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Identifying Vancomycin as an Effective Antibiotic for Killing *Borrelia burgdorferi*

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ABSTRACT *Borrelia burgdorferi* is the causative agent of Lyme borreliosis. Antibiotic therapy of early acute infection is effective for most patients, but 10 to 20% go on to develop posttreatment Lyme disease syndrome (PTLDS). The nature of PTLDS remains unknown, but currently approved antibiotics for the treatment of Lyme disease do not appear to impact these symptoms after they have developed. We reason that minimizing the time the pathogen interacts with the host will diminish the probability of developing PTLDS, irrespective of its nature. This calls for an efficient eradication of the pathogen during acute infection. In search of a superior killing antibiotic, we examined approved antibiotics for their ability to kill *B. burgdorferi*. Vancomycin proved more effective in killing the pathogen *in vitro* than ceftriaxone, the standard of care for disseminated *B. burgdorferi* infection. Both compounds were also the most effective in killing stationary-phase cells. This is surprising, given that inhibitors of cell wall biosynthesis are known to only kill growing bacteria. We found that peptidoglycan synthesis continues in stationary-phase cells of *B. burgdorferi*, explaining this paradox. A combination of vancomycin and gemifloxacin sterilized a stationary-phase culture of *B. burgdorferi*. Examination of the action of antibiotics in severe combined immunodeficient (SCID) mice showed that doxycycline, a standard of care for uncomplicated acute infection, did not clear the pathogen. In contrast, both ceftriaxone and vancomycin cleared the infection. A trial examining the early use of more potent antibiotics on the development of PTLDS may be warranted.

KEYWORDS *Borrelia burgdorferi*, Lyme disease, antimicrobial activity

Lyme borreliosis is the most common vector-bone disease in the United States, with more than 300,000 cases reported annually (1). The geographic range of ticks carrying *Borrelia burgdorferi*, the causative agent of Lyme disease, has greatly expanded over the last decade (2). In the United States, most cases of Lyme disease are caused by the spirochete *Borrelia burgdorferi*. Other spirochetes, including *Borrelia duttonii*, *Borrelia garinii*, and *Borrelia afzelii*, cause similar infections in Europe and Asia (3). Antibiotic treatment at the early localized stage of Lyme disease, usually with oral doxycycline, cures the disease in most patients. When the pathogen starts to enter the bloodstream and disseminate, it causes carditis, arthritis, meningitis, and peripheral neuropathies (4, 5). Arthritis in particular can persist from months to years (6, 7). While most Lyme disease patients are cured after 2 to 4 weeks of antibiotic treatment, in the case of Lyme disease arthritis, a second course of antibiotic treatment is often recommended (6, 8, 9). In addition, 10 to 20% of patients continue to experience symptoms, such as fatigue and muscle and joint pain, a condition known as posttreatment Lyme disease syndrome (PTLDS) (9, 10).

The nature of PTLDS is currently unknown. Attempts to isolate the pathogen from patients with PTLDS have not been successful (11–14). At the same time, there is

TABLE 1 Activities of antibiotics against *B. burgdorferi* B31 and N40

Antibiotic name	MIC (μg/ml)[a]		Target	Drug class
	B31	N40		
Doxycycline	0.25	0.25	Ribosome	Tetracycline
Amoxicillin	0.06	0.06	Penicillin-binding proteins	β-Lactam
Ceftriaxone	0.03	0.12	Penicillin-binding proteins	β-Lactam
Vancomycin	0.25	0.25	D-Ala-D-Ala	Glycopeptide
Telavancin	32	32	D-Ala-D-Ala	Lipoglycopeptide
Dalbavancin	64	64	D-Ala-D-Ala	Lipoglycopeptide
Fosfomycin	>50	>50	MurA	Novel class
D-Cycloserine	64	32	D-Ala-D-Ala synthesis	D-Alanine analogue
Tunicamycin	32	>64	Synthesis of lipid disaccharide intermediate	Nucleoside antibiotics
Bacitracin	32	64	Undecaprenyl pyrophosphate	Cyclic peptides

