Persisters of *B. burgdorferi*, the causative agent of Lyme disease

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Abstract of Dissertation

*Borrelia burgdorferi* is the causative agent of Lyme disease, which affects an estimated 300,000 people annually in the United States. When treated early, the disease usually resolves, but left untreated, can result in symptoms such as arthritis, carditis, and encephalopathy. Treatment of the late stage disease may require multiple courses of antibiotic therapy. Given that antibiotic resistance has not been observed for *B. burgdorferi*, the reason for the recalcitrance of late stage disease to antibiotics is unclear. In other chronic infections, the presence of drug-tolerant persisters has been linked to recalcitrance of the disease. Hence, my goal was to examine if *B. burgdorferi* forms persisters in vitro and, if so, to determine ways to eradicate persisters.

In this thesis, I show that *B. burgdorferi* forms persisters in vitro. These persisters are tolerant to killing by high doses of antibiotics and are formed in a growth-phase dependent manner. I also show that *B. burgdorferi* persisters could be eradicated in vitro by using a novel pulse dosing method using ceftriaxone, one of the drugs currently used to treat late disseminated Lyme disease or by using Mitomycin C, an anticancer agent. Additionally, previous work has shown that compounds that are specific to a given bacterium might also be active against its persisters, Hence, I reasoned that an antimicrobial that specifically kills *B. burgdorferi* may also be efficacious against its persisters. Thus, I developed a high-throughput screen to identify compounds from a compound library that are active only against *B. burgdorferi*. From a pilot screen of 10,000 compounds, I have identified 2 compounds that have an MIC of less than 5 µg/ml against *B. burgdorferi*, a therapeutic index higher than 10, and show better in vitro efficacy against *B. burgdorferi* than ceftriaxone, one of the drugs used to treat Lyme
disease. These results validated the screen paving the path for screening additional compound libraries.

Study of persisters of *B. burgdorferi* and discovery of a drug regimen or novel compound that targets persisters of *B. burgdorferi* will lead to a better understanding of Lyme disease and a better treatment for Post Treatment Lyme Disease Syndrome (PTLDS).
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List of Abbreviations

ADEP: acyldepsipeptide
Amox: amoxicillin
ATCC: American type/tissue culture collection
ATP: adenosine triphosphate
Bb: Borrelia burgdorferi
Bf: Bacteroides fragilis
Bl: Bifidobacterium longum
BSK: Barbour-Stoenner-Kelly
Bt: Bacteroides thetaiotaomicron
C: carbon
Cd: Clostridium difficile
Cef: ceftriaxone
cfu: colony forming units
CLSI: clinical and laboratory standards institute
CMRL: Connaught medical research laboratories
CO₂: carbon dioxide
Compd.: Compound
Dapt: daptomycin
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
Dox: doxycycline
Ec: Escherichia coli
Ef: Enterococcus faecalis
EM: erythema migrans
EMEM: eagle’s minimum essential medium
FBS: fetal bovine serum
FDA: federal drug administration
FSS: fatigue severity scale
Gemi: gemifloxacin
GSI SCL-90: general severity index for symptom checklist -90
ppGpp: guanosine tetraphosphate
GVHD: graft-versus-host-disease
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hip: high-persister
HLA: human lymphocyte antigen
hLFA-1: human leukocyte function-associated antigen-1
HTS: high-throughput screen
IC50: inhibitory concentration 50
IDSA: Infectious Diseases Society of America
IFN-gamma: interferon- gamma
IV: intravenous
LD: Lyme disease
_Lr_: Lactobacillus reuteri
MBC: minimum bactericidal concentration
MCS: mental component summary
MHB: Mueller Hinton broth
MHC: major histocompatibility complex
MIC: minimum inhibitory concentration
MMC: mitomycin C
ND: not determined
O2: oxygen
OD: optical density
PCR: polymerase chain reaction
PCS: physical component summary
PTLDS: post treatment Lyme disease syndrome
RA: rheumatoid arthritis
RTPCR: reverse transcription polymerase chain reaction
_Sa_: Staphylococcus aureus
SAM: Schaedler anaerobe media
SF-36: 36-item short form health survey
Spec: spectinomycin
TA: toxin/antitoxin
TI: therapeutic index
TMTC: too many to count
TNF: tumor necrosis factor
UTI: urinary tract infection
Vanc: vancomycin
VAS: visual analog scale
Chapter 1: Introduction
1.1 Lyme disease (LD)

LD is the most commonly reported vector-borne disease in the United States with an estimated 300,000 cases annually [1]. It is caused by a number of species from the Spirochaetae family, broadly referred to as *B. burgdorferi* sensu lato. While *B. burgdorferi* is the most prevalent species causing LD in the United States, other species like *B. afzelii* and *B. garnii* also cause LD in Europe and Asia [2]. Recently, a new species named *B. mayonii* was reported to cause rare cases of LD in the United States [3]. LD was first reported as a cluster of juvenile rheumatoid arthritis in Lyme, Connecticut. After what is noted as a great example of positive interplay between the community, academic medicine, and public health workers, these clusters of symptoms seen in areas of Connecticut were associated with the appearance of the cutaneous rash called erythema migrans (EM) [4], which seemed to form after the bite of a tick belonging to the genus *Ixodes* [5]. A notable accomplishment very early on was the identification that EM can be cleared with antibiotics like penicillin leading to prevention or lessening of subsequent arthritis [6]. Eventually, Willy Burgdorfer isolated *B. burgdorferi* *in vitro* [7] and two separate groups isolated *B. burgdorferi* from patients with LD [8, 9]. The successful *in vitro* cultivation of *B. burgdorferi* has led to a great deal of research on its physiology, pathogenesis, genetics, and ecology in diverse hosts.

1.2 *Borrelia burgdorferi*, the causative agent of LD

*B. burgdorferi* has a spiral or wave-like morphology with a bundle of flagella in the periplasmic space [10]. While the general organization of cell membranes in *B. burgdorferi* resembles that of gram-negative bacteria, the outer membrane of this bacterium is unique given it lacks lipopolysaccharides [11], instead having lipoproteins [12] and cholesterol containing lipid
rafts [13]. Additionally, it has a distinctive genome consisting of a linear chromosome and multiple linear and circular plasmids [14]. The plasmids are known to harbor essential genes such as the gene that encodes telomere resolvase (resT) required for chromosome and linear plasmid maintenance [15, 16]. It is an auxotroph for amino acids, nucleotides and fatty acids. Since it lacks enzymes for the tricarboxylic acid cycle and oxidative phosphorylation, it relies on glycolysis for energy [17]. Additionally, as opposed to other pathogenic bacteria, \textit{B. burgdorferi} does not seem to require iron for growth since the intracellular concentration of iron in \textit{B. burgdorferi} is well below physiologically relevant concentrations. Similarly, with the exception of BB0690, a Dps-like bacterioferritin orthologue, its genome does not encode for typical bacterial protein that contain iron such as cytochromes or catalases [17, 18]. In addition, enzymes that normally use iron as cofactors have substituted manganese for iron as cofactor [19].

\textit{B. burgdorferi} is maintained in the environment within an enzootic cycle that involves small vertebrates -- small rodents and birds -- and ticks. Upon the bite of an infected nymph or adult tick \textit{B. burgdorferi} establishes long-term infections of years to lifelong in its natural hosts such as mice [20]. These infected vertebrates then serve as reservoirs of \textit{B. burgdorferi} that can be acquired by uninfected larval ticks upon blood meal, thus propagating the infection cycle, after larval molting to nymph and feeding on the next uninfected host. Occasionally, infected ticks (predominantly nymphs) can incidentally transmit \textit{B. burgdorferi} to humans.

\textbf{1.3 Symptoms and treatment}

After the bite of an infected tick, it can take between 3 to 30 days for symptoms to appear. After transmission, \textit{B. burgdorferi} disseminates from the site of the tick bite to other sites causing a multi-organ infection. It infects the heart, joints, and central nervous system thereby
causing symptoms like carditis, arthritis, and meningitis. Progression of LD can occur over the following stages:

a. Early/acute LD

b. Late LD

c. Post treatment LD

a. Early/acute LD: This is characterized by the appearance of the characteristic bull’s eye rash, which is also known as EM. This may be accompanied by flu-like symptoms such as fever, fatigue, headache, arthralgia, myalgia and/or neck pain. The typical EM presents as an expanding annular ring with a central clearing and measures from 5 to 30 cm in diameter. The EM rash is, however, seen only in up to 80% of patients with early LD [21]. The rash may present as atypical often resembling insect bite [22, 23] complicating the diagnosis of LD. The CDC recommends a two-tiered test for clinical diagnosis of Lyme disease. The diagnosis protocol involves testing for IgM or IgG (together or separately) followed by immunoblotting for important B. burgdorferi antigens. Serology testing at early stage of LD is not recommended since the IgM response takes about 2-4 weeks to mature leading to about 60% of false-negative diagnosis in the first 2-4 weeks after infection [23]. IgG response takes about 4 weeks to mature. Early LD is treated with a 10-21 day course of oral doxycycline, amoxicillin, or cefuroxime axetil [24].

After infection, B. burgdorferi enters the bloodstream and spreads throughout the body to cause a multi-organ infection. This is the early-disseminated stage of LD and includes neurological and cardiac involvement. Neurological and cardiovascular symptoms are seen in about 15% and 4-8% of patients, respectively [23]. Patients with neurological symptoms such as meningitis, facial palsy, or radiculopathy are treated with a 14-28 day course of intravenous (IV) ceftriaxone or oral doxycycline [24]. Patients with cardiac involvement may be treated with 14-
21 day course of oral or IV antibiotics. Infectious Diseases Society of America (IDSA) recommends treatment with ceftriaxone for Lyme patients hospitalized with cardiac symptoms [24].

b. Late LD: Inflammatory oligoarticular arthritis often represents the late stage of LD. About 60% of untreated patients experience intermittent attacks of arthritis months after infection [25]. It usually affects the large weight-bearing joints like the knees and can range in severity from mild to severe [21, 26]. A 28-day course of oral antibiotics similar to the one used for early Lyme patients with EM is recommended for patients with Lyme arthritis without neurological involvement and a 2-4 weeks course of IV ceftriaxone is recommended for patients suffering from Lyme arthritis with neurological involvement. A subset of patients with Lyme arthritis, however, does not respond to the recommended treatment regimen. In such cases, retreatment with 2-4 weeks of oral or IV antibiotics as used above is recommended [24]. Not all cases of recurrent Lyme arthritis are resolved by antibiotics. There are recommended treatment algorithms that can be followed in these cases [27-29]. Some individuals with Lyme arthritis may show evidence of *B. burgdorferi* DNA in their synovial fluid after standard antibiotic treatment and are treated with additional 1- or 2-month courses of antibiotics [29]. A smaller minority of such patients referred to as having “antibiotic-resistant/refractory Lyme arthritis continue to have arthritis with synovial fluid that is negative for *B. burgdorferi* by PCR. These patients are treated with anti-inflammatory agents such as methotrexate or tumor necrosis factor (TNF) inhibitors [27]. Treatment resistant Lyme arthritis is thought to be caused by either immune response to leftover Borrelial antigens after the apparent clearance of the spirochete or by autoimmune response [30, 31].
Borrelia antigens have been reported to persist in mouse tissues after treatment with antibiotics and have been hypothesized to also be present in humans [31, 32]. The amber theory of Lyme arthritis proposes that dead B. burgdorferi and Borrelial debris gets enmeshed in the host-derived fibrous or collagenous matrix during the course of its dissemination in the host after infection [31]. If this Borrelial debris gets released into the synovia long after the spirochete is removed from the body, it may elicit an inflammatory response leading to recurrent arthritis. This theory is based on the visualization of a dead but intact spirochete that was immotile, appeared to be enmeshed in a matrix of unknown nature and could not be subcultured, in the synovial fluid of a patient with Lyme arthritis before antibiotic treatment and on the observation that experimental introduction of Borrelial outer surface lipoproteins into the joint of rats results in severe arthritis [31]. However, in the absence of direct evidence suggesting that fossilized Borrelial debris results in treatment resistant Lyme arthritis in humans, other hypotheses need to be considered.

An autoimmune pathomechanism of treatment resistant Lyme arthritis has also been proposed [30]. Genetic predisposition to development of antibiotic refractory Lyme arthritis has been described. Patients bearing human leukocyte antigen (HLA)-DR4, are found to be genetically prone to developing antibiotic resistant Lyme arthritis [33]. HLA-DR is a class II major histocompatibility complex (MHC) cell surface receptor usually found on antigen presenting cells such as dendritic cells, macrophages, and B cells. HLA-DR4 is a HLA-DR serotype with the capacity to present a Borrelial epitope derived from OspA. OspA (outer surface protein A) is a major antigen of B. burgdorferi that is highly expressed during transmission from mammalian hosts to tick and during tick colonization but is repressed during transmission from ticks to mammals. However, some patients with long-term infection with B. burgdorferi
seroconvert to OspA for unknown reasons [34]. An immunodominant peptide of OspA shows sequence homology to human leukocyte function-associated antigen-1 (hLFA-1), which may serve as an autoantigen [33, 35]. Hence, in genetically prone individuals an inflammatory response is initiated when *B. burgdorferi* at the joints expresses OspA leading to recruitment of OspA-reactive, IFN-gamma producing T cells into synovia. This causes an upregulation of MHC-II expression on antigen presenting cells and hLFA-1 on T cells and macrophages. Phagocytosis of apoptotic T cells by macrophages can result in presentation of LFA-1 by class II MHC molecules. Since hLFA-1 shows strong sequence homology to OspA, some of the T cells reactive to OspA may also recognize epitopes derived from hLFA-1. After the eradication of live *B. burgdorferi*, OspA-primed T cells may remain active due to presentation of the cross-reactive hLFA-1 peptide [30]. While there is evidence supporting the molecular mimicry between the immunodominant epitope of OspA and HLA-I, much needs to be done to directly demonstrate the pathological potential of OspA-specific T cells in Lyme arthritis [36].

It seems very likely that an immune response is the cause of antibiotic refractory Lyme arthritis in patients with the synovial fluid negative by PCR for *B. burgdorferi*. However, given that no antibiotic resistance has been reported in *B. burgdorferi* for any antibiotic used against Lyme disease, it is baffling why multiple long courses of antibiotics are required for treatment of Lyme arthritis.

c. Post Treatment LD Syndrome (PTLDS): Even though LD symptoms can be severe, the prognosis for an uncomplicated case of LD is favorable. In most cases of LD, symptoms can be resolved with antibiotic treatment. However, about 10- 20% patients affected with LD exhibit symptoms like persistent fatigue, musculoskeletal pain, and cognitive abnormalities after 6
months or longer post-antibiotic treatment [22, 37]. As proposed by the IDSA, if these symptoms are accompanied by a functional decline, patients are considered to meet clinical criteria for PTLDS [38]. Prolonged antibiotic treatment is inadvisable for treatment of PTLDS [24]. Four clinical studies were performed to determine the efficacy of long-term antibiotic treatment in patients with PTLDS (Table 1).

