.00
Artemisinin	2	>0.9	<0.1	---	0.85	0.15	0.20

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by “---”.

Table 11. Proportion of viable and killed cells after 7-day treatment with high activity doxycycline-containing combinations against amoxicillin-treated persistent *Borrelia burgdorferi* when compared to no drug control

Drug Treatment	Controls						
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.78	0.22	0.27			
Daptomycin	10	0.70	0.30	0.11			
Daptomycin + Cefoperazone + Doxycycline	10	0.42	0.58	---			
Drug Treatment	Doxycycline + Drug treatment				Cefuroxime + Doxycycline + Drug treatment		
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Rifabutin	2	>0.9	<0.1	---	0.58	0.42	---
Ciprofloxacin	3	>0.9	<0.1	---	0.77	0.23	0.26
Pyrimethamine	2	0.81	0.19	0.13	>0.9	<0.1	---
Methylene Blue	4	0.85	0.15	0.26	0.83	0.17	0.15
Artemisinin	2	>0.9	<0.1	---	0.86	0.14	0.15
Fluconazole	6	>0.9	<0.1	---	0.87	0.13	0.23

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---

Hydroxychloroquine

Hydroxychloroquine is chloroquine derivative that is believed to function by altering cellular metabolism.⁷⁵ Though used commonly against malarial infections, the exact mechanism for action against prokaryotes is still undetermined, as its primary target in eukaryotic cells are membrane-bound vesicles, which inhibits the parasite's ability to break down hemoglobin.⁷⁵ Hydroxychloroquine is a highly penetrative weak lipophilic base that is able to penetrate through plasma membranes and accumulate intracellularly in eukaryotes. Therefore the drug may be able to penetrate the bacterial cell membrane and accumulate within the cytoplasm.⁷⁶ Once intracellular, hydroxychloroquine's alkaline nature attracts acids into the cytoplasm, which can alter the ion gradient, prevent efficient nutrient uptake and destabilize bacterial enzymes.⁷⁶ The presence of permeant bases such as hydroxychloroquine can rapidly overwhelm the bacterial internal pH homeostasis mechanisms and effect DNA integrity and bacterial protein structure.⁶⁸

Hydroxychloroquine only has activity against stationary phase *Borrelia* populations when used in combination with free radical producing and protein synthesis inhibiting drugs (Table 12) This increase in activity is likely due to a decrease in intracellular pH affecting both DNA and protein structure. Genes implicated in the maintenance of DNA stability have also been found to be upregulated in doxycycline-induced persistent *Borrelia* populations, suggesting this is an important mechanism for maintenance of persisters.⁶⁰

Table 12. Proportion of viable and killed cells after 7-day treatment with high activity hydroxychloroquine-containing combinations against stationary phase *Borrelia burgdorferi* when compared to no drug control

Drug Treatment	Controls						
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.97	0.03	0.05			
Daptomycin	10	0.88	0.12	0.08			
Daptomycin + Cefoperazone + Doxycycline	10	0.64	0.36	---			
Drug Treatment	Hydroxychloroquine + Drug treatment				Cefuroxime + Hydroxychloroquine + Drug treatment		
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Azithromycin	2	>0.9	<0.1	---	0.80	0.20	0.01
Artemisinin	2	>0.9	<0.1	---	0.88	0.12	0.04
Nitrofurantoin	2	>0.9	<0.1	---	0.90	0.10	0.03

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by “---”.

The high activity hydroxychloroquine-containing drug combinations against persistent populations were involved in inhibiting metabolism and DNA transcription (Table 13). In fact, the triple combination of hydroxychloroquine, cefuroxime and rifabutin was the most highly active drug combination against persistent *Borrelia*. As

previously discussed, nutrient uptake has been shown to be an upregulated process in both doxycycline and amoxicillin treated persister cells, suggesting its importance in the maintenance of persisters. It is possible that the alteration of the intracellular pH disrupted the ion transport gradient, preventing the cells from achieving the necessary nutrients in their dormant state. When coupled with drugs that alter DNA transcription, transmembrane proteins may also be affected, further preventing the cells from maintaining their necessary nutrient and ion balance.⁷⁷ This would also explain why fluconazole was effective in combination with cefuroxime and hydroxychloroquine, as the prevention of ergosterol synthesis would cause further membrane instability.