[a]MICs are the mode values.

evidence suggesting that some residual bacteria or bacterial products may be present after antibiotic treatment in several animal models of Lyme disease (15–18). In well-studied chronic infections, such as those associated with biofilms, or in the case of tuberculosis, recalcitrance of the infection to antibiotic treatment is linked to the presence of a subpopulation of dormant drug-tolerant persister cells (19). Similar to all other pathogens studied, *B. burgdorferi* forms persister cells tolerant to killing by antibiotics *in vitro* (20). Whether persisters contribute to the pathology of PTLDS is unclear. However, effective early control of the organism would be expected to reduce both the probability of the development of persisters and shedding of bacterial products, such as proteins and DNA/RNA that may induce inflammation. If the development of PTLDS is related to the presence of inflammatory products, more effective antibiotic regimens for early killing of bacteria may improve outcomes. Rapid eradication requires that an antibiotic kill both growing and nongrowing forms of the pathogen. The goal of this study was to identify more effective bactericidal antibiotics than those in the current regimen for treating Lyme disease.

RESULTS

Cell wall-acting antibiotics vancomycin and β-lactams have potent killing activity against *B. burgdorferi*. Stationary-phase *B. burgdorferi* contains a larger fraction of persister cells and is more tolerant to killing by antibiotics than exponential-phase cells (20). In the search for a superior killing antibiotic, we examined approved antibiotics for their ability to kill *B. burgdorferi* in our previous study (20). Surprisingly, β-lactams, which are known to only kill growing cells, show good killing of stationary-phase cells of *B. burgdorferi* (20). Both amoxicillin and ceftriaxone, antibiotics used to treat Lyme disease, kill the majority of stationary-phase *B. burgdorferi* cells. Given the unusual susceptibility of *B. burgdorferi* to β-lactams, we decided to test additional cell wall-acting antibiotics, including the glycopeptide class. Antibiotics that target different steps of peptidoglycan synthesis were selected, and their MICs were tested against *B. burgdorferi* strains B31 and N40 (Table 1). Among these additional compounds, only vancomycin showed potent activity against both *B. burgdorferi* strains, with an MIC of 0.25 μg/ml against both strains (Table 1), in agreement with previous reports (20). Vancomycin is a glycopeptide that inhibits peptidoglycan synthesis by binding to the terminal D-alanyl–D-alanine moieties of the *N*-acetylmuramic acid/*N*-acetylglucosamine (NAM/NAG) peptides. However, the other two glycopeptide class antibiotics, telavancin and dalbavancin, were only weakly active against *B. burgdorferi*, with MICs of 32 and 64 μg/ml, respectively (Table 1). We previously tested vancomycin and found that it efficiently kills *B. burgdorferi* (20). We undertook a more detailed examination of this compound in the present study and find that at the human maximum drug concentration (C_{max}) after dosing according to clinical recommendations, vancomycin (40 μg/ml) (21) killed more exponential-phase *B. burgdorferi* cells than did doxycycline (2.5 μg/ml) (22) in 5 days, with only 10 to 100 cells surviving. Ceftriaxone (257 μg/ml) (23)
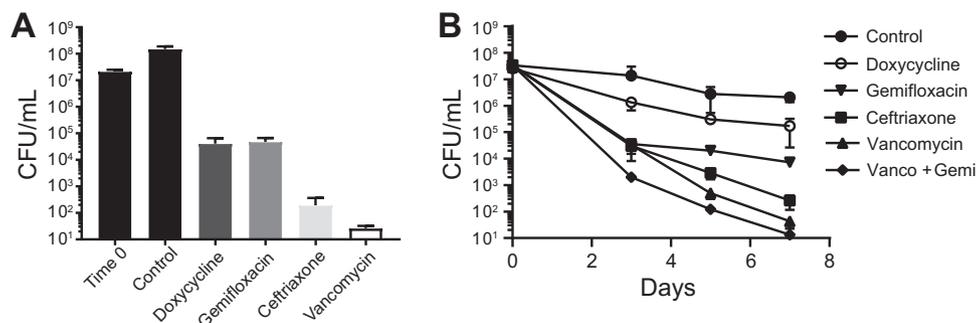
FIG 1 (A) Killing of exponential-phase *B. burgdorferi* (B31 5A19) with antibiotics. Late-exponential *B. burgdorferi* cultures were exposed to doxycycline (2.5 μg/ml), ceftriaxone (257 μg/ml), gemifloxacin (2.3 μg/ml), or vancomycin (40 μg/ml) for 5 days. (B) Time-kill of stationary-phase *B. burgdorferi* culture. Stationary-phase *B. burgdorferi* cultures were exposed to ceftriaxone (257 μg/ml), vancomycin (Vanco; 40 μg/ml), and/or gemifloxacin (Gemi; 2.3 μg/ml) for 7 days. Aliquots were taken at the indicated times and plated for CFU counts. The limit of detection is indicated by the *x* axis; there are 3 biological replicates for each experiment.