Klempner et al. conducted two randomized placebo-controlled trials to assess the efficacy of prolonged antibiotic treatment in patients with PTLDS [39]. In these studies, 78 seropositive patients and 51 seronegative patients were treated with 1 month of IV ceftriaxone followed by 60 days of oral doxycycline administration. Both studies were terminated early because an interim planned analysis determined that it was highly unlikely that a significant difference between the antibiotic and placebo group will be observed. Similarly, in a randomized, double-masked study conducted by Krupp, et al., 55 patients with PTLDS characterized by chronic fatigue 6 months after standard antibiotic therapy were treated with IV ceftriaxone or placebo for 28 days [40]. PTLDS patients treated with ceftriaxone showed improvement in fatigue but not cognitive functions compared to placebo group. In addition, adverse events related to antibiotic treatment were observed. Hence, this study concluded that treatment with additional IV antibiotics is not beneficial for patients with PTLDS. Fallon et al. investigated the efficacy of long-term IV antibiotic administration in 37 Lyme disease patients with cognitive impairment after standard antibiotic therapy [41]. Patients were randomly assigned to 10 weeks of IV ceftriaxone or placebo treatment. Improvement in cognitive functions and severe fatigue, pain, and impaired physical functioning and durability of benefit was assessed at 12 and 24 weeks after treatment. Cognitive functions were improved in patients after antibiotic treatment at 12 weeks but this effect was not sustained after 24 weeks. However, the secondary measures like severe fatigue,
pain, and physical functioning was improved and sustained in antibiotic treated patients up to 24 weeks after treatment. This study concluded that long-term IV ceftriaxone treatment does not result in sustained improvement of cognitive functions in PTLDS patients and reported adverse events related to antibiotic administration in 26% patients. However, it is important to note that higher rate of adverse events was expected because this study used a long course of IV antibiotics.

Multiple groups that reached varying conclusions have evaluated these studies. One group argues that the results of these studies were not informative of the true efficacy of long term antibiotic treatment tested because of low sample size, sub-optimal study design, and use of impractical criteria to consider an observed effect as significant and concluded that prolonged courses of antibiotics are likely to be helpful [42]. Contrary to this, another group argues that based on the results of these studies retreatment of patients with parenteral antibiotics cannot be justified because of underestimation of placebo effect [43]. There is, however, consensus that LD patients experience debilitating symptoms after standard antibiotic therapy and there is a need for a better treatment option for patients with PTLDS.
<table>
<thead>
<tr>
<th>Trial/ Enrollment</th>
<th>Treatment</th>
<th>Outcome measurement/ measurement months</th>
<th>Outcome</th>
<th>Conclusion</th>
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<tbody>
<tr>
<td>Klempner et. al. 2001/ Seropositive (n=78) and seronegative (n=51) with musculoskeletal pain, cognitive impairment, radicular pain, and paresthesias that interfered with functioning per patient self-report</td>
<td>30 days IV ceftriaxone followed by 60 days oral doxycycline</td>
<td>SF-36 physical component summary (PCS); Mental component summary (MCS)/ 3 months</td>
<td>No statistically significant improvement observed in patients treated with antibiotics vs. placebo</td>
<td>The tested antibiotic regimen is not effective for treatment of PTLDS patients presenting with musculoskeletal pain, cognitive impairment and radicular pain and paresthesia</td>
</tr>
<tr>
<td>Krupp, et. al. 2003/ patients with a history of LD and ongoing symptoms of severe fatigue validated by a Fatigue Severity Scale (FSS-11) score ≥ 4.0. (n=55)</td>
<td>30 days IV ceftriaxone</td>
<td>Fatigue measured by the FSS-11, mental speed using an alphabet arithmetic (A-A) test, and clearance of outer surface protein A (OspA) from the cerebrospinal fluid/ 6 months</td>
<td>Clinical improvement in fatigue measured by FSS-11 (18.5% on placebo versus 64% on ceftriaxone (p =0.01)). Other measurements were not statistically significant</td>
<td>Findings did not support antibiotic treatment. Improved fatigue may have been biased because of potential unmasking Beneficial effect on fatigue was outweighed by the lack of effect on the other primary endpoints and the high number of adverse events.</td>
</tr>
<tr>
<td>Fallon et al. 2008/ Subjects had memory impairment on subjective and objective assessment tools despite having previously received a minimum of 3 weeks of IV ceftriaxone (n=37)</td>
<td>10 weeks IV ceftriaxone</td>
<td>Primary outcome: Cognitive change over time. Secondary outcome: SF-36 PCS and MCS scores, fatigue (FSS-11), pain (VAS), depression (Beck), anxiety (Zung), and global psycho-pathology (GSI SCL-90) / 12 and 24 weeks</td>
<td>Improved cognitive change in antibiotic treated group compared to placebo after 12 weeks, which was not sustained at 24 weeks. Improved secondary outcome of fatigue and pain sustained over 24 weeks.</td>
<td>Lack of durable cognitive improvement and the risk of adverse events led to conclude that 10 weeks of ceftriaxone was not an effective strategy</td>
</tr>
</tbody>
</table>
The treatment regimen for PTLDS is of much debate because the actual pathogenesis of PTLDS is highly debated. The cause of PTLDS is a topic of much controversy because PTLDS patients do not show clear evidence for the presence of the pathogen. While some argue that PTLDS, similarly to treatment resistant Lyme arthritis, is driven by an immune mechanism, others believe that it is caused by persistent infection by *B. burgdorferi*.

A number of studies in animal models including dogs, mice, and monkeys have been performed to ascertain if *B. burgdorferi* persists in animal hosts after antibiotic treatment [44-48]. A common theme has emerged from these animal studies: *B. burgdorferi* cannot be cultured from antibiotic treated hosts; however, transcripts of Borrelial genes and intact spirochetes can be recovered using RTPCR and xenodiagnoses, respectively. In a study conducted to understand the safety of using ticks to detect live *B. burgdorferi* in patients with PTLDS (xenodiagnosis), Borrelial DNA was recovered from ticks fed on one patient with PTLDS further strengthening the case for presence *B. burgdorferi* in patients with PTLDS [49]. It has been speculated that these leftover spirochetes are drug-tolerant persister cells [45, 46].

1.4 Persister cells

“Persister cells” represent a small subpopulation of dormant bacteria that survive antibiotic treatment. Persisters are distinct from resistant cells because unlike antibiotic resistant cells they lack a specialized resistance mechanism to prevent interaction of the antibiotic with the target molecules [50]. The bactericidal effect of most antibiotics relies on their interaction with active cellular target to produce corrupt products. For example, fluoroquinolones bind to and inhibit the re-ligation step to introduce double strand breaks in DNA that result in cell death [51]. In the case of resistance the drug-target binding is inhibited. In case of persister formation the responsible mechanisms shuts down cellular targets leading to tolerance [50, 52].
Persisters have been found to exist in vitro in a wide range of clinically relevant bacterial pathogens, including *Staphylococcus aureus*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, *Mycobacterium tuberculosis*, and urinary tract infection (UTI) strains of *Escherichia coli* [53-57]. Presence of persisters can explain why some infections are difficult to treat in the absence of detectable resistance mechanisms. Many chronic infections including *P. aeruginosa* infection in patients with cystic fibrosis, UTI, osteomyelitis, endocarditis and infection of indwelling devices and catheters are caused by antibiotic susceptible pathogens and are associated with bacterial biofilms [50, 52]. Biofilms are surface attached microbial populations encased in extracellular polymeric matrix and represent a well-studied case of bacterial immune evasion and a paradigm for understanding chronic infections. The biofilm and planktonic stationary phase cells of *P. aeruginosa* are equally susceptible to antibiotics, with a small sub-population of surviving persisters [58]. Hence, it is proposed that antibiotics kill regular cells in a biofilm but dormant persisters survive, and when the concentration of antibiotic drops, they resuscitate and repopulate the biofilm [50]. This is supported by the finding that high-persister (hip) *P. aeruginosa* are selected for over the course of repeated antibiotic treatment in patients with cystic fibrosis, a genetic disorder associated with biofilm-mediated chronic airway infection [55]. Similarly, hip *Candida albicans* are selected during the course of prolonged antibiotic treatment in patients with oral thrush [59]. Additionally, a recent publication showed that commensal and clinical isolates of *E. coli* obtained from patients with UTI also possess the high persister *hipA7* allele [60].

Immune evasion is another hallmark of persister mediated chronic infection. The immune system can effectively remove sessile cells from the blood and many of the tissues, and this accounts for the efficacy of antibiotics, including bacteriostatic compounds, in treating
uncomplicated infections. When the immune response is limited, the result is often a chronic infection [50]. In tuberculosis, dormant cells are likely responsible for the need of a lengthy treatment of the acute stage and for the latent form of the disease. *Mycobacterium tuberculosis* hides from the immune system in macrophages or in granulomas [61]. Similarly, internalization of *Salmonella* by human cells was accompanied by a sharp increase in persisters and tolerance to antibiotics [54].

Persisters may be formed stochastically or through dedicated mechanisms [50, 62]. The mechanism of persister formation has been predominantly linked to dormancy [63, 64]. Genetic screening using different approaches was used to identify candidate persister genes but they did not yield one single mechanism [50, 65-69]. This highlighted the redundant nature of the mechanism of persister formation. Gene expression profiles obtained from persisters selected after lysing susceptible cells with ampicillin revealed the role of chromosomally encoded toxin/antitoxin (TA) modules and other proteins that inhibit vital cellular functions in formation and multidrug tolerance of persisters [66]. Chaperones (*dnaJ* and *dnaK*), global regulators (*fis, hns, dksA, hnr*) and genes involved in nucleotide metabolism (*apaH* and *yigB*) were found to contribute to persister formation in a screen of the ordered deletion library of *E. coli* [65]. Similarly, genes involved in carbon metabolism were implicated in persister formation in a screen of the overexpression library and Tn-seq of *E. coli* identified genes involved in motility and amino acid synthesis as being responsible for tolerance to aminoglycosides [68, 69].

The mechanism of persister formation through TA modules is the best studied in *E. coli*. TAs are common prokaryotic systems composed of a stable toxin and an unstable antitoxin that binds and inactivates the toxin [70]. Under stress, the antitoxin is degraded by proteases leading to free toxins in the cell capable of targeting cell processes including DNA replication,
translation and cell division to induce growth arrest or cell death. HipBA is the most studied TA system involved in persister formation. HipA is the toxin of a TA module and hipB is the cognate antitoxin. *hipA7* allele was originally described as being involved in persister formation through a screen of mutagenized strains of *E. coli* where this allele was found to increase persisters by 100 fold [71]. When free, HipA, a serine threonine kinase, phosphorylates the glutamyl tRNA synthetase to inactivate its function leading to inhibition of protein synthesis and growth arrest/ tolerance [72]. A recent study reported that tolerance increases in a small population of *E. coli* stochastically overexpressing HipA by using single-cell analysis and that *hipA7* increases tolerance by interrupting the binding between HipA and HipB [60]. *hipA7* mutants were also shown to be present in *E. coli* isolates from patients with UTI [60] providing a link between the molecular mechanism of persister formation and the clinical manifestation of the disease. Additionally, a model of persister formation proposes that *hipA7* facilitates persistence by inducing the synthesis of guanosine tetraphosphate (ppGpp), an alarmone mediating bacterial stringent response [72, 73].

Other TA modules have also been implicated in persister formation. An inducible mechanism of persister formation involving the TisB/istR TA system has been described for *E. coli* [52]. Antibiotic induced DNA damage initiates SOS response leading to TisB toxin expression. TisB binds to the membrane and disrupts the proton motive force leading to a decrease in intracellular adenosine triphosphate (ATP) levels and thereby formation of dormant, drug-tolerant persisters. Expression of other toxins like RelE has also been shown to increase tolerance to antibiotics [66]. Additionally, the study of the mechanism of persister formation in *Staphylococcus aureus* in exponential phase has identified persisters as cells that enter stationary
phase early. These cells have low intracellular ATP levels leading to diminished activity of antibiotic targets that results in tolerance [74].

*B. burgdorferi* does not encode TA modules [75]. It has also not been reported to be intracellular or hide in macrophages. However, *B. burgdorferi* has an impressive armamentarium to avoid clearance by the immune system, including antigenic variation of surface components, resistance to complement-mediated killing by factor-H binding, and decreasing exposure of immunogenic antigens like flagella [76]. In this regard, LD resembles other chronic infections where the pathogen is protected from the immune system, yet a systematic study to determine if *B. burgdorferi* forms persisters had not been performed.

**1.5 Dissertation goals**

My goal was to test if *B. burgdorferi* forms persisters *in vitro* and, if successful, to determine possible means to eradicate persisters.

Using a series of time-, dose-, and growth phase-dependent killing experiments, I showed that *B. burgdorferi* forms persisters *in vitro*. These cells are tolerant to killing by high doses of antibiotics and are formed in a growth-phase dependent manner. I also showed that *B. burgdorferi* persisters could be eradicated *in vitro* by using a novel pulse-dosing regimen with ceftriaxone, one of the drugs currently used to treat late Lyme disease or by using mitomycin C, an anticancer agent. These findings were published in *Antimicrobial Agents and Chemotherapy* in May 2015 [77].

Additionally, previous work has found that compounds selective against a genus of bacteria may also be active against the persisters of that genus [78, 79]. Consequently, I performed a compound screen to identify antibiotics specific against *B. burgdorferi*. I adapted the colorimetric assay for MIC determination to perform a high-throughput screen of a
commercially available small compound library. A comparative screen was performed to identify the compounds acting selectively against *B. burgdorferi* [78]. Compounds were first screened for activity against *B. burgdorferi*. Positive hits from this screen were then screened for activity against a panel of bacteria including gut commensals. Compounds that had activity only against *B. burgdorferi* were selected for further characterization. This approach helps exclude toxic compounds and compounds that target general bacterial metabolism [78]. In a pilot study, I screened 10,000 compounds for efficacy against *B. burgdorferi* and counter-screened the primary hits against a panel of bacteria including gut microbes and mammalian cell lines, yielding two compounds that have specific activity against *B. burgdorferi* and exhibit low toxicity to mammalian cells. These compounds show better killing of *B. burgdorferi* than ceftriaxone, one of the drugs used to treat late LD.