Table 13. Proportion of viable and killed cells after 7-day treatment with high activity hydroxychloroquine-containing combinations against amoxicillin-treated persistent *Borrelia burgdorferi* when compared to no drug control

Drug Treatment	Controls						
	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error			
No Drug Control	0	1.00	0.00	---			
Amoxicillin	5	0.78	0.22	0.27			
Daptomycin	10	0.70	0.30	0.11			
Daptomycin + Cefoperazone + Doxycycline	10	0.42	0.58	---			
Azithromycin + Drug treatment					Cefuroxime + Azithromycin + Drug treatment		
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	Viable Cells	Killed Cells	Std. Error
Rifabutin	2	0.65	0.35	---	0.57	0.43	---
Fluconazole	6	>0.9	<0.1	---	0.72	0.28	0.16
Pyrimethamine	2	>0.9	<0.1	---	0.77	0.23	0.22
Azithromycin	2	>0.9	<0.1	---	0.80	0.20	0.28
Artemisinin	2	>0.9	<0.1	---	0.85	0.15	0.30
Rifaximin	2	>0.9	<0.1	---	0.86	0.14	0.28
Methylene Blue	4	0.87	0.13	0.31	0.88	0.12	0.21

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

Rifabutin

Rifabutin is a spiro-piperidyl-rifampin derivative that inhibits DNA transcription by binding to the beta-subunit of the DNA-RNA polymerase.⁶³ Rifabutin is one of the most lipophilic and highly penetrative rifampin derivatives, allowing for increased intracellular penetration over rifaximin, another rifampin derivative used in the screen.⁶³ The prevention of RNA synthesis can result in an inhibition of protein synthesis as well as prevent the cell from regulating gene expression. Amoxicillin-treated *Borrelia* persisters have been shown to upregulate 342 genes and downregulate 174 genes compared to non-treated *Borrelia* populations.⁶⁰ Without the ability to regulate gene expression in response to this drug-induced stress, the bacteria will likely be less able to maintain its persistent state.

Rifabutin does best against stationary phase bacteria when used when in combination with free radical producing and protein synthesis inhibiting drugs (Table 14). This increase in antibacterial activity is likely due to an additive effect on protein inhibition, as these drugs would all damage proteins at different stages in the production process. This would allow for a more complete inhibition of protein synthesis without competition for binding sites or cellular targets.

Table 14. Proportion of viable and killed cells after 7-day treatment with high activity rifabutin-containing combinations against stationary phase *Borrelia burgdorferi* when compared to no drug control

	Controls				
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Standard Error	
No Drug Control	0	1.00	0.00	---	
Amoxicillin	5	0.97	0.03	0.05	
Daptomycin	10	0.88	0.12	0.08	
Daptomycin + Cefoperazone + Doxycycline	10	0.64	0.36	---	
	Rifabutin + Drug treatment			Cefuroxime + Rifabutin + Drug treatment	
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Viable Cells	Killed Cells
Nitrofurantoin	2	>0.9	<0.1	0.75	0.25
Methylene Blue	4	>0.9	<0.1	0.75	0.25
Azithromycin	2	>0.9	<0.1	0.78	0.22

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

Rifabutin containing combinations have some of the highest activity against persistent *Borrelia* populations in this screen, especially when used with drugs that

decrease protein stability and function (Table 15). While rifabutin-containing combinations have higher antibacterial activity when combined with cefuroxime, methylene blue and fluconazole have lower antibacterial activity when used with the drug. Both of these drugs have high activity on the bacterial membrane. It is possible that the addition of cefuroxime may hinder the activity of drugs that interact with the plasma membrane either as a drug target or for penetration into the cell. Cefuroxime's disruptive effect on the bacterial cell wall could result in changes in the *Borrelia* plasma membrane, especially if the cell wall becomes perforated. This change in the cell wall could alter aspects of the plasma membrane making it harder for the other drugs to act on the lipids.

Rifabutin has high activity against amoxicillin-treated *Borrelia* persists when in combination with many different drugs. Rifabutin's inhibition of RNA synthesis likely has an additive effect when used in combination with cefuroxime, nitrofurantoin, and doxycycline, which act on the protein synthesis pathway. This follows a trend seen with other drug combinations that suggests protein synthesis may be a key pathway to target against *Borrelia* persists. The high activity of rifabutin with fluconazole and hydroxychloroquine also suggests that regulation of cellular homeostasis and nutrient transport could also be a key target for persistent *Borrelia*. By inhibiting the ability of the cell to regulate gene expression, the bacteria will likely be unable to adequately cope with stressors such as increased intracellular pH, nutrient imbalance or the oxidative damage caused by artemisinin and methylene blue.