also showed good potency, with <1,000 cells surviving (Fig. 1A). Vancomycin also showed superior killing against stationary-phase *B. burgdorferi* cells at a C_{max} of 40 μg/ml, with <100 cells surviving (Fig. 1B).

We also tested several combinations of antibiotics with vancomycin to determine if it was possible to achieve complete eradication of a stationary-phase culture. For this, we tested vancomycin with compounds from the other two classes of highly bactericidal antibiotics, aminoglycosides and fluoroquinolones. *B. burgdorferi* had low susceptibility to fluoroquinolones, with only gemifloxacin having good potency, at an MIC of 0.125 μg/ml. Gemifloxacin alone showed modest killing of *B. burgdorferi* (Fig. 1B). Importantly, a combination of vancomycin and gemifloxacin at their C_{max} (2.33 μg/ml for gemifloxacin [24]) completely eradicated a stationary-phase culture (Fig. 1B).

Cell wall targeting in stationary-phase *B. burgdorferi*. We reasoned that remodeling of the cell wall may continue in stationary-phase *B. burgdorferi*, which would explain the vulnerability of this pathogen to inhibitors of peptidoglycan synthesis. In order to determine whether cell wall synthesis occurs in stationary phase, we monitored the incorporation of a fluorescent analog of D-alanine, (R)-2-amino-3-[(7nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino] propanoic acid hydrochloride (NADA), into peptidoglycan. After 24 h of incubation, a strong fluorescence signal was observed at the middle and at both poles of exponentially growing cells. Apparently, one pole is new and is bright because it carries label from the division plane (middle of the cell), and the new pole incorporates a new label (Fig. 2A). It was recently reported that peptidoglycan synthesis starts at the middle of growing *B. burgdorferi* cells, based on the incorporation of fluorescent analogs of D-alanine tracked for an hour after the addition of the label (25). Incorporation at the pole, which we observe in addition to the synthesis of peptidoglycan, in the middle of the cell requires a longer time. Interestingly, NADA incorporated into the peptidoglycan of stationary-phase cells as well (Fig. 2A). In contrast to exponentially growing cells, the label incorporated at only one of the cell poles and to a lesser degree at the middle of the cell. This clearly shows a difference with the dividing cells of the growing culture. A time course of label incorporation measured by fluorescence spectroscopy of a bulk population confirmed the presence of significant peptidoglycan synthesis at stationary phase, although it was expectedly less than in exponentially growing cells (Fig. 2D). We were interested to examine how this pattern of peptidoglycan synthesis compares to a typical bacterium that becomes tolerant to killing at stationary phase. According to our previous findings, *Staphylococcus aureus* acquires complete tolerance to cell wall-acting inhibitors at stationary phase (26, 27). There was rapid incorporation of NADA into exponentially growing cells of *S. aureus*, which essentially stopped at stationary phase (Fig. 2C).