This study is the first to show that *B. burgdorferi* forms drug-tolerant persister cells *in vitro*. It is yet to be determined if persisters exist *in vivo* and contribute to the pathogenesis of PTLDS. The high-throughput screen validated in this study can be used to screen larger compound libraries to identify better treatment options for LD.
1.6 References


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Chapter 2: *Borrelia burgdorferi*, the causative agent of Lyme disease, forms drug-tolerant persister cells.

Bijaya Sharma, Autumn V. Brown, Nicole E. Matluck, Linden T. Hu and Kim Lewis

Borrelia burgdorferi, the causative agent of LD, forms drug-tolerant persister cells.

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Running title: Persisters of Borrelia burgdorferi

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2.1 Abstract

*B. burgdorferi* is the causative agent of LD, which affects an estimated 300,000 people annually in the US. When treated early, the disease usually resolves, but left untreated, can result in symptoms such as arthritis and encephalopathy. Treatment of the late stage disease may require multiple courses of antibiotic therapy. Given that antibiotic resistance has not been observed for *B. burgdorferi*, the reason for the recalcitrance of late stage disease to antibiotics is unclear. In other chronic infections, the presence of drug-tolerant persisters has been linked to recalcitrance of the disease. In this study, we examined the ability of *B. burgdorferi* to form persisters. Killing of growing cultures of *B. burgdorferi* with antibiotics used to treat the disease was distinctly biphasic, with a small subpopulation of surviving cells. Upon regrowth, these cells formed a new subpopulation of antibiotic-tolerant cells, indicating that these are persisters rather than resistant mutants. The level of persisters increased sharply as the culture transitioned from exponential to stationary phase. Combinations of gemifloxacin with cell envelope active drugs improved killing. Daptomycin, a membrane-active bactericidal antibiotic, killed stationary phase cells, but not persisters. Mitomycin C, an anti-cancer agent that forms adducts with DNA, killed persisters and eradicated both growing and stationary cultures of *B. burgdorferi*. Finally, we examined the ability of pulse-dosing an antibiotic to eliminate persisters. After addition of ceftriaxone, the antibiotic was washed away, surviving persisters were allowed to resuscitate, and antibiotic was added again. Four pulse-doses of ceftriaxone killed persisters, eradicating all live bacteria in the culture.
2.2 Introduction

All pathogens studied to date form persisters, dormant variants of regular cells which are tolerant to killing by antibiotics. The ability to produce persisters explains the puzzling recalcitrance of chronic infections to antibiotics that are effective against the same pathogen in vitro. Indeed, many chronic infections are caused by drug-susceptible pathogens [1, 2]. The immune system can effectively remove sessile cells from the blood and many of the tissues, and this accounts for the efficacy of antibiotics, including bacteriostatic compounds, in treating uncomplicated infections. When the immune response is limited, the result is often a chronic infection [2]. Biofilms are a well-studied case of immune evasion and serve as a paradigm for understanding chronic infections. In biofilms, cells are protected from the large components of the immune system by a surface exopolymer [3-5]. Antibiotics kill the regular cells, but dormant persisters survive, and when the concentration of antibiotic drops, they resuscitate and repopulate the biofilm [2]. This scenario is supported by our finding of high-persister (hip) *Pseudomonas aeruginosa* selected in the course of prolonged antibiotic treatment [6]. Isolated from patients with late-stage cystic fibrosis, hip mutants can produce 1000 times more persisters than the parent strain; this indicates that selection for increased tolerance (rather than resistance) provided the pathogen with a survival advantage. Similarly, hip mutants are selected during treatment of oral thrush caused by *Candida albicans* [7]. In *Salmonella typhimurium*, entrance of pathogens into human cells where they are protected from the immune system is accompanied by a sharp increase in persister formation and tolerance to killing by antibiotics [8]. In tuberculosis, dormant cells are likely responsible for the need of a lengthy treatment of the acute stage and for the latent form of the disease. *Mycobacterium tuberculosis* hides from the immune system in macrophages or in granulomas [9].
B. burgdorferi causes LD with 300,000 estimated cases annually in the United States alone [10]. When treated early with antibiotics, the disease usually resolves [11, 12]. If treatment is delayed, the pathogen spreads throughout the body and can cause meningitis, arthritis, and carditis. Meningitis and carditis are mostly self-limited, but Lyme arthritis can persist for years [13, 14]. A substantial proportion of patients receiving their first course of antibiotics for Lyme arthritis do not respond fully to a 28-day course of treatment. In such cases, retreatment with additional courses of antibiotics is recommended [13, 15, 16]. B. burgdorferi avoids immune attack by antigenic variation of surface components and by decreasing exposure of antigens [17-19]. In this regard, LD resembles other chronic infections where the pathogen is protected from the immune system, and persister cells may enable it to survive treatment with antibiotics. In E. coli, the model organism for the study of persisters, dormant cells are formed primarily through expression of TA modules. Toxins confer dormancy by either inhibiting protein synthesis or by decreasing the energy level of cells [20-22]. TA modules are widely spread among bacteria, and are copiously present in some pathogens. E. coli has more than 30 TA modules and M. tuberculosis over 75 [23, 24]. Interestingly, there are apparently no TA modules in the genome of B. burgdorferi [25]. Virtually nothing is known about persisters in this species. In this study, we report formation of drug-tolerant persisters in B. burgdorferi and describe possible approaches to their elimination.

2.3 Materials and methods

2.3.1 Bacterial strains and growth conditions: Borrelia burgdorferi B31 5A19 that had been passaged five times in vitro was kindly provided by Dr. Monica Embers [26]. B. burgdorferi was grown in BSK-II liquid media in a microaerophilic chamber (34°C, 3% O₂, 5% CO₂). Cultures
ware started by thawing -80°C glycerol stocks of *B. burgdorferi* (titer approximately $10^7$ cfu/mL) and diluting 1:20 into fresh BSK-II media.

BSK-II liquid medium was prepared according to protocol received from Monica Embers’ lab by adding the following ingredients to 400 ml of deionized water and mixing thoroughly: 20 g bovine serum albumin (Sigma), 2 g neopeptone (Fluka), 0.8 g yeastolate (BD), 4 g HEPES sodium salt (Sigma), 2.4 g 10X CMRL (US Biologicals), 0.28 g sodium citrate (Fisher), 0.32 g sodium pyruvate (Sigma), 2 g glucose (Fisher), 0.16 g N-acetyl-glucoasmine (Sigma), 0.88 g sodium pyruvate (Sigma). The pH of the medium was adjusted to 7.6 and 24 ml of rabbit serum (Sigma) was added to the media. The medium was then filtered through a 0.22 µm filter.

Semi-solid plating was used to obtain cfu counts [27]. First, BSK 1.5X medium for semi-solid plating was prepared as in Samuels 1995 [27]. The following ingredients were added to 1 L of deionized water (LabChem, Inc) and mixed thoroughly: 8.33 g neopeptone (Fluka), 4.22 g yeastolate (BD), 9.99 g HEPES acid (Fisher), 8.33 g glucose (Fisher), 1.22 g sodium citrate (Fisher), 1.33 g sodium pyruvate (Sigma), 0.670 g N-acetyl-glucoasmine (Sigma), 7.66 g sodium bicarbonate (Sigma). The pH of the media was adjusted to 7.5 and then 83.25 g of bovine serum albumin (Sigma) was added. The medium was stirred for one hour then filtered using a 0.22 µm filter. 1.5X BSK-II was stored at 4°C and used within 7 days of preparation. On the day of plating, 125 ml of 1.5X BSK was mixed with 6 ml rabbit serum and 19 ml 1X CMRL (97.89 mg/mL) and equilibrated to 55°C. 1.7% agarose (Lonza) was melted and equilibrated to 55°C. When all ingredients had equilibrated to 55°C, 1.7% agarose was added to 1.5X BSK at a ratio of 2:1 (BSK:agarose) to create BSK agarose. 8 ml of BSK agarose was dispensed into 60mm Petri dishes as bottom agar and allowed to solidify. For top agar, 100 µl of the given dilution of
*B. burgdorferi* was mixed with 5 ml of 55°C BSK agarose and poured onto the bottom agar plates and allowed to solidify. The plates were incubated in zip lock bags in microaerophilic chamber (34°C, 3% O_2, 5% CO_2) for at least 21 days to obtain visible colonies.

### 2.3.2 Antimicrobial agents:
Amoxicillin (Sigma), doxycycline hydrochloride (MP Biomedicals), ceftriaxone disodium salt hemi (heptahydrate) (Sigma), and vancomycin hydrochloride (Sigma) were dissolved in water. Mitomycin C (Sigma), gemifloxacin mesylate (Tecoland Corporation), and spectinomycin dihydrochloride pentahydrate (RPI) were dissolved in DMSO. Daptomycin cyclic lipopeptide (Sigma) was dissolved in a 5 µg/ml solution of calcium chloride. Stock solutions of antibiotics were aliquoted and stored at -20°C until use. Antibiotics did not undergo freeze-thaw cycles.

### 2.3.3 Killing experiments:
*B. burgdorferi* was cultured in liquid BSK-II media for 3 days to late-exponential growth phase or for 5 days to stationary phase. Antibiotics were then added to the culture. The cultures were incubated in the microaerophilic chamber (34°C, 3% O_2, 5% CO_2). At a given time point, an aliquot of the culture was washed twice by centrifuging the culture at 13.2k rpm for 5 minutes and resuspending the pellet in an equal volume of fresh BSK-II medium. The cultures were then serially diluted in fresh BSK-II media. 100 µl of the appropriate dilution was mixed with 5 ml of BSK agarose and poured as top agar. Plates were incubated in the microaerophilic chamber until visible colonies appeared (at least 21 days).

### 2.3.4 Growth-persister experiments:
Cultures of *B. burgdorferi* were started as described above. At each time point, an aliquot of a growing culture was removed, diluted, and plated for
cfu counts to generate the growth curve. A second aliquot (1 mL or 3 mL) was removed at the same time and challenged for five days with the indicated antibiotic. After five days, an aliquot of challenged culture was removed, washed twice, diluted, and plated for cfu counts to generate the persister curve.

2.3.5 Minimum Inhibitory Concentration (MIC) tests: A slightly modified version of the broth microdilution [28] was used. *B. burgdorferi* was grown in liquid culture for three days to reach exponential phase and then back diluted 1:10 into fresh BSK-II media to make the inoculum solution. All antibiotics were prepared as stock solutions in solvent (water or DMSO) based on the concentration to be tested and diluted in two fold increments in a 96 well stock plate. 2 µl per well of the antibiotic stock solution was transferred to the 96 well MIC plate to which 198 µl of the *B. burgdorferi* inoculum solution was added (final inoculum of approximately 10^6 cells/well). Media, growth, and vehicle controls were included on each plate. The MIC plate was covered with Breatheasy Film (Diversified Biotech) and incubated in the microaerophilic chamber (34°C, 3% O_2, 5% CO_2) for 72 hours. The lowest concentration of antibiotics that showed inhibition of growth was interpreted as the MIC. All MIC assays were repeated at least twice in triplicate.

2.4 Results

2.4.1 Characterization of *B. burgdorferi* persisters

The presence of persisters is indicated by a biphasic killing pattern in a time-dependent killing experiment. The bulk of the population is rapidly killed, followed by a slower rate of death in a subpopulation of tolerant cells [29, 30]. In order to determine whether *B. burgdorferi* forms persisters, time-dependent killing experiments were performed with antibiotics commonly
prescribed to patients with LD. Doxycycline is a bacteriostatic protein synthesis inhibitor; amoxicillin and ceftriaxone inhibit bacterial cell wall synthesis and are bactericidal for many bacteria. Minimum inhibitory concentrations (MICs) of doxycycline, amoxicillin, and ceftriaxone were determined (Table 2.1). Levels of antibiotics close to what is achievable with standard clinically prescribed treatment dosing were chosen to evaluate persister formation in *B. burgdorferi*, and we used colony forming unit (cfu) count to determine viability.

**Table 2.1**: Selected antibiotics tested against *B. burgdorferi*.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Class</th>
<th>MIC (µg/ml)</th>
<th>Max. serum concentration (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin</td>
<td>β-lactam</td>
<td>0.06</td>
<td>7.6 [31]</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>Cephalosporin</td>
<td>0.01</td>
<td>256.9 [32]</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>Tetracycline</td>
<td>0.25</td>
<td>2.6- 5.9 [33]</td>
</tr>
<tr>
<td>Gemifloxacin</td>
<td>Fluoroquinolone</td>
<td>0.125</td>
<td>2.33 [34]</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>Aminoglycoside</td>
<td>2</td>
<td>140-160 [35]</td>
</tr>
<tr>
<td>Daptomycin</td>
<td>Lipopeptide</td>
<td>12.5-25</td>
<td>55-133 [36]</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>Glycopeptide</td>
<td>0.25</td>
<td>40 [37]</td>
</tr>
<tr>
<td>Mitomycin C</td>
<td>Antitumor antibiotic</td>
<td>0.2</td>
<td>3.2 [38]</td>
</tr>
</tbody>
</table>

The MIC was determined by broth microdilution method.
Amoxicillin (6 μg/ml, 100X MIC) and ceftriaxone (3 μg/ml, 300X MIC) at clinically achievable levels killed the majority of cells in the first day, after which a slow phase of death followed for the next 6 days (Figure 2.1A). This characteristic biphasic pattern of killing is consistent with the presence of drug-tolerant persister cells.

Previous studies have shown that the persister fraction in other bacteria remains relatively unchanged even as the antibiotic level increases. We sought to determine if \textit{B. burgdorferi} persisters behaved similarly in a dose-dependent killing experiment. As the concentration of amoxicillin and ceftriaxone increased, the fraction of surviving cells remained largely unchanged (Figures 2.1B-D). Doxycycline is a bacteriostatic antibiotic, but at higher concentrations appeared to effectively kill \textit{B. burgdorferi} (Figure 2.1D). Again, the fraction of surviving cells did not change significantly with increasing levels of the compound. Thus, \textit{B. burgdorferi} forms persisters capable of surviving very high concentrations of antibiotics, which exceed what is clinically achievable.
FIG 2.1 Killing of *B. burgdorferi* by antibiotics. (A) Time-dependent killing. Antibiotics were added to an exponentially growing culture, samples were taken over time, washed, diluted, and plated in semi-solid BSK-II media for cfu counts. The culture was treated with amoxicillin (Amox) (6 µg/ml) or ceftriaxone (Cef) (3 µg/ml) (n=9). (B-D) Dose-dependent killing. A late-exponential culture of *B. burgdorferi* culture was exposed to antibiotics for 5 days, and surviving cells were determined by cfu count. The culture was treated with amoxicillin (B), ceftriaxone (C), or doxycycline (Dox) (D) (n=6). Error bars represent standard error.