Table 15. Proportion of viable and killed cells after 7-day treatment with high activity rifabutin-containing combinations against amoxicillin-treated persistent *Borrelia burgdorferi* when compared to no drug control

	Controls				
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Std. Error	
No Drug Control	0	1.00	0.00	---	
Amoxicillin	5	0.78	0.22	0.27	
Daptomycin	10	0.70	0.30	0.11	
Daptomycin + Cefoperazone + Doxycycline	10	0.42	0.58	---	
	Rifabutin + Drug treatment			Cefuroxime + Rifabutin + Drug treatment	
Drug Treatment	Conc. (µg/mL)	Viable Cells	Killed Cells	Viable Cells	Killed Cells
Hydroxychloroquine	2	0.65	0.35	0.57	0.43
Nitrofurantoin	2	>0.9	<0.1	0.57	0.43
Doxycycline	3	>0.9	<0.1	0.58	0.42
Methylene blue	4	0.59	0.41	0.76	0.24
Fluconazole	6	0.63	0.37	>0.9	<0.1
Artemisinin	2	>0.9	<0.1	0.69	0.31

*Viability of residual cells remaining after drug treatment determined via epifluorescent microscopy. Drug combinations that showed high activity in the SYBR Green I/PI rapid viability test were examined microscopically for residual viable cells remaining after treatment. The average of triplicate microscopic values was taken for each high activity combination. These drug combinations were repeated against a different *Borrelia burgdorferi* culture and a second average residual viable cells remaining value was determined. The average residual viable cells remaining for each biological replicate was transformed into a proportion of residual viable cells remaining in comparison to the replicate's no drug control value to account for inter-plate variation. The average of these two proportions are presented with accompanying standard error. The no drug control standard is shown as a proportion of 1.00, equating to a 100% viable cell baseline against which all drug combinations were measured. Some drugs were only tested against one replicate, and therefore do not have an accompanying standard error, represented by "---".

CONCLUSIONS

The drug combination of daptomycin + doxycycline + cefoperazone has been found to be highly active against an *in vitro* persister model using stationary phase *Borrelia*.³⁵ In this study, this drug combination was found to be highly effective against both stationary phase and amoxicillin-treated persisters. However, there were drug combinations consisting of better tolerated and orally available drugs that had similar activity against these *Borrelia* populations *in vitro*. These highly active drug combinations can provide insight into better treatment of *Borrelia in vitro* by identifying key pathway combinations to target for future treatments. The drug combinations with the highest activity against stationary phase populations were drug combinations of free radical producing drugs along with protein synthesis inhibitors and cell wall disruptors, including; cefuroxime + artemisinin + azithromycin, cefuroxime + methylene blue + azithromycin, cefuroxime + methylene blue + nitrofurantoin, and cefuroxime + artemisinin + nitrofurantoin.

The addition of cefuroxime to the drug combinations generally helped the antibacterial activity of these combinations against stationary phase populations, with a few exceptions. This data seems to suggest that cell wall penetration is a key aspect for drug treatment against stationary phase *Borrelia in vitro*. However, drugs associated with plasma membrane phospholipids such as hydroxychloroquine and fluconazole had higher activity against persisters, suggesting that membrane permeability may play a more important role in killing of persisters than stationary phase *Borrelia*.

The drug combinations with the highest activity against amoxicillin-treated *Borrelia* persisters tended to include RNA synthesis inhibitors and either protein

synthesis inhibitors or intracellular homeostasis regulators. These combinations included: cefuroxime + rifabutin + hydroxychloroquine, cefuroxime + nitrofurantoin + rifabutin, cefuroxime + doxycycline + rifabutin, and rifabutin + fluconazole. The combinations with the highest activity against persistent *Borrelia* contained drugs that affected DNA integrity and nutrient uptake, such as hydroxychloroquine, fluconazole, and ciprofloxacin, rather than protein maintenance.

However, the definitive mechanism of action of these drugs has not been determined against persistent *Borrelia*. More research needs to be done to confirm the mechanisms of these drugs within these populations. More replicates will also need to be done of these drug combinations for further confirmation of the drug combination's activity *in vitro*. These drug combinations would need further study both *in vitro* and *in vivo* in animal models before use in the clinic. However, this study does suggest that optimal treatment of Lyme patients may be different for untreated and treatment-resistant PTLDS patients. Overall, the use of combination therapy of readily available oral drugs for use in the treatment of Lyme disease merits further investigation, both *in vitro* and *in vivo*.

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