Peptidoglycan synthesis was efficiently inhibited by vancomycin and ceftriaxone (Fig. 2A and B). Some incorporation of label could still be observed at the middle of a
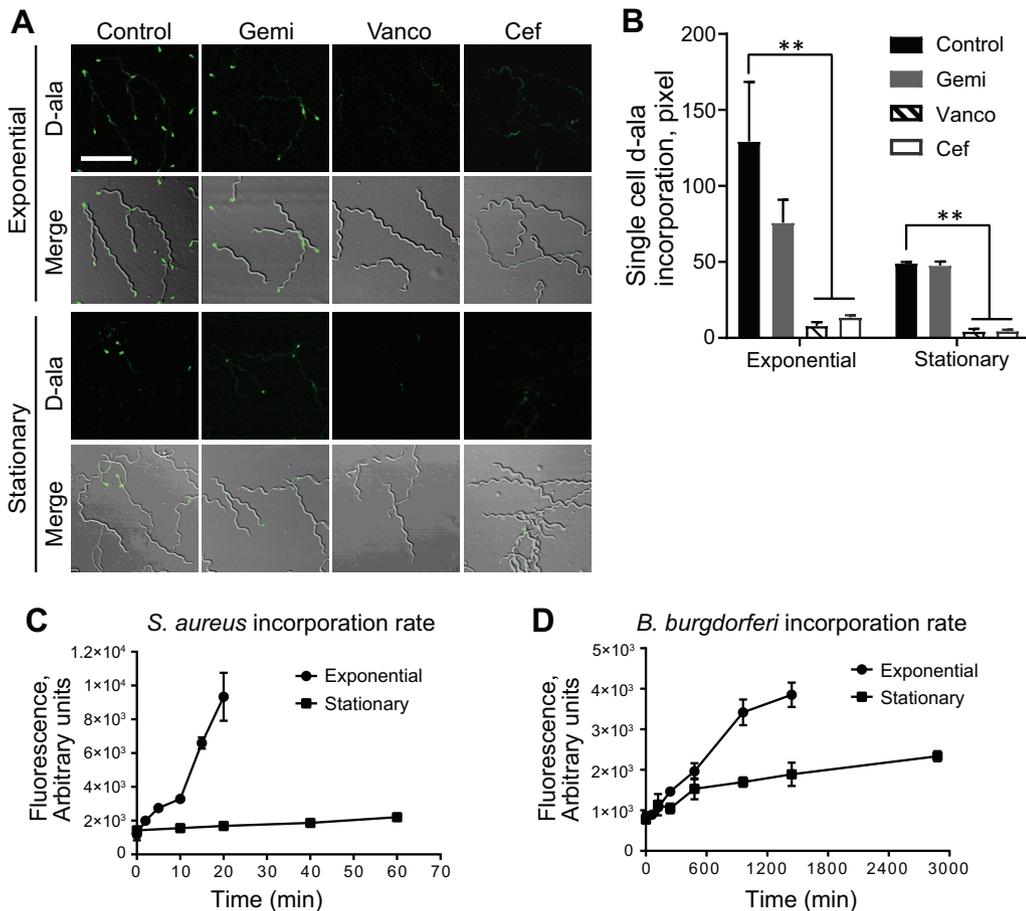
FIG 2 Vancomycin inhibits *B. burgdorferi* B31 5A19 cell wall synthesis in both exponential- and stationary-phase cells. (A) D-Ala (NADA) incorporation of drug-treated exponential- and stationary-phase *B. burgdorferi*. *B. burgdorferi* cells were treated with antibiotics at 1× MIC, vancomycin (Vanco; 0.25 μg/ml), gemifloxacin (Gemi; 0.12 μg/ml), ceftriaxone (Cef; 0.03 μg/ml), or no-drug control (ND), and then incubated with fluorescent D-ala (NADA). Fluorescent images were taken with a confocal microscope. Scale bar = 10 μm. (B) Fluorescence intensity of single-cell D-ala (NADA) incorporation. The average single-cell pixel intensity was measured by Image J using more than 3 confocal fluorescent images from each treatment group. (C) Kinetics of fluorescent D-Ala incorporation into *S. aureus*. (D) Kinetics of fluorescent D-Ala incorporation into *B. burgdorferi*. **, significant difference between groups ($P < 0.05$).