Density-dependent formation is a common feature of persisters reported for all pathogens examined so far, including *E. coli, Staphylococcus aureus, P. aeruginosa*, and *M. tuberculosis* [6, 39-41]. In order to test this property in *B. burgdorferi*, samples from a growing culture were removed over time, exposed to a lethal dose of antibiotic for 5 days, and then plated for cfu. There was a characteristic dip in persister levels in the early log phase, which is probably due to the resuscitation of dormant cells carried over from the inoculum (Figure 2.2). At mid-log phase,
there is a sharp increase in persister levels, which continues as the density of the culture rises. In *E. coli*, once the culture reaches stationary state, complete tolerance is achieved for β-lactams that only kill growing cells [42]. In *B. burgdorferi*, we observe a very different picture – both amoxicillin and ceftriaxone kill stationary cells fairly well, yet the fraction of persisters continues to increase. One possibility is that this “stationary” culture actually represents a steady state where some cells die and others grow.

**FIG 2.2** Growth-dependent persister formation in *B. burgdorferi*. Growth in BSK-II medium was determined by CFU count. Persister levels were determined by taking samples from the growing culture, exposing to antibiotic for 5 days, and counting cfu. (a) Amoxicillin (Amox) (6 µg/ml) (*n* = 6); (b) ceftriaxone (Cef) (3 µg/ml) (*n* = 6). Error bars represent standard errors.

Next, we tested whether the *B. burgdorferi* cells surviving antibiotic treatment are drug-tolerant persisters or resistant mutants. For this, colonies produced by the surviving cells were regrown and tested for MIC. The amoxicillin and ceftriaxone MIC remained unchanged, showing that surviving cells had not acquired or developed a genetic mechanism for antibiotic resistance. The population grown from the surviving cells produced the same level of persisters as the original population (Figure 2.3). These experiments show that *B. burgdorferi* forms typical persister cells.
FIG 2.3 Persister formation is not heritable. Colonies recovered from a persister experiment before and after antibiotic treatment were used to inoculate fresh BSK-II media. The colonies were allowed to grow for 3 days and treated with the same antibiotic used in the original persister experiment for 5 days. Persister levels of the colonies recovered after antibiotic treatment (Persisters) were not significantly different than the colonies recovered before antibiotic treatment (Control). N=5. Error bars represent standard error. Amox = amoxicillin, Cef = ceftriaxone.

2.4.2 Eradication of B. burgdorferi Persisters

Drug combinations. Some antibiotics act synergistically, such as sulfonamide and trimethoprim, polymixin and gentamicin, aminoglycosides and β-lactams [43], and we wanted to see if a combination of compounds known to be active against B. burgdorferi will increase efficiency of killing both regular and persister cells.

All possible two-drug combinations of amoxicillin, ceftriaxone, and doxycycline were tested with a late-exponential phase culture in a time-dependent killing experiment and found to be no more effective than the drugs used individually in killing of B. burgdorferi (Figure 2.4A). Doxycycline actually inhibited the action of amoxicillin. We have shown previously that fluoroquinolones and aminoglycosides can kill non-growing cells [44, 45], and we next tested
these compounds against *B. burgdorferi*. The pathogen is generally poorly susceptible to compounds from these classes. However, the *B. burgdorferi* MICs for gemifloxacin (fluoroquinolone) and spectinomycin (aminoglycoside) are within achievable human dosing levels so we chose to test them [34, 35, 46, 47] (Table 2.1). Both gemifloxacin and spectinomycin were ineffective in killing *B. burgdorferi* at tested concentrations (Figure 2.4B). Combining these compounds also did not improve killing (Figure 2.4B).

**FIG 2.4** Killing of *B. burgdorferi* with drug combinations. (a) Time-dependent killing of late exponential *B. burgdorferi* cultures exposed to the indicated antibiotics in combination. Amoxicillin (Amox) (6 µg/ml), ceftriaxone (Cef) (3 µg/ml), and doxycycline (Dox) (2.5 µg/ml) (n=6). (b) Killing of late exponential *B. burgdorferi* exposed to gemifloxacin (Gemi) (1.5 µg/ml) and/or spectinomycin (Spec) (160 µg/ml) singly or in combination (n=6). An aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid BSK-II media for cfu counts. Error bars represent standard error.

**Experimental compounds.** We sought to examine some novel potential antimicrobial agents. We recently showed that acyldepsipeptide (ADEP4), an activator of the ClpP protease, effectively kills persisters in *S. aureus* [48]. In the presence of ADEP4, the protease cleaves mature proteins, forcing the cell to self-digest. However, ADEP4 did not have significant activity against *B. burgdorferi* (not shown), which may be due to poor penetration.

We then considered whether knowledge of *B. burgdorferi* biology might be exploited to predict vulnerability to existing approved compounds. *B. burgdorferi* lives under microaerophilic
conditions, where the capacity for energy generation is limited by comparison to aerobic organisms. Daptomycin is the only approved membrane-acting antibiotic that disrupts the proton motive force. The *B. burgdorferi* MIC to daptomycin was fairly high, 12-25 µg/ml (Table 2.1), in accordance with published data [49]. Daptomycin was highly bactericidal against *B. burgdorferi*, but a remaining subpopulation of persisters survived (Figure 2.5), suggesting that *B. burgdorferi* persisters can tolerate a drop in the energy level. Next, we tested vancomycin. This large glycopeptide antibiotic binds to lipid II, precursor of peptidoglycan, on the outside of the cytoplasmic membrane. Vancomycin is highly effective against Gram-positive bacteria, but does not penetrate across the outer membrane of Gram-negative species. Surprisingly, the vancomycin MIC with *B. burgdorferi* is low, 0.25 µg/ml, similarly to Gram-positive species. *B. burgdorferi* has an outer membrane; the basis for this anomaly is unclear. Vancomycin effectively killed growing cells of *B. burgdorferi*, but not persisters, and was comparable to ceftriaxone (not shown). We also tested teixobactin, a compound we recently discovered, which also binds lipid II [50]. At 1.2 kDa, teixobactin is considerably smaller than vancomycin (1.8 kDa), but it did not exhibit good activity in killing *B. burgdorferi* (not shown).
FIG 2.5 Killing of *B. burgdorferi* by daptomycin. Time dependent killing of stationary phase *B. burgdorferi* exposed to daptomycin (81 µg/mL) (n=3). Error bars represent standard error.

**Prodrugs.** Growth under microaerophilic conditions suggests vulnerability to compounds whose action depends specifically on a low oxygen environment. Nitroaromatic compounds such as metronidazole are prodrugs that are converted into reactive drugs by bacterial nitroreductases. These enzymes are expressed under anaerobic or microaerophilic conditions, and target pathogens living in these environments (i.e. *Helicobacter pylori, Clostridium difficile, E. coli*). We found that some nitroaromatic compounds like nitrofurantoin are effective in killing *E. coli* persisters [51]. However, we did not detect homologs of nitroreductases in the genome of *B. burgdorferi*. The MIC for nitroaromatic compounds (nitrofurantoin, nitrofurazone, and metronidazole) was too high to make them useful agents for killing *B. burgdorferi* persisters (data not shown).

Another compound that depends on a reductive environment for action is the prodrug mitomycin C. Upon entering the cell, mitomycin C is reduced into an active drug which then forms covalent adducts with DNA [52]. Originally discovered in a screen for antibiotics,
mitomycin C is now used as an anticancer agent. Cancers often create a microaerophilic environment, which, together with rapid cell division, accounts for the relatively selective action of mitomycin C against them. Functional RecBC and RecFOR pathways are required to repair DNA damaged by mitomycin C in *E. coli* [52]. Interestingly, according to genomic data, *B. burgdorferi* lacks the genes of the RecFOR pathway [53], further suggesting vulnerability to this compound.

Mitomycin C eradicated a late exponential culture of *B. burgdorferi* within 24 hours, with no detectable persisters remaining (Figure 2.6A). This was observed with a low, clinically achievable dose of the compound - 1.6 µg/ml, or 8X MIC. In a dose-dependent experiment, eradication of a late exponential culture was achieved within 5 days with a 0.8 µg/ml (4X MIC) dose of the compounds (Figure 2.6B). Finally, mitomycin C was tested against a stationary culture of *B. burgdorferi*. Surprisingly, eradication was achieved with a low dose of 4X MIC within 24 hours (Figure 2.6C). It appears that a stationary population is more susceptible to this compound than an exponentially growing one.
FIG 2.6 Killing of *B. burgdorferi* by mitomycin C (MMC). (A,C ) Time-dependent killing of *B. burgdorferi*. Three independent cultures of *B. burgdorferi* either at late exponential phase (A) or stationary phase (C) of growth were treated with MMC: 0.8 µg/ml (4X MIC) or 1.6 µg/ml (8X MIC). (B) Dose-dependent killing of late exponential cultures of *B. burgdorferi* culture after 5-day exposure to increasing concentrations of MMC. An aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid BSK-II media for cfu counts. N=6. The x-axis is the limit of detection. An asterisk represents eradication to the limit of detection.

**Pulse-dosing.** Apart from identifying compounds capable of killing persisters, it may also be possible to eliminate them with conventional bactericidal antibiotics using pulse-dosing. Based on our results, the level of persisters is lowest during early exponential growth (Figure 2.2). We reasoned that allowing growth to resume and then re-treating them as they enter exponential phase could kill persisters surviving an antibiotic challenge. Eradication of the culture could then be achieved after several rounds of killing and regrowth. To test this, a culture of *B. burgdorferi*
was exposed to amoxicillin or ceftriaxone. The surviving persisters were allowed to resuscitate for a short period of time in fresh media, and then exposed to antibiotic again for a second round of killing. Persisters were substantially diminished after four rounds of killing with amoxicillin, and were eradicated below the limit of detection after four rounds of killing with ceftriaxone (Figure 2.7). Additionally, we found that a ceftriaxone solution stored under experimental conditions (in BSK-II media at 34°C, 3% O₂, 5% CO₂) does not lose activity, as measured by MIC against *B. burgdorferi*, for up to 20 days. The activity of amoxicillin measured similarly, however, dropped 20-fold over 20 days, which suggests degradation over time. The resulting MIC was still lower than the concentration used in killing experiments. This pulse-dosing experiment shows that a population of the pathogen can be eradicated with conventional antibiotics commonly used to treat the disease.

**FIG 2.7** Pulse dosing results in effective killing of *B. burgdorferi* persisters. Late exponential cultures of *B. burgdorferi* were treated with (a) ceftriaxone (Cef) (3 µg/ml) or (b) amoxicillin (Amox) (6 µg/ml) for 5 days. This represents the first round of killing. The cultures were then washed and allowed to recover in fresh BSK-II media for 24 hours. They were then treated again with amoxicillin (6 µg/ml) or ceftriaxone (3 µg/ml) for a further 5 days to give the second round of killing. This was repeated for a total of four rounds of killing with a 24 hours period of growth in fresh media between each round. Bars represent the average of at least three independent
cultures (n=3-6) and error bars represent standard error. The x-axis is the limit of detection. An asterisk represents eradication to the limit of detection.

Exploiting vulnerabilities of *B. burgdorferi* to design drug combinations. Based on our experience testing antibiotics belonging to different classes against *B. burgdorferi*, it became evident that the two biggest vulnerabilities of *B. burgdorferi* are DNA damage and damage to the cell envelope. Hence, we reasoned that a combination of antibiotics targeting DNA and cell envelope would be highly efficacious against *B. burgdorferi*.

Among the DNA damaging agents are drugs like mitomycin C and fluoroquinolones that have different modes of action. While mitomycin C forms adducts with DNA and causes interstrand-crosslinking of DNA [52], fluoroquinolones inhibit the action of DNA gyrase and topoisomerases by binding them to create an irreversible complex thereby inhibiting the DNA rejoining capability of these enzymes and resulting in double strand DNA breaks [54]. Among drugs acting on the cell envelope are drugs like amoxicillin and ceftriaxone that are routinely used to treat LD and other drugs like vancomycin and daptomycin that have excellent *in vitro* activity against *B. burgdorferi* but are not used for treatment of LD. Beta lactam antibiotics like amoxicillin and ceftriaxone (cephalosporin) act by blocking the cross-linking of peptidoglycan units by inhibiting the action of transpeptidases that catalyzes the formation of peptide bonds between the immature peptidoglycan units [55]. Vancomycin is a glycopeptide that inhibits the cell wall synthesis by binding with the D-Ala-D-Ala C-terminus of the pentapeptide, thus blocking the addition of late precursors by transglycosylation to the nascent peptidoglycan chain and preventing subsequent cross-linking by transpeptidation [55]. Daptomycin acts by inserting its lipophilic tail into the bacterial cell membrane causing membrane depolarization and an influx of potassium ion. This leads to arrest of DNA, RNA, and protein synthesis and eventually cell death [56].
All cell wall acting drugs described above have good efficacy against *B. burgdorferi* resulting in about 4 logs of killing of a late logarithmic phase culture (Figures 2.1 and 2.5) when used singly. Mitomycin C can eradicate a culture of *B. burgdorferi* (Figure 6). However, mitomycin C is toxic and is not a practical choice for treatment of LD. Gemifloxacin is not as active as mitomycin C but results in about 3 logs of killing of *B. burgdorferi* when used singly (Figure 2.4B). We reasoned that combining DNA damaging antibiotic to a cell envelope active drug would result in superior killing of *B. burgdorferi*.

We tested combinations of mitomycin C at sub inhibitory concentrations with cell envelope active antibiotics like amoxicillin, ceftriaxone, vancomycin, or daptomycin. All tested combinations of mitomycin C with the cell envelope active drugs were not more efficacious than the drugs used singly (Data not shown).

Similarly, we tested combinations of fluoroquinolone (gemifloxacin) with amoxicillin, ceftriaxone, vancomycin, or daptomycin. Gemifloxacin has a low MIC against *B. burgdorferi* and relatively high bioavailability in the body (Table 2.1). Combination of gemifloxacin with amoxicillin or ceftriaxone resulted in one log more killing than either drug used singly (Figure 2.8A and 2.8B). Again, combination of gemifloxacin with daptomycin resulted in three logs more killing than either drug used singly (Figure 2.8D). Vancomycin as well as combination of vancomycin with gemifloxacin resulted in near eradication of the culture when used singly (Figure 2.8C). Treatment with combination of gemifloxacin with ceftriaxone, vancomycin, or daptomycin resulted in killing below the limit of detection. Hence, combinations of gemifloxacin, a DNA damaging agent, with cell envelope acting drugs like amoxicillin, ceftriaxone, or daptomycin seem to be more efficacious against *B. burgdorferi in vitro*, than any
of the drugs used singly. These experiments need to be repeated against stationary phase *B. burgdorferi*.

**FIG 2.8** Killing of *B. burgdorferi* with combination of fluoroquinolone with cell envelope active drugs. Time-dependent killing of late exponential *B. burgdorferi* cultures exposed to gemifloxacin (1.5 µg/ml) and/or (A) amoxicillin (Amox) (6 µg/ml), (B) ceftriaxone (Cef) (3 µg/ml), (C) vancomycin (Vanc) (40 µg/ml), (D) daptomycin (Dapt) (81 µg/ml). (n=3). An aliquot was taken at indicated time points, washed, diluted, and plated on semi-solid BSK-II media for cfu counts. Error bars represent standard error. Asterisk represents killing below the limit of detection.
2.5 Discussion

The presence of drug-tolerant persisters can explain the recalcitrance of chronic infections to antimicrobial therapy, especially in cases when the disease is caused by a susceptible pathogen. While some chronic infections are ancient – leprosy, syphilis, tuberculosis – many cases in developed countries are consequences of otherwise successful medical intervention. Various indwelling devices (catheters, prostheses, heart valves) provide a substratum for biofilms that protect persisters from the immune system [2]. Even in bacterial infections that are routinely successfully treated with antibiotics, there is dependence upon the host immune system to control persisting bacteria that are not eradicated by antibiotics. The role of the immune system becomes evident when these same infections involve immunocompromised hosts and antibiotic eradication of the infection becomes much more difficult.

*B. burgdorferi* is a pathogen that can affect immunocompetent hosts. It establishes long term infections of years to lifelong in both its natural (i.e. mice) and incidental (i.e. humans) hosts in the absence of antibiotic therapy [14, 57]. Treatment in the early stages of disease results in good outcomes. Delays in diagnosis and treatment lead to sequelae that may require additional treatment. For example, patients who develop arthritis, which typically begins after 1 month of untreated infection, often do not respond fully to a first course of 28 days of antibiotics [58]. The majorities of these patients have evidence of *B. burgdorferi* DNA in their synovial fluid and will respond to additional one or two month courses of antibiotics [13, 16]. A smaller minority of patients referred to, as “antibiotic resistant Lyme arthritis” will continue to have arthritis with synovial fluid that is PCR negative for *B. burgdorferi* DNA. These patients typically respond to anti-inflammatory agents such as methotrexate or TNF-inhibitors. Both these groups of patients
should be distinguished from the highly controversial group of patients with “chronic LD” that exhibit fatigue, myalgias and arthralgias without objective evidence of disease. For the first group of Lyme arthritis patients responsive to antibiotics, given that there is no reported resistance to clinically used tetracyclines, β-lactams, and cephalosporins in the pathogen, the need for lengthy courses of therapy is unclear. The presence of persister cells is one possible explanation. Other chronic infections like *P. aeruginosa* and *Candida albicans* in patients with cystic fibrosis and oral thrush, respectively, are caused by antibiotic susceptible pathogens and persister cells are thought to be relevant for these infections *in vivo*.

We found that similar to other pathogens, the pattern of killing of *B. burgdorferi* by bactericidal antibiotics is biphasic, with a small subpopulation of surviving persisters. These surviving clones are not resistant mutants; upon regrowth they form a new persister subpopulation. Also similar to *E. coli*, *S. aureus*, and other pathogens, the level of persisters increases as the culture deviates from strictly exponential growth, reaching a maximum at stationary state. This is probably due to a deterioration of growth conditions resulting in increasing numbers of dormant cells. However, of note, the stationary state in *B. burgdorferi* is atypical, as amoxicillin and ceftriaxone continue to kill the majority of cells despite an increase in the level of persisters in the population. Cell-wall acting antibiotics do not normally kill non-growing cells. The ability of β lactams to kill non-growing cells has also been observed in *M. tuberculosis* where a combination of meropenem and a β lactamase inhibitor was able to kill viable but non-replicative cells [59]. The authors speculate that peptidoglycan remodeling continues in these non-replicating cells allowing for the activity of the β lactam. This is a possible explanation of the killing we observe of stationary phase *B. burgdorferi* with amoxicillin and ceftriaxone.
In a recently published study, Iyer, et al. [60] treated two different strains of *B. burgdorferi* with ceftriaxone and were unable to detect live *B. burgdorferi* by subculture in liquid medium. However, the cell density in that study was $10^7$ cells/ml, and according to our data, persister levels in this early exponential culture are low. In some of the biological replicates treated with ceftriaxone, we have not been able to recover live cells. At higher cell densities, the presence of persisters is unambiguous.

We recently described efficient killing of persisters in *S. aureus* [48] and in *E. coli* [51] by ADEP4 and nitrofuran produgs respectively, and tested these compounds against *B. burgdorferi*. ADEP4, an activator of the Clp protease, causes massive protein degradation in *S. aureus*, killing regular cells and persisters. However, ADEP4 was not active against *B. burgdorferi*. We also reported that nitrofuran produgs are effective in killing *E. coli* persisters. Nitrofurans are reduced by bacterial nitroreductases into generally reactive compounds, explaining their activity against persisters. Nitroreductases are expressed under anaerobic or microaerophilic conditions. *B. burgdorferi* is a microaerophilic organism, but does not have obvious homologs of a nitroreductase, and nitrofurans we tested were fairly inactive.

We also tested daptomycin, a lipopeptide that acts by increasing $K^+$ permeability of the membrane. Being in a low-energy (microaerophilic) environment, the pathogen may be vulnerable to membrane-acting compounds. Daptomycin killed the majority of cells in a stationary culture, but the level of surviving persisters was comparable to that of a stationary culture treated with ceftriaxone. In a recent publication daptomycin was reported to kill *B. burgdorferi* persisters more effectively than regular cells [49]. This conclusion was based on equating stationary cells with persisters. As follows from our experiments, a stationary culture harbors a small subpopulation of persisters. The actual level of stationary cells apparently
surviving treatment by daptomycin in that study was very high, 28%, as determined by live/dead staining. Under similar conditions, we detect about $10^3$ (0.002%) surviving persisters by cfu count. It appears that live/dead staining may be over reporting the level of live \textit{B. burgdorferi} cells.

Another weakness of the pathogen is its apparently limited ability for DNA repair. Based on the genome, \textit{B. burgdorferi} lacks recFOR. In \textit{E. coli}, both RecBC and RecFOR are required for repair of DNA damage caused by an anticancer drug. Mitomycin C at a low, clinically achievable dose (8X MIC), eradicated \textit{B. burgdorferi} persisters in both exponential and stationary cultures within 24 hours. A highly reduced environment activates mitomycin C, and this contributes to its selective action in microaerophilic tumors. While the killing of persisters by mitomycin C is impressive, given the toxicity of this drug, this is more of a proof-of-principle for a compound exploiting the weaknesses of this pathogen rather than a clinically useful agent. Treatment with mitomycin C can result in serious negative side effects and it should not be used for treatment of LD. This agent will be useful to examine the possible contribution of persisters to the disease in an animal model of infection.

One common strategy for improving elimination of infective agents is to combine existing compounds. For example, β lactams and aminoglycosides are known to synergize with each other to achieve effective killing of \textit{Enterococci} [61]. We tested combinations of standard antibiotics used in treatment of LD as well as a combinations of a fluoroquinolone and an aminoglycoside, compounds that often synergize and are capable of killing non-growing cells. However, there was no synergy in killing \textit{B. burgdorferi} with any of the tested combinations. We reasoned that a combination of antibiotics targeting two vulnerabilities of \textit{B. burgdorferi} would lead to elimination of this bacterium \textit{in vitro}. Hence, we tested combinations of mitomycin C or
gemifloxacin with cell wall envelope active drugs: amoxicillin, ceftriaxone, vancomycin, or daptomycin. Combinations of sub-inhibitory concentration mitomycin C with the cell envelope active drugs were no more effective than the drugs used singly. However, combinations of gemifloxacin with vancomycin, ceftriaxone, or daptomycin resulted in eradication of late-exponential phase cultures of *B. burgdorferi*. After some optimization, these combinations can be tested for efficacy in an animal model of Lyme disease, and if successful, in humans.

Another peculiar feature of *B. burgdorferi* and a weakness of the pathogen is the lack of development of resistance to any antibiotic used to treat LD. Even attempts to raise mutants resistant to amoxicillin and ceftriaxone *in vitro* have been unsuccessful. Joseph Bigger proposed an interesting strategy for elimination of persisters in 1944, in the first publication describing these cells [62]. The rationale is to add antibiotic to kill off regular cells; wash it away; allow the culture to start regrowing, at which point persisters will resuscitate. Reintroducing antibiotics will kill the regrowing bacteria. The argument against pulse dosing is that this protocol invites resistance development. Given that this is not a concern for *B. burgdorferi*, pulse dosing may be an effective strategy and we performed pulse dosing with amoxicillin and ceftriaxone. Persisters were eradicated with ceftriaxone in four pulses. These experiments form the basis for testing pulse dosing in an animal model, and if successful, in humans.

While we have identified the presence of *B. burgdorferi* persisters in cultures of the organism, the mechanisms by which they are able to survive remain unknown. There are multiple pathways of persister formation in other bacteria. The study of persisters so far identified redundant TA modules as a main component responsible for persister formation in *E. coli* and *S. typhimurium* [8, 20, 22]. TA modules are widely spread among bacteria, but are surprisingly absent from the genome of *B. burgdorferi*. Other components leading to persister
formation in *E. coli* have been detected as well - the stringent response [63], various metabolic processes [64, 65], global regulators, and protein stabilizing chaperones [65]. Future work will determine if these or other processes are involved in persister formation in *B. burgdorferi* and if persisters play a role in the pathogenesis of LD in humans.

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### 2.6. Contributions

Bijaya Sharma and Autumn V. Brown: Designed and performed the experiments, wrote the manuscript

Nicole E. Matluck: Performed experiments

Linden T. Hu and Kim Lewis: Provided valuable advice and wrote the manuscript
2.7 References


Chapter 3: Development of a high-throughput screen to identify *B. burgdorferi* specific compounds
3.1 Abstract

Lyme disease is the most commonly reported vector borne disease in the United States. It is caused by the spirochete, *Borrelia burgdorferi*. 10-20% of patients experience symptoms after treatment with standard course of antibiotics. In other chronic infections, presence of drug-tolerant persisters has been linked to recalcitrance of the disease. Previously, we showed that *B. burgdorferi* forms persisters *in vitro*. If present *in vivo*, these persisters may cause treatment failure in a sub-population of Lyme patients. Previous studies have shown that compounds that are specific to a given bacteria may also be active against its persisters. Hence, an antimicrobial that specifically kills *B. burgdorferi* may be active against its persister and hence address the problem of recalcitrance of disease. Thus, in this study we developed a high-throughput screen to identify compounds from a compound library that are active only against *B. burgdorferi*. In a pilot study, we screened 10,000 compounds from a commercially available small compound library of which 45 were active against *B. burgdorferi*. Counter screening against a panel of commensal bacteria identified 22 of the 45 hits as being active only against *B. burgdorferi*. Of the 22 compounds, 2 have an MIC of less then 5 µg/ml against *B. burgdorferi*, low cytotoxicity against mammalian cell lines, and show better *in vitro* efficacy against *B. burgdorferi* compared to ceftriaxone. The results of the pilot study validate the screen paving the path for screening of additional compound libraries.

3.2 Introduction

LD is caused by the spirochete *B. burgdorferi*. Upon transmission by the bite of a tick belonging to the genus *Ixodes*, it can result in symptoms ranging from fever and fatigue to arthritis, carditis, and meningitis. For acute LD, typical treatment involves taking oral doxycycline or amoxicillin for 14-21 days, while for late disseminated LD, a 28-day course of
intravenous ceftriaxone is recommended [1]. While the majority of individuals see resolution of symptoms with these two treatment regimens, about 10-20% of Lyme patients experience symptoms months to years after antibiotic treatment [2]. Patients that do not respond to antibiotic treatment can be separated into two groups: (1) Patients with unresolved Lyme arthritis and (2) Patients with other non-specific symptoms like fatigue, musculoskeletal pain, and cognitive abnormalities (widely known as PTLDS). Among patients that have unresolved Lyme arthritis, those who test positive for *B. burgdorferi* are given an additional 1-2 months course of oral or IV antibiotics (doxycycline, amoxicillin, or ceftriaxone) and those who test negative for *B. burgdorferi* are treated with anti-inflammatory agents [3]. There is, however, no universally accepted treatment guideline for patients with PTLDS. Given that *B. burgdorferi* is susceptible to antibiotics used in the clinic and upon infection *B. burgdorferi* activates both innate and adaptive immune response, the relapse of symptoms is puzzling. Some researchers argue that PTLDS is caused by an autoimmune reaction, while others suspect symptoms are due to persistent *B. burgdorferi* infection [4].

*B. burgdorferi* has been shown to persist in monkeys, dogs, and mice after antibiotic treatment by using a variety of methods including RTPCR, immunofluorescence, and anti-*B. burgdorferi* serum testing [4-9]. However, *B. burgdorferi* has not been cultured from an antibiotic treated host yet. Xenodiagnosis has been used to show that uninfected ticks can acquire *B. burgdorferi* DNA from infected hosts that have undergone antibiotic treatment, further strengthening the evidence for ongoing *B. burgdorferi* infection in hosts (mice, dogs, monkeys, and humans) after antibiotic treatment [8-10].

Some have speculated that these leftover spirochetes are drug-tolerant persister cells [4, 8]. Persisters are drug-tolerant phenotypic variants of an isogenic population and have been
reported to be present in a variety of pathogenic bacteria including *Staphylococcus aureus*, *S. typhimurium*, *P. aeruginosa*, *M. tuberculosis*, and UTI strains of *E. coli* [11-15]. Antibiotics kill regular cells leaving drug-tolerant persisters, which regrow after the antibiotic stress is removed and cause the relapse of symptoms. This is supported by previous work in the Lewis lab in which they found that hip mutants of *P. aeruginosa* and *C. albicans* are selected for in patients with cystic fibrosis and oral thrush, respectively, over the course of prolonged antibiotic treatment [11, 12]. We showed that *B. burgdorferi* forms persisters *in vitro*. If present *in vivo*, this may explain the sometimes-chronic nature of LD.