cell but not at the poles. Single-cell florescence intensity decreased 16-fold and 11-fold in exponential and stationary phases, correspondingly, after incubation with vancomycin, as determined by quantitative single-cell analysis (Fig. 2B). Label incorporation into stationary cells was not affected in a control incubated with gemifloxacin, a DNA synthesis inhibitor. In the exponential-phase cells, bright fluorescence was observed only on one of the poles, similar to stationary phase (Fig. 2A), likely due to the growth inhibition by gemifloxacin. Taken together, these experiments show that peptidoglycan synthesis continues in stationary-phase cells of *B. burgdorferi*, explaining the unusual vulnerability of this pathogen to cell wall synthesis inhibitors.

Vancomycin efficiently clears *B. burgdorferi* in a mouse model of infection. Given the potent killing activity of vancomycin against *B. burgdorferi*, we examined its efficacy *in vivo* and compared it to the two standard-of-care compounds, doxycycline and ceftriaxone. Apart from its ability to kill the pathogen, vancomycin reduces cell wall stiffness and decreases the rate of motility of *B. burgdorferi*, which improves its capture by phagocytes (28).

The N40 strain used in the mouse infection model is also sensitive to doxycycline, ceftriaxone, and vancomycin. The MICs of doxycycline, ceftriaxone, and vancomycin against the N40 strain are 0.25, 0.12, and 0.25 μg/ml, respectively. Vancomycin was introduced into infected C3H mice at 110 mg/kg of body weight every 12 h by

TABLE 2 *B. burgdorferi* N40 cultures from skin and DNA copies from bladder of both wild-type and SCID mice after 5 days of treatment[a]

WT treatment group	Whole-ear culture result[b]	No. of bladder DNA copies[c]
WT		
Saline	+	40
	+	25
	+	20
	+	32
	+	105
	+	53
Doxycycline	−	0
	−	0
	−	0
	−	0
	−	0
	−	0
Ceftriaxone	−	0
	−	0
	−	0
	−	0
	−	0
	−	0
Vancomycin	−	0
	−	0
	−	0
	−	0
	−	0
	−	0
SCID		
Saline	+	609
	+	1,032
	+	1,043
	+	1,667
	+	1,776
	+	1,327
Doxycycline	+	7
	+	9
	+	9
	−	6
	−	0
	+	4
Ceftriaxone	−	0
	−	0
	−	0
	−	0
	−	0
	−	0
Vancomycin	−	0
	−	0
	−	0
	−	0
	−	0
	−	0

[a]The following doses were given every 12 h for 5 days: doxycycline, 50 mg/kg; ceftriaxone, 156 mg/kg; and vancomycin, 110 mg/kg.
[b]+, *B. burgdorferi* cells were detected in the culture; −, no *B. burgdorferi* cells were detected in the culture.
[c]DNA copies were normalized to 200 ng of tissue DNA.

subcutaneous injection. Vancomycin eliminated the infection after 5 days of treatment, similarly to doxycycline and ceftriaxone (Table 2). An issue with this standard model is that the inhibition of growth alone may be sufficient for substantial clearing of the infection, since the remaining cells will be eliminated by the immune system. In order to better differentiate between antibiotics with varied capabilities to kill and taking into account that not all human patients will mount a robust immune response, we decided to use severe combined immunodeficient (SCID) mice as a model. SCID mice are

TABLE 3 *B. burgdorferi* N40 cultures from ear punches during antibiotic treatment in wild-type and SCID mouse infection models[a]

Mouse group	Treatment group	No. with positive ear punch culture			
		Day 1	Day 2	Day 3	Day 4
WT	Saline	5	4	4	5
	Doxycycline	1	0	0	0
	Ceftriaxone	0	0	0	0
	Vancomycin	0	0	0	0
SCID	Saline	6	4	4	6
	Doxycycline	6	5	4	4
	Ceftriaxone	0	0	0	0
	Vancomycin	0	0	0	0

[a]The following doses were given every 12 h for 5 days: doxycycline, 50 mg/kg; ceftriaxone, 156 mg/kg; and vancomycin, 110 mg/kg. $n = 6$ per treatment group.

deficient for both B and T lymphocytes, which results in a higher *B. burgdorferi* burden (29).