It is also possible that PTLDS is caused by an autoimmune reaction. Highly immunogenic *borrelial* antigens have been shown to persist in mouse models after killing *B. burgdorferi* and are hypothesized to cause the long-term symptoms [16, 17]. This theory, however, has not been validated. Recently, autoimmune diseases such as Lupus and Rheumatoid arthritis (RA) have been linked to antibiotic use and to impoverishment of the microbiome associated with a Western life-style [18]. In patients with Lupus, the dysbiosis of microbiome leads to a “leaky” gut epithelium. This results in disruption of the gut epithelium, allowing intestinal microbes and metabolites to enter into circulation and cause chronic disease [19]. RA has also been linked to a disrupted microbiome- particularly loss of a *Prevotella sp*. Interestingly, in a mouse model of RA, introduction of *Prevotella histicola* resulted in reduction of both incidence and severity of arthritis [20]. In Graft-Versus-Host-Disease (GVHD), where immune rejection occurs after transplantation, bone marrow transplantation is followed by a depletion of the microbiome in both humans and mice [21, 22]. The link of the autoimmune disease to microbiome is a recent development and plausible in the case of LD where the standard therapy is between 14 to 28-day course of antibiotics.
The current treatment regimen for LD is sub-optimal because it results in relapses in about 10-20% of patients. This highlights the need of a better treatment option. If treatment failure is causing the presence of drug-tolerant persisters and/or a dysbiosis in the gut microbiome, then a narrow spectrum antimicrobial capable of killing persisters of *B. burgdorferi*, while causing minimal disruption to the microbiome, is the ideal treatment option for LD.

Drug screens performed in the Lewis lab have shown that drugs that are specific to a certain microorganism may also be active against its persisters [23, 24]. These selective compounds are interesting because they must possess a specific target in the pathogen not shared by their fellow prokaryotes, inherently making them non-toxic to distantly related human cells. For example, hpi1 is a small compound identified to be selective against *Helicobacter pylori* with activity against its persisters, has low toxicity to human cells, and excellent efficacy in mouse model of infection [23]. Similarly, lassomycin, a cyclic peptide, is *M. tuberculosis* specific with activity against *M. tuberculosis* persisters and is currently under clinical development [24].

I reasoned that a *B. burgdorferi* specific compound might be active against its persisters. Genome analysis of *B. burgdorferi* revealed that about 29% of genes on the chromosome and 90% of genes on the plasmid are unique to *B. burgdorferi* [25, 26]. The high number of unique genes is likely due to the specialized life-style of *B. burgdorferi*, wherein it adapts efficiently between different kinds of hosts and growth conditions. Again, though not saturating, transposon mutagenesis has identified 66% of genes in the chromosome and 43% genes in the plasmid of *B. burgdorferi* as essential [27]. It is likely that some of the chromosomal and plasmid genes that are unique to *B. burgdorferi* are also essential. If true, this provides an opportunity to discover a compound that hits one of these unique targets in *B. burgdorferi* making it possible to
discover *B. burgdorferi* specific compounds from collections of compounds that have failed to produce broad-spectrum antibiotics. Moreover, a species-specific compound will not harm the human gut microbes, whose importance in human health is increasingly appreciated with recent discoveries of the possible role of gut microbiome in diseases including but not limited to colorectal cancer, rheumatoid arthritis, obesity, diabetes, and cardiovascular diseases [28]. Hence, I adapted the colorimetric assay for MIC determination to perform a high throughput screen of the Enamine library, a commercially available small compound library [29]. A comparative screen was done to identify *B. burgdorferi* specific compounds and in the process exclude toxic compounds and compounds that target general bacterial metabolism [30]. A primary screen of the library was performed to identify compounds effective against *B. burgdorferi*. The hits obtained from the primary screen were then screened against other microorganisms (*Clostridium difficile, Escherichia coli, Bacteroides thetaiotaomicron, Bacteroides fragilis, Enterococcus faecalis, Lactobacillus reuteri, Staphylococcus aureus, and Bifidobacterium longum*). Compounds with activity against only *B. burgdorferi* were further examined *in vitro* for minimum bactericidal concentration (MBC), toxicity against human cell lines, and killing kinetics against *B. burgdorferi*. To our knowledge, a screen of compound libraries to identify *B. burgdorferi* specific compounds has not been done. It should be noted that FDA approved antimicrobials have been screened for activity against stationary phase *B. burgdorferi* [31]. However, these compounds are broad-spectrum, leading to deleterious effects in the microbiome.

In this pilot study, I screened 10,000 compounds from the Enamine library, a commercially available small compound library, of which 45 were active against *B. burgdorferi*. Counter screening against a panel of commensal bacteria identified 22 of the 45 hits as being
active only against *B. burgdorferi*. Of the 22 compounds, 2 have a MIC of less than 5 µg/ml against *B. burgdorferi*, a therapeutic index of more than 10 against mammalian cell lines, and a better *in vitro* efficacy against stationary phase *B. burgdorferi* compared to ceftriaxone, an antibiotics used clinically against LD that has the best *in vitro* efficacy against *B. burgdorferi* in our studies so far. The results of the pilot study validate the screen, paving the path for screening of additional compound libraries.

### 3.3 Materials and methods

#### 3.3.1 Bacterial strains and growth conditions

*Borrelia burgdorferi* B31 5A19 was kindly provided by Dr. Monica Embers [32]. This strain had been passaged five times *in vitro*. *B. burgdorferi* was grown in BSK-II liquid media in a microaerophilic chamber (34°C, 3% O₂, 5% CO₂). Cultures were started by thawing -80°C glycerol stocks of *B. burgdorferi* (titer approximately 10⁷ cfu/mL) and diluting 1:20 into fresh BSK-II media.

BSK-II liquid medium was prepared according to protocol received from Monica Embers’ lab by adding the following ingredients to 400 ml of deionized water and mixing thoroughly: 20 g bovine serum albumin (Sigma), 2 g neopeptone (Fluka), 0.8 g yeastolate (BD), 4 g HEPES sodium salt (Sigma), 2.4 g 10X CMRL (US Biologicals), 0.28 g sodium citrate (Fisher), 0.32 g sodium pyruvate (Sigma), 2 g glucose (Fisher), 0.16 g N-acetyl-glucoasmin (Sigma), 0.88 g sodium pyruvate (Sigma). The pH of the medium was adjusted to 7.6 and 24 ml of rabbit serum (Sigma) was added to the media. The medium was then filtered through a 0.22 µm filter.

Semi-solid plating was used to obtain cfu counts [33]. First, BSK 1.5X medium for semi-solid plating was prepared as in Samuels 1995 [33]. The following ingredients were added to 1 L
of deionized water (LabChem, Inc) and mixed thoroughly: 8.33 g neopeptone (Fluka), 4.22 g yeastolate (BD), 9.99 g HEPES acid (Fisher), 8.33 g glucose (Fisher), 1.22 g sodium citrate (Fisher), 1.33 g sodium pyruvate (Sigma), 0.670 g N-acetyl-glucosamine (Sigma), 7.66 g sodium bicarbonate (Sigma). The pH of the media was adjusted to 7.5 and then 83.25 g of bovine serum albumin (Sigma) was added. The medium was stirred for one hour then filtered using a 0.22 μm filter. 1.5X BSK-II was stored at 4°C and used within 7 days of preparation. On the day of plating, 125 ml of 1.5X BSK was mixed with 6 ml rabbit serum and 19 ml 1X CMRL (97.89 mg/mL) and equilibrated to 55°C. 1.7% agarose (Lonza) was melted and equilibrated to 55°C. When all ingredients had equilibrated to 55°C, 1.7% agarose was added to 1.5X BSK at a ratio of 2:1 (BSK:agarose) to create BSK agarose. 8 ml of BSK agarose was dispensed into 60mm Petri dishes as bottom agar and allowed to solidify. For top agar, 100 μl of the given dilution of B. burgdorferi was mixed with 5 ml of 55°C BSK agarose and poured onto the bottom agar plates and allowed to solidify. The plates were incubated in zip lock bags in microaerophilic chamber (34°C, 3% O2, 5% CO2) for at least 21 days to obtain visible colonies.

*Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29148, *Bifidobacterium longum* ATCC BAA-999, *Clostridium difficile* CD196, *Lactobacillus reuteri* ATCC 23272, *Escherichia coli* ATCC 700927 and *Enterococcus faecalis* ATCC 47077 were cultivated in Schaedler Anaerobe Media (SAM). *Staphylococcus aureus* 8325-4 was cultivated in Mueller Hinton Broth (MHB). *Clostridium difficile* CD196 was obtained from the Sonenshein lab, Tufts University. All strains were grown anaerobically in an anaerobic chamber (Coy Lab Products, USA) at 37°C, with an atmosphere of 5% hydrogen, 10% carbon dioxide, and 80% nitrogen gas.
3.3.2 High-throughput screen for hits against *B. burgdorferi*

The high-throughput screen (HTS) was performed in 96-well microtiter plates (Corning Costar 3370) with compounds added under aerobic conditions. *B. burgdorferi* B31 5A19 was grown in BSK-II media for 3 days to mid to late exponential phase, diluted 1:5 in fresh BSK-II media which resulted in approximately 1X 10^6 cfu/mL and introduced to assay wells containing compounds. Each plate contained a positive control column (0.6 µg/mL amoxicillin, final concentration) and a negative control column (1% DMSO v/v, final concentration). Plates were incubated in the microaerophlic chamber (34°C, 3% O_2, 5% CO_2) for 120 hours and scored by eye for bacterial growth or by measuring optical density (OD) at 540 nm by a BioTek Synergy Mx (Biotek, USA). A Z'-factor of 0.71 was achieved indicating the HTS was a robust assay and the results were reliable. The Z'-factor was calculated using the equation: \(-3\times(\sigma_p+\sigma_n)/|\mu_p-\mu_n|\) (\(\mu_p\) and \(\sigma_p\): mean and standard deviation of the positive control; \(\mu_n\) and \(\sigma_n\): mean and standard deviation of the negative control).

3.3.3 Minimal Inhibitory Concentration (MIC) measurements for *B. burgdorferi*

A slightly modified version of the broth microdilution [34] was used. *B. burgdorferi* was grown in liquid culture for three days to reach exponential phase and then back diluted 1:5 into fresh BSK-II media to make the inoculum solution. All compounds were prepared as stock solutions in solvent (DMSO) based on the concentration to be tested and diluted in two fold increments in a 96 well stock plate. 2 µl per well of the antibiotic stock solution was transferred to the 96 well MIC plates to which 198 µl of the *B. burgdorferi* inoculum solution was added (final inoculum of approximately 10^5 cells/well). Media, growth, and vehicle controls were included on each plate. Each plate contained a positive control row of amoxicillin MIC starting at 0.92 µg/ml diluted in two fold increments down the row. The MIC plates were covered with
Breatheasy Film (Diversified Biotech) and incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂) for 120 hours. The lowest concentration of antibiotics that showed inhibition of growth was interpreted as the MIC. All MIC assays were repeated at least twice in triplicate.

### 3.2.4 MIC measurements for commensal strains

Standard MIC assays were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, all strains were grown under anaerobic conditions overnight to stationary phase at 37 °C with shaking (110 rpm). The cultures were diluted 1:10 in fresh media, grown for 1.5 hour at 37 °C with shaking (110 rpm), diluted 1:100 to an OD of approximately 0.01 and 196 µL of the bacterial culture dilution was inoculated into 96-well plates containing 4 µL of 2-fold serial dilutions of compounds. All wells contained a final DMSO concentration of 2% v/v. Plates were incubated at 37 °C for 20-24 hours and scored by eye and by OD measurement at 600 nm for bacterial growth. All experiments were repeated at least two times in triplicate.

### 3.3.5 Minimal Bactericidal Concentration (MBC) measurements

Minimal bactericidal concentrations assays were performed to find the lowest concentration of compound that kills more than 99.9% of the initial bacterial inoculum. Briefly, *B. burgdorferi* (1×10⁶ cfu/mL) was incubated anaerobically with serially diluted compounds in 96-well plates (Corning Costar 3370). After 120 hours of incubation, the contents of 1× MIC, 2× MIC and 4× MIC wells were taken, washed 2X in BSK-II media, serial diluted in BSK-II media, and plated for cfu counts. The plates were incubated microaerophilically (34°C, 3% O₂, 5% CO₂) for 21 days before counting cfus. All experiments were repeated at least two times in triplicate.
3.3.6 Human cell cytotoxicity

Cryopreserved FaDu cells (ATCC HTB-43) and Hep G2 cells (ATCC HB-8065) were purchased from ATCC (American Tissue Culture Collection). Cells were suspended in Eagle’s Minimum Essential Medium (ATCC 30-2003) supplemented with 10% fetal bovine serum (FBS, ATCC 30-2020) and seeded into a 75cm² tissue culture flask (BD Falcon 353136) according to the manufacturer’s recommendations. Cells were incubated at 37 °C and 5% CO₂ until ~70% confluency. The cells were trypsinized and diluted in media (10% FBS supplemented EMEM, filter sterilized) such that the final count is 2X 10⁵ cells/ml. 100 µl of diluted cell suspension was added to wells of clear-bottomed black 96-well plates (Corning Costar 3603) and incubated overnight or till ~70% confluency. 2-fold serial dilutions of compounds were prepared in a separate plate in media and then added to FaDu and Hep G2 plates. The plates were incubated at 37 °C with 5% CO₂ for 3 days. At this point, 5 µl of 3 mM Alamar Blue was added to each well of the assay plate and incubated for 3-5 hours at 37 °C with 5% CO₂. The fluorescence (544/590) was read with a BioTek Synergy Mx. The lowest concentration of each compound in a dilution series that produced more than 50% death is reported as the cytotoxic concentration (IC₅₀). DMSO concentration was kept constant in all compound-containing wells (2% v/v).