In this new model, doxycycline failed to clear the infection in the majority of mice after 5 days of administration (Table 2). Both ceftriaxone and vancomycin cleared the infection after two doses in the first day of administration based on an analysis of single ear punch cultivation (Table 3). To better compare the efficacy between vancomycin and ceftriaxone, mice were sacrificed after only one or two doses, and larger amounts of tissues were collected. A whole ear of every mouse was used for cultivation analysis. The spleen, bladder, and heart were collected for whole-DNA extraction and quantitative PCR (qPCR) analysis. After 1 dose, in the ceftriaxone group, 5 out of 7 mice remained culture positive, while in the vancomycin group, 3 out of 7 mice were positive. After 2 doses, both groups only had 1 culture-positive mouse (Table 4). *B. burgdorferi* DNA could still be detected from some tissues based on qPCR of the *flaB* gene of culture-negative mice, ranging from a few to hundreds of copies (Table 4). For example, after 2 doses of ceftriaxone, *B. burgdorferi* could be detected in an ear punch culture of only one of seven mice. Of the culture-negative mice, 5 were positive by PCR from all tissues, and the sixth mouse was positive for PCR only from the heart tissue. Similarly, after 2 doses of vancomycin, only 1 out of 7 mice was culture positive. However, of the culture-negative mice, 4 mice were positive by PCR from at least one tissue, while 2 mice were PCR negative for all tested tissues. These signatures may either represent live pathogens or components that had not been cleared from tissues. Both two-way analysis of variance (ANOVA) (ceftriaxone groups versus vancomycin groups) and *t* tests (ceftriaxone versus vancomycin, 1 dose and 2 doses separately) were performed to compare the efficacies of ceftriaxone and vancomycin. However, there was no statistically significant difference between treatments by the two antibiotics ($P > 0.05$).

DISCUSSION

In the current study, we sought to identify antibiotics with good bactericidal properties, and ideally, with an ability to kill *B. burgdorferi* persisters. All bacteria studied to date form persister cells *in vitro* in response to antibiotic exposure. Our current understanding of persisters is still limited, but considerable progress has been made in recent years, providing both a link to chronic infections and molecular mechanisms for the formation of these drug-tolerant cells. High-level persister mutants (hip) have been identified in *Pseudomonas aeruginosa* from patients with cystic fibrosis (30), in clinical isolates of *Mycobacterium tuberculosis* (31), and in *Escherichia coli* from patients with urinary tract infection (32). We recently found that a stochastic decrease in ATP levels is the main mechanism of persister formation in *S. aureus* (26) and *E. coli* (33). These species are unrelated, suggesting a broad conservation of this mechanism, which may be present in *B. burgdorferi* as well.

We identified two compounds that are capable of killing both growing and persister cells. Acyldepsipetide (ADEP) dysregulates the Clp protease in Gram-positive bacteria,

TABLE 4 *B. burgdorferi* N40 cultures from skin and DNA copies from tissues of SCID mice after one or two doses of 110 mg/kg vancomycin or 156 mg/kg ceftriaxone[a]

Treatment group	Whole-ear culture result[b]	No. of DNA copies in[c]:		
		Spleen	Bladder	Heart
Ceftriaxone, 1 dose	−	14	38	309
	+	55	870	132
	+	0	30	136
	+	72	11	177
	+	38	56	189
	+	38	800	99
	−	46	14	80
Ceftriaxone, 2 doses	−	24	26	182
	+	382	368	95
	−	48	13	10
	−	48	80	96
	−	95	80	131
	−	0	0	204
	−	12	12	48
Vancomycin, 1 dose	−	53	0	0
	−	0	0	0
	−	0	0	0
	+	12	15	258
	+	5	104	128
	+	22	97	370
	−	9	12	14
Vancomycin, 2 doses	−	35	0	0
	−	0	0	44
	+	138	48	367
	−	0	0	0
	−	0	0	0
	−	0	26	60
	−	67	10	40