3.3.7 Antimicrobial agents

All compounds and antibiotics were dissolved in DMSO. Amoxicillin, erythromycin, metronidazole, and vancomycin hydrochloride were purchased from Sigma. Stock solutions of antibiotics were aliquoted and stored at -20°C until use.
3.3.8 Killing experiments

*B. burgdorferi* was cultured in liquid BSK-II media for 3 days to late-exponential growth phase or for 5 days to stationary growth phase. Antibiotics were then added to the culture. The cultures were incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂). At a given time point, an aliquot of the culture was taken, washed twice by centrifuging the culture at 13.2k rpm for 5 minutes, and the pellets were resuspended in an equal volume of fresh BSK-II medium. The cultures were then serially diluted in fresh BSK-II media. 100 µl of the appropriate dilution was mixed with 5 ml of BSK agarose and poured as top agar. Plates were incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂) until visible colonies appeared (at least 21 days). All assays were done in triplicate. All killing kinetics experiments were followed for 7 days. For dose-dependent killing experiments, cultures were treated with compounds for 5 days.

3.3.9 Resistant mutant isolation

*B. burgdorferi* was grown in BSK-II media. To isolate the resistant mutant and determine frequency of resistant mutant development, 10⁷ to 10⁸ cells were plated in semi solid BSK medium with the compound/antibiotic of choice. Spectinomycin was used as a control since the resistant mutant frequency for spectinomycin has been reported to be about 5-7 x 10⁶ [35]. Spectinomycin was used at the concentration of 6 µg/ml, 20 µg/ml, and 30 µg/ml, compound 3239 was used at 2.5 µg/ml and 20 µg/ml, and compound 8384 was used at 0.12 µg/ml, 0.25 µg/ml, 0.5 µg/ml, 1 µg/ml, and 2 µg/ml. Plates were incubated in the microaerophilic chamber (34°C, 3% O₂, 5% CO₂) for upto 21 days before colonies were counted. Nine, compound 3239 resistant mutants, five, compound 8384 resistant mutants, and ten, spectinomycin resistant mutants were isolated and grown in 5 ml BSK-II media with given compound/antibiotics. Glycerol stocks were made using these cultures and used as seed for further experiments.
For resistant mutant frequency calculation, cfus on plates without compounds were considered as the total number of cells plated. Cfus on plates with compounds were considered resistant mutants. Frequency of resistance was calculated according to the following formula.

\[
\text{Frequency of resistance} = \frac{\text{Total number of resistant cfus}}{\text{Total number of cells plated}}
\]

The isolated mutants were confirmed to be resistant to the given compound/antibiotic by testing the MIC of given compound/antibiotic against the resistant mutant.

3.4 Results

3.4.1 Development of a high-throughput screen (HTS) against *B. burgdorferi*

The colorimetric assay for MIC determination was adapted to perform a HTS of the Enamine compound library [29]. Briefly, compounds were manually transferred from library plates to dry test plates under aerobic conditions. *B. burgdorferi* B31 5A19 grown to mid/late exponential phase was diluted 1:5 in fresh BSK-II media and introduced to assay plates containing compounds. Plates were incubated in the microaerophilic chamber for 120 hours and scored by eye for bacterial growth. Growth of *B. burgdorferi* in the culture medium does not result in a detectable increase in turbidity. However, when *B. burgdorferi* grows it ferments glucose decreasing the pH of the media such that phenol red indicator present in the media changes color from red to yellow. This color change can be observed visually or by reading OD at 540 nm. Therefore, among the wells of a 96 well plate with test compounds, a hit is a well that does not change color from red to yellow. A Z’-factor of 0.71 was achieved, which indicated the HTS was robust and the results were reliable. Z’ is the statistical measurement of effect size and is used to judge if a high-throughput screen is robust enough to warrant further attention. For small molecule screening assays, the following Z’-factor values indicate assay fitness [36]:

- \(1 > Z’ > 0.9\): an extremely robust assay
- 0.9 > Z’ > 0.7: a robust assay
- 0.7 > Z’ > 0.5: an acceptable assay, but identification of positives will benefit significantly from any improvement
- 0.5 = Z’: the minimum recommended for high-throughput small molecule screen

In the pilot study, I screened 10,000 compounds from the Enamine library of which 45 were active against *B. burgdorferi* (hit rate= 0.45%). As previous lab members- Chao Chen, Laura Fleck and Michael Lafleur had screened the library against *C. difficile, E. coli, Bacillus anthracis,* and *H. pylori,* I was able to use data from these previous screens as the initial counter-screen for *B. burgdorferi* selectivity. Of the 45 compounds active against *B. burgdorferi,* 23 were not identified as hits in previous screens. An additional counter screen was performed against *S. aureus* with the 23 compounds. Of the 23 compounds, 1 compound hit *S. aureus.* The remaining 22 *B. burgdorferi* specific hits were subjected to a panel of eight bacteria: *B. fragilis, B. thetaiotaomicron, B. longum, C. difficile, L. reuteri, E. coli* and *E. faecalis,* to identify *B. burgdorferi*-specific compounds (Table 3.1). Of the eight bacteria, *B. thetaiotaomicron, B. longum* and *B. fragilis* are known to be abundant gut microbes [37] and *E. coli, S. aureus, C. difficile,* and *E. faecalis* are clinically significant microbes. A schematic of the screening procedure is shown in figure 3.1.
FIG 3.1 A schematic of the screening process. The HTS was originally developed by Dr. Gabriele Casadei and followed up by Dr. Chao Chen in the Lewis lab. Compounds were manually transferred from library plates to assay plates aerobically. B. burgdorferi (1×10^6 cfu/mL) was then dispensed into the assay plates. The plates were incubated microaerophilically. Hits were scored by eye observation based on color change of media from red to yellow on wells with positive growth or by measuring OD at 540 nm. Primary hits were counter screened against a panel of bacteria in the anaerobic environment and prioritized for further study.

Minimum inhibitory concentrations of these 22 B. burgdorferi specific compounds were determined against B. burgdorferi and the commensal strains (Table 3.1). Of the 22 compounds, 6 compounds were chosen for further investigation because they had an MIC of 5 µg/ml or less against B. burgdorferi and did not hit the commensal strains (Table 3.2). Structures of the 6 prioritized compounds are shown in figure 3.2.
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Compd.: Compound; Bb: B. burgdorferi; Sa: S. aureus; Ec: E. coli; Ef: E. feacalis; Bf: B. fragilis; Bl: B. longum; Cd: C. difficile; Bt: B. thetaotaomicron; Lr: L. reuter
Minimum bactericidal concentration (MBC) assay was performed with the 6 compounds. Of the 6 compounds, 4 were bactericidal and 2 were bacteriostatic (Table 3.2). Compound 3239 stood out because it eradicated the culture to below the limit of detection with only 2X MIC concentration (Table 3.2). To be considered for drug development the compound should not be toxic to human cells. Hence, cytotoxicity assays were carried out against 2 types of human cell lines- FaDu and HepG2. A cytotoxicity assay yields the IC50 value for the given compound against the tested cell line. IC50 is the lowest concentration of each compound in a dilution series that produces more than 50% death of a given mammalian cell line. I then calculated the therapeutic index (TI), the ratio of IC50 to the MIC of the active compounds. Compound 8384 stood out because it has a low MIC of 0.625 µg/ml, is bactericidal, and has a TI higher than 40 against FaDU and HepG2 cells (Table 3.2). Hence, compounds 3239 and 8384 were further studied for activity against B. burgdorferi in vitro.

Table 3.2 MBC of priority compounds against B. burgdorferi, cytotoxicity of priority compounds against mammalian cells and their therapeutic index

<table>
<thead>
<tr>
<th>Compound</th>
<th>MBC</th>
<th>Bactericidal or Bacteriostatic?</th>
<th>MIC (µg/ml)</th>
<th>FaDU IC50 (µg/ml)</th>
<th>TI</th>
<th>HepG2 IC50 (µg/ml)</th>
<th>TI</th>
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<td>Bacteriostatic</td>
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MBC: Minimum Bactericidal Concentration; MIC: Minimum Inhibitory Concentration; TI: Therapeutic Index= IC50/ MIC
FIG 3.2 Molecular structures of the prioritized Enamine hits in table 3.2. These compounds had an MIC of 5 µg/ml or less against *B. burgdorferi* and did not have activity against the panel of bacteria used for counter screening in this study.

### 2.4.2 *In vitro* killing activity of Enamine compounds 3239 and 8384

Preliminary time-dependent killing experiments were performed with compound 3239 against logarithmic and stationary phase cultures of *B. burgdorferi*. Actively growing cultures of *B. burgdorferi* used in the MBC experiments were eradicated below the limit of detection when treated with 4X MIC (10 µg/ml) of compound 3239. Thus, late log phase cultures and stationary phase cultures of *B. burgdorferi* were treated with 4X MIC of compound 3239 (Figure 3.3). Control cultures were treated with 300X MIC of ceftriaxone (0.3 µg/ml). Additional controls were left untreated. Treatment of logarithmic phase cultures of *B. burgdorferi* with 4X MIC of compound 3239 resulted in 4 logs of killing as opposed to 5 logs of killing observed when treated with 300X MIC of ceftriaxone (Figure 3.3A). Treatment of stationary phase cultures of *B. burgdorferi* with 4X MIC resulted in 3 logs of killing, a level similar to what is seen after treatment with 300X MIC of ceftriaxone (Figure 3.3B). Ideally, killing kinetic experiments are followed for 7 days to be able to observe the biphasic pattern of killing characteristic of persister
formation (See chapter 2, figure 2.1). Stationary phase killing with compound 3239 could, however, only be followed till day 3 because of contamination (Figure 3.3B).

**FIG 3.3** Time-dependent killing of *B. burgdorferi* by compound 3239. Compounds were added to cultures, samples were taken over time, washed, diluted, and plated in semi-solid BSK-II media for cfu counts. The culture was treated with compound 3239 at 10 µg/ml (4X MIC) or ceftriaxone (Cef) at 3 µg/ml (300X MIC). (A) Killing of log phase cultures (n=6), (B) Killing of stationary phase cultures (n=2-6). Error bars represent standard error.

Similarly, preliminary time-dependent killing experiments were performed with compound 8384 against stationary phase cultures of *B. burgdorferi*. Cultures of *B. burgdorferi* were treated with 10X MIC (6.25 µg/ml) of compound 8384. This represented a lethal concentration of the compound. Control cultures were treated with 300X MIC of ceftriaxone (0.3 µg/ml). Additional controls were left untreated. Treatment of stationary phase cultures of *B. burgdorferi* with 10X MIC of 8384 resulted in 3 logs of killing as opposed to 4 logs of killing observed when treated with 300X MIC of ceftriaxone (Figure 3.4).

These results were encouraging because treatment with low relative concentration of both 3239 and 8384 resulted in killing comparable to that observed when cultures are treated with a high relative concentration of ceftriaxone.
FIG 3.4 Time-dependent killing of *B. burgdorferi* by compound 8384. Compounds were added to stationary phase cultures of *B. burgdorferi*. Samples were taken over time, washed, diluted, and plated in semi-solid BSK-II media for cfu counts. The culture was treated with compound 8384 at 6.25 µg/ml (10X MIC) or ceftriaxone (Cef) at 3 µg/ml (300X MIC). n=6. Error bars represent standard error.

Given the encouraging results obtained from the pilot killing experiments, dose dependent killing experiments wherein cultures are treated with similar relative levels of compound 3239, compound 8384, or ceftriaxone were done to compare efficacy of compound 3239 and 8384 to that of ceftriaxone. Stationary phase cultures of *B. burgdorferi* were treated with 1X, 10X, and 100X MIC of compound 3239, 1X, 10X, and 100X MIC of compound 8384 or 1X, 10X, and 100X MIC of ceftriaxone for 5 days. The killing duration of 5 days was chosen because 5 days is the mid-point of the persister plateau observed when *B. burgdorferi* is treated with antibiotics like amoxicillin and ceftriaxone (see chapter 2, figure 2.1A). Aliquots of the cultures were plated for cfu counts before and 5 days after antibiotic treatment.

Treatment with 1X MIC of both ceftriaxone and compound 3239 did not kill stationary phase *B. burgdorferi* (Figure 3.5A). However, while treatment with 10X MIC of ceftriaxone did not result in any killing of stationary phase *B. burgdorferi*, administration of 10X MIC of compound 3239 resulted in 6 logs of killing of the culture (*p*=0.003) (Figure 3.5B). Again, while
3 logs of killing of stationary phase *B. burgdorferi* cultures were seen upon administration of 100X MIC of ceftriaxone, stationary phase *B. burgdorferi* cultures were killed below the limit of detection upon treatment with 100X MIC of 3239. Given that the TI of 3239 is about 10, the observation that treatment with 10X MIC of 3239 resulted in 6 logs of killing of stationary phase cultures is promising.

Similarly, while treatment with 1X MIC of ceftriaxone resulted in no killing of *B. burgdorferi*, treatment with 1X MIC of 8384 resulted in 2.5 logs of killing of stationary phase *B. burgdorferi* \((p=0.017)\) (Figure 3.5D). Administration of 10X MIC of compound 8384 resulted in 6 logs of killing of the *B. burgdorferi* compared to no killing observed when treated with 10X MIC of ceftriaxone, \((p=0.0036)\) (Figure 3.5E). Better killing efficacy with 10X MIC of 8384 was observed in this experiment (Figure 3.5EB) compared to previous experiments testing the same concentration of 8384 against stationary phase *B. burgdorferi* (Figure 3.4). This discrepancy was likely because 8384 tends to precipitate out of solution after a freeze thaw cycle. Upon noticing this, fresh solution of 8384 was prepared for the dose dependent experiment. At 100X MIC, ceftriaxone resulted in 4 logs of killing while 8384 resulted in 5 logs of killing \((p=0.00036)\) (Figure 3.5F). Killing with compound 8384 was 1 log less when used at 100X MIC compared to 10X MIC. This difference is, however, not statistically significant \((p=0.069)\). Decreased killing with a higher concentration of the compound may be due to non-target-specific binding, resulting in premature termination of processes like transcription and translation, making the cells dormant and hence tolerant to killing by antibiotics.

From these results, we can conclude that compounds 3239 and 8384 have better *in vitro* efficacy against stationary phase cells of *B. burgdorferi* than ceftriaxone.
FIG 3.5 Dose-dependent killing of B. burgdorferi with ceftriaxone and compound 3239 and 8384. Stationary phase cultures of B. burgdorferi culture were exposed to given compounds for 5 days, and surviving cells were determined by cfu count. The cultures were treated with (A) 1X MIC of ceftriaxone (0.01 µg/ml) and compound 3239 (2.5 µg/ml), (B) 10X MIC of ceftriaxone (0.1 µg/ml) and compound 3239 (25 µg/ml), and (C) 100X MIC of ceftriaxone (1 µg/ml) and compound 3239 (250 µg/ml), (D) 1X MIC of ceftriaxone (0.01 µg/ml) and compound 8384 (0.625 µg/ml), (E) 10X MIC of ceftriaxone (0.1 µg/ml) and compound 8384 (6.25 µg/ml), and (F) 100X MIC of ceftriaxone (1 µg/ml) and compound 8384 (62.5 µg/ml). n=3. Error bars represent standard error of the mean. Asterisk denotes killing below limit of detection.

2.4.3 Resistant mutant isolation

The standard technique to identify the target of a novel antimicrobial is to isolate resistant mutants, and I sought to do this for both of my B. burgdorferi specific lead compounds, 3239 and 8384. To accomplish this a high density of B. burgdorferi was plated on media with different concentrations of compound 3239 or compound 8384. Spectinomycin was used as a control because the resistant mutant frequency of B. burgdorferi against spectinomycin has been reported to be about 6E-06 [35]. Resistant mutant isolation was successful for spectinomycin and
compound 3239 at all concentrations tested (Table 3.3), and spectinomycin had a high resistant mutant frequency of $10^{-5}$ to $10^{-6}$, which was concurrent with what was previously reported [35].

Compound 3239 had a resistant mutant frequency from $10^{-5}$ to $10^{-6}$ (Table 3.3). Nine resistant mutant colonies were taken and grown in BSK-II media with 3239 and stocked as glycerol stocks. MIC of 3239 against these resistant mutants was determined. Seven mutants had an MIC of 64 µg/ml and two had an MIC of 2-4 µg/ml. To identify the molecular target of compound 3239, some of these mutants have been sent for whole genome sequencing, to identify the mutations responsible for resistance. We are currently awaiting the sequencing data.

In addition, compound 8384 had a low resistant mutant frequency of $10^{-8}$ (Table 3.3). *B. burgdorferi* was able to grow in the presence of 0.12 µg/ml and 0.25 µg/ml of compound 8384. This suggests that 8384 has a higher agar MIC than broth MIC. This is usually observed when the given compound exhibits low solubility in a solvent. Indeed, 8384 tends to precipitate out of solution after a freeze thaw cycle. Resistant mutants were only seen on plates with 0.5 µg/ml of compound 8384 and there were no colonies on plates with concentrations higher than 0.5 µg/ml.

The resistant mutants seen on plates with 0.5 µg/ml of compound 8384 have been passaged in BSK-II media with the same concentration of compound 8384. If and when these colonies grow in this selective media, they will be stocked as glycerol stocks and MIC of 8384 against these isolates will be determined to confirm that they are resistant to compound 8384. Confirmed resistant mutants will be sent for whole genome sequencing to identify the molecular target of compound 8384.
Table 3.3. Resistant mutant frequency of spectinomycin, compound 3239, and compound 8384 for *B. burgdorferi* B31 5A19 strain

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µg/ml)</th>
<th>Number of cells plated</th>
<th>Number of colonies recovered</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spectinomycin</td>
<td>6</td>
<td>1.20E+08</td>
<td>5.80E+01</td>
<td>6.90E-06</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>5.50E+07</td>
<td>2.70E+01</td>
<td>5.00E-05</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>1.20E+08</td>
<td>1.07E+02</td>
<td>5.10E-06</td>
</tr>
<tr>
<td>Compound 3239</td>
<td>2.5</td>
<td>1.20E+08</td>
<td>2.20E+02</td>
<td>1.83E-06</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.20E+08</td>
<td>2.90E+01</td>
<td>2.42E-07</td>
</tr>
<tr>
<td>Compound 8384</td>
<td>0.12</td>
<td>5.50E+07</td>
<td>TMTC</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>5.50E+07</td>
<td>TMTC</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>5.50E+07</td>
<td>5</td>
<td>8.00E-08</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.50E+07</td>
<td>0</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.50E+07</td>
<td>0</td>
<td>ND</td>
</tr>
</tbody>
</table>

TMTC: Too Many To Count; ND: Not Determined

3.5 Discussion

All antibiotics used to treat LD are broad-spectrum antimicrobials, capable of damaging the gut microbiome, and we found these compounds are not effective against persisters of *B. burgdorferi* [38]. It is plausible that PTLDS or chronic LD is caused by drug-tolerant persister cells and/or due to a dysbiosis in microbiome associated with long-term antibiotic use that leads to an autoimmune disease. Thus, a narrow spectrum antimicrobial capable of eradicating persisters of *B. burgdorferi* will be an ideal therapeutic, as it will be able to circumvent both of these potential chronic Lyme-inducing mechanisms.

There are a number of advantages to screening for species selective compounds. One of the biggest hurdles of using a HTS of compound libraries to identify useful antimicrobials is the
likelihood of identifying generally toxic compounds like detergents and inhibitors of general metabolism as positive hits. This hurdle can be overcome by eliminating these compounds early in the screening process by prioritizing compounds that do not show activity against other bacteria. Counter-screening against a panel of bacteria also eliminates the chance of rediscovering known broad-spectrum antibiotics. Additionally, unlike broad-spectrum antimicrobials, a selective compound should not damage the gut microbiome, which is increasingly proving to be important for human health.

I performed a pilot screen of 10,000 compounds from the Enamine library that yielded two compounds with specific activity against B. burgdorferi and exhibit low toxicity to mammalian cells. The two priority hits, Enamine compounds 3239 and 8384, show better in vitro killing of stationary phase B. burgdorferi than antibiotics currently used to treat LD. Drugs like amoxicillin, ceftriaxone, and doxycycline are known to cause changes in colonic microflora often resulting in reduction of bacteria like Lactobacillus, Bifidobacterium, and Bacteroides species, enabling an outgrowth of Clostridium difficile and fungal species [39-41]. In addition to having better in vitro efficacy, these B. burgdorferi specific compounds may be safe for the human microbiome. Both compound 3239 and compound 8384 are fairly large compounds with molecular weight of 447.9 g/mol and 453.6 g/mol respectively. They are also hydrophobic. Recovery of large hydrophobic compounds during a screen for species selective compounds is a trend we have seen in similar screens in our lab. We do not have an explanation for why this trend exists. Currently, we do not know the mode of action of either compound 3239 or compound 8384. However, preliminary experiments done to isolate mutants to both compounds have yielded several colonies, which are currently being investigated.
The present treatment of LD is not optimal. It is extremely long and often not efficacious. Hence, there is room for development of more efficacious therapeutics. Novel antimicrobials developed for specific eradication of *B. burgdorferi* can be optimized for shorter dosing regimen to achieve improved cure rates in Lyme. In addition to identifying two potential hits to follow up on, this study paves the path for additional drug screens to identify compounds that target *B. burgdorferi*, which may result in a better drug against LD, and reduction of cases of PTLDS.

### 3.6 Contributions

Bijaya Sharma: Designed and performed the experiments

Nicole E. Matluck, Alicyn Reverdy, Rebecca Schilling, Wendy Wu: Performed experiments

Michael Lafleur and Kim Lewis: Provided valuable advice
3.7 References


Chapter 4: Conclusion and future directions
4.1 Conclusion

I showed for the first time that *B. burgdorferi*, the causative agent of Lyme disease, forms drug-tolerant persisters *in vitro* and that these cells could be eradicated using a novel pulse-dosing regimen with ceftriaxone, one of the drugs clinically used against Lyme disease. In addition, persisters of *B. burgdorferi* can also be eradicated by treatment with mitomycin C, an anticancer agent. Additionally, I developed and validated a high-throughput screen to identify *B. burgdorferi* specific compounds from compound libraries.

This work may be of great value to the field of Lyme disease for a number of reasons. First, the cause of PTLDS has been debated for over three decades, and some researchers argue that persistent *B. burgdorferi* infection may be causal. My work shows that the antibiotics used clinically against Lyme disease are not able to eradicate a *B. burgdorferi* culture -- instead a subpopulation of drug tolerant cells survive. If such a subpopulation is able to survive antibiotic treatment *in vivo*, it could explain the treatment failure observed in 10-20% of people treated for Lyme disease. I showed that *B. burgdorferi* persisters could be eradicated *in vitro* by pulse dosing with ceftriaxone. This pulse-dosing regimen can be tested *in vivo* as a possible treatment regimen for PTLDS. Additionally, the HTS that I validated can be used to identify narrow spectrum antimicrobials against Lyme disease.

The biggest pitfall of the study is that it is done *in vitro* and hence lacks the immune components that would otherwise be present *in vivo* to aid in clearing infection. Hence, *in vitro* efficacy would not always translate to *in vivo* efficacy. Whether pulse dosing works *in vivo* remains to be seen. Despite its excellent *in vitro* killing efficacy, mitomycin C is toxic and should not be used for treatment of Lyme disease. Similarly, the colorimetric assay used for the
drug screen is quick and easy but it would identify compounds that lower the pH of the medium as a positive hit thus increasing the chance of getting false positives. Despite these pitfalls, these studies provide the background to begin investigating persisters in vivo and discover a better treatment option for LD.

4.2 Future directions

Beyond the implications my work has had for the field of Lyme, there are several exciting avenues to further expand upon my work. This includes identifying the mechanisms of persister formation of B. burgdorferi, investigating whether persisters of B. burgorferi exist in vivo, and finding an optimal treatment regimen for Lyme disease (whether improving dosing regimens of approved drugs or screening for novel therapeutics).

4.2.1 Identification of the mechanism of persister formation in B. burgdorferi

a. Tn seq: Tn-seq is a powerful tool that allows a comparative fitness analysis of a population of individual transposon mutants exposed to a selective pressure. This is done by quantitatively identifying the frequency of each transposon insertion site (usually multiple per gene) in a pooled transposon library before and after exposure to selective pressure. By identifying mutants that are increased or decreased after selection, and comparing those levels to the parent strain exposed to the same selection, calculations of the fitness of mutants containing a specific transposon insertion can be determined. Tn-seq has not only been used to identify genes required for lung infection by Hemophilus influenza [1] and genes required for colonization of intestinal tract by Bacteroides thetaiotaomicron [2], but also to identify population bottlenecks during infection with B. burgorferi [3].

Tn-seq can be performed to identify genes required for tolerance to different classes of antibiotics. The frequency of different mutants in the population before (input) and after (output)
antibiotic treatment can be determined using massive parallel sequencing. The frequency of the mutants in the input and output population can then be compared to identify the mutant strains that decreased considerably in frequency, with the hypothesis that mutants in the genes that are involved in persister formation will not survive antibiotic treatment as well as the parent strain does (thus having a lower frequency in the output population). All candidate persister genes can then be confirmed by creating clean deletion strains and/or strains overexpressing the candidate persister genes, and performing persister assays (kill curves). A decrease in persister levels in a mutant and an increase in an overexpressing strain will validate a candidate gene. The identification of persister genes and/or pathways will likely lead to identification of the mechanism(s) of persister formation in B. burgdorferi.

b. Transcriptome analysis: Transcriptome analysis has previously been used for E. coli and M. tuberculosis [4, 5] to identify the potential mechanisms of persister formation. This method allows us to get a complete picture by revealing the functional elements of the genome. An advantage of transcriptome analysis over Tn-seq is that it allows us to measure the expression of essential genes that are not represented as mutants in a transposon library. A strain of B. burgdorferi carrying degradable GFP can be used to sort out persisters for transcriptome analysis. Dim cells of such strain will indicate cells exhibited lower levels of translation, meaning dormant (persister) cells. These cells can be sorted out and their transcriptomes analyzed to obtain candidate persister genes. The candidate persister genes can be validated via gene deletion and overexpression, as discussed above. The transcriptome analysis can also be used to assess if the candidate persister genes identified by Tn-seq are differentially expressed in persisters.
4.2.2 Survey of tick and clinical B. burgdorferi isolates for persister levels

The Lewis lab previously found that long-term antibiotic treatment of clinical UTI isolates of E. coli leads to a selection of high-persister phenotypes, due to specific point mutations in the hipA gene [6]. As it is possible that a similar phenomenon is occurring for B. burgdorferi, it would be fascinating to compare the levels of persisters in B. burgdorferi strains collected from wild ticks to those collected from human samples. To accomplish this, a screen of a collection of B. burgdorferi isolates obtained from wild ticks and skin biopsies from patients with EM for hip mutants can be performed. The goal will be to determine what percentages of such isolates have hip phenotypes. If the percentage of hip phenotypes recovered among tick isolates and human isolates correlate, it could provide an explanation for the variability in clinical course seen in patients. This will enable us to investigate a link between recalcitrance of Lyme disease and drug tolerance. Additionally, it would be interesting to see if any of the genes identified in our Tn-seq screen or transcriptome analysis have certain point mutations in clinical isolates. This could be performed by comparative whole-genome analysis, or sequencing these genes individually.

4.2.3 Find an optimal treatment regimen for Lyme disease

a. Using pulse dosing of ceftriaxone: I showed that persisters of B. burgdorferi could be eradicated in vitro by using a novel pulse-dosing regimen with ceftriaxone. The reasoning behind using pulse dosing is that there is the least amount of persisters present in a culture when bacteria are starting to grow. Consequently, if timed correctly and antibiotic is administered during such a window, persisters should be eradicated after a few pulses of the antibiotics. Preliminary experiments I performed show this is true, where I treated cultures of B. burgdorferi with ceftriaxone for 5 days, washed away the antibiotics, and allowed it to grow for 1 day before treating with antibiotics again. I observed eradication of the culture after four pulses of
ceftriaxone. This regimen can be optimized to achieve eradication with just two or three pulses of ceftriaxone by empirically determining the optimal “off time” required by *B. burgdorferi* to just start growing *in vitro*.

Pulse-dosing with ceftriaxone is being tested for efficacy in an animal model of Lyme disease. Since, *in vitro* data is not always translatable *in vivo*, the regimen will likely have to be optimized for efficacy *in vivo* as well. If successful, this will not only potentially provide a better treatment option for patients with Lyme disease but also provide some evidence supporting the pathogenic nature of PTLDS.

**b. Expanding the high-throughput screen to identify *B. burgdorferi* specific compounds.**

The hits from the pilot screen that I performed can be further validated *in vitro*, and tested for efficacy in animal model of Lyme disease. In addition, the high-throughput screen that I validated can be expanded to include thousands of compounds from different compound libraries. If we continue to have similar hit rates as our pilot screen, such an effort will yield a substantial number of *B. burgdorferi* specific hits that can be further characterized for efficacy *in vitro*, in animals, and then in humans. This is the first time a drug is being specifically developed to target Lyme disease and has the potential to result in a better treatment options for Lyme patients.
4.3 References