[a]Mice were sacrificed 24 h after the second dose.
[b]+, *B. burgdorferi* cells were detected in the culture; −, no *B. burgdorferi* cells were detected in the culture.
[c]DNA copies were normalized to 200 ng of tissue DNA.

forcing the cell to self-digest (27), and lassomycin activates futile ATP hydrolysis by the ClpP1P2C1 protease in *M. tuberculosis* (34). However, these compounds are inactive against *B. burgdorferi* (data not shown).

The role of persisters in *B. burgdorferi* infection remains unknown. The majority of patients infected with *B. burgdorferi* who are treated with standard antibiotics do well with traditional antibiotic regimens that are not very effective against *B. burgdorferi* persisters *in vitro*. This suggests that *in vivo*, persisters are probably cleared by the host immune system.

A major unanswered question is whether persistence of the organisms or its components is linked to the development of PTLDS. If PTLDS is linked to either persistence of the organism or to the persistence of particles from killed organism, effective control of the infection may have an impact on the development of PTLDS. An additional and more obvious consideration is that PTLDS, irrespective of its nature, is ultimately caused by the infection, and minimizing the exposure of the patient to the pathogen will prevent PTLDS. With this in mind, we undertook a search of compounds with superior activity against *B. burgdorferi*.

In our previous study, we focused on potential vulnerabilities of the pathogen that could be targeted by antibiotics (20). *B. burgdorferi* has a limited ability to repair DNA, and we found that mitomycin C, which forms DNA adducts, kills regular and persister cells of the pathogen. While mitomycin C is an FDA-approved drug, it is a toxic anticancer agent unlikely to be introduced as an antibiotic. We also serendipitously found that β-lactams, including ceftriaxone, were able to kill the majority of cells in a stationary-phase culture. This was unexpected, since cell wall-acting antibiotics have

been known to act only against rapidly growing bacteria (35). In the current study, we tested additional cell wall-acting compounds and found that vancomycin was better than ceftriaxone in killing *B. burgdorferi in vitro* when killing was observed over a longer time period than previously reported (20). Of note, *B. burgdorferi* does not develop resistance to antibiotics, at least in part due to the lack of human-to-human transmission of the pathogen. The probability of resistance development to vancomycin is particularly low due to its action against a nonprotein target (lipid II, precursor of peptidoglycan) and would not be a concern for an infection caused by *B. burgdorferi*.

Vancomycin in combination with gemifloxacin completely eradicated a stationary-phase culture. We sought to determine the nature of this vulnerability to cell wall-acting antibiotics. Tracking the incorporation of a fluorescently labeled D-alanine, we found that peptidoglycan continues to be synthesized in nongrowing stationary-phase cells of *B. burgdorferi*, accounting for this susceptibility paradox.

We attempted to test the relative efficacies of doxycycline, ceftriaxone, and vancomycin *in vivo* but found that the standard mouse model of Lyme disease is poorly suited for this purpose. Doxycycline cleared the infection of wild-type (WT) mice after 1 day of treatment, similarly to ceftriaxone and vancomycin. We then turned to SCID mice and found that doxycycline failed to clear the infection even after 5 days of treatment. In contrast, vancomycin and ceftriaxone both cleared the infection rapidly even in the immunocompromised mice.

It would be difficult to justify the use of intravenous antibiotics, such as vancomycin and ceftriaxone, for the early treatment of *B. burgdorferi* infection given the added risks and difficulty in administration and monitoring compared with oral doxycycline. However, it is tempting to speculate that the persistence of bacteria or their products that has been seen in animals after treatment may trigger ongoing inflammatory responses in some patients, resulting in PTLDS. It is unknown what factors predispose patients to the development of PTLDS and why only some patients are susceptible. One hypothesis is that patients with immunodeficiencies may fare poorly with drugs, such as doxycycline, similar to what was seen with our immunocompromised mice. Indeed, Strle et al. have shown that an inactivating mutation in Toll-like receptor 1 (TLR1) is associated with persistent arthritis in patients with Lyme arthritis after antibiotic treatment (36). These patients may benefit from early use of more bactericidal antibiotics that kill both growing and stationary bacteria, as studies of intravenous (i.v.) ceftriaxone administered after the establishment of PTLDS have not shown a benefit compared with a placebo (12, 37, 38). Unfortunately, there is currently no good test, genetic or otherwise, to identify patients at higher risk for developing PTLDS *a priori* who may benefit from more aggressive therapy with intravenous antibiotics. There are several markers reported that may be related to PTLDS, such as increased immune response to VlsE membrane-proximal epitopes in PTLDS patients (39), innate acute C-reactive protein (40), T-cell chemokine CCL19 (41), and greater frequency of the TLR1-1805GG polymorphism (42). It seems possible that a combination of several markers may provide a basis for identifying high-risk patients for PTLDS. This will justify clinical trials to test the ability of highly bactericidal antibiotics, such as ceftriaxone and vancomycin, to diminish the incidence of PTLDS.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Borrelia burgdorferi sensu stricto* strain B31 (clone 5A19) (43) was kindly provided by Monica Embers at the Tulane National Primate Research Center and was used for all *in vitro* studies. *B. burgdorferi sensu stricto* strain N40 (clone D10E9) (44) was kindly provided by John Leong at Tufts-New England Medical Center Hospital and was used for MIC tests and all infection studies in mice. Cultures were started by thawing −80°C glycerol stocks of *B. burgdorferi* (titer, approximately 10^7 CFU/ml) and diluting 1:20 into fresh BSK-II medium. Bacteria were cultured in a microaerophilic chamber (34°C, 3% O_2, 5% CO_2). Semisolid plating was used to obtain CFU counts (45). The plates were incubated in zip-lock bags in a microaerophilic chamber for at least 21 days to obtain visible colonies.

MIC measurements for *B. burgdorferi*. A modified version of the broth microdilution method was used. *B. burgdorferi* was grown in liquid culture for 7 days to reach stationary phase and then diluted 1:5 into fresh BSK-II medium to make the inoculum solution. All compounds were prepared as stock solutions in solvent (dimethyl sulfoxide [DMSO]), based on the concentration to be tested, and diluted

in 2-fold increments in a 96-well stock plate. Two microliters per well of the antibiotic stock solution was transferred to the 96-well MIC plates, to which 198 μl of the *B. burgdorferi* inoculum was added (final inoculum, approximately 10^5 cells/well). Medium, growth, and vehicle controls were included in each plate. Each plate contained a positive-control row of amoxicillin starting at 0.92 μg/ml diluted in 2-fold increments down the row. The plates were covered with Breathe-Easy film, incubated in the microaerophilic chamber for 7 days, and scored visually for medium color change from pink to yellow of the phenol red present in the medium. The lowest concentration of antibiotics that prevented color change was interpreted as the MIC. All MIC assays were repeated at least twice in triplicate.

Time-kill experiments. Late-exponential-phase or stationary-phase *B. burgdorferi* B31 cultures were incubated with or without antibiotics at their C_{max} for 7 days. Samples taken at day 0, 3, 5, and 7 were washed 3 times using fresh BSK-II medium to remove antibiotics. Then, the washed samples were serially diluted and mixed in semisolid agar plates for CFU counts (45). All the plates were incubated in the microaerophilic chamber for 21 days before colony counting.

Fluorescent D-ala incorporation assay. Fluorescent D-alanine analog (R)-2-amino-3-[(7nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino] propanoic acid hydrochloride (NADA) was synthesized by Acme Bioscience, Inc. (46, 47). To obtain the NADA incorporation curve, *S. aureus* NCTC 8325 and *B. burgdorferi* B31 cultures were incubated with 0.1 mM NADA in the presence of 1% DMSO (25). All the cultures were protected from light. Samples were taken at different time points, washed 3 times, and resuspended in phosphate-buffered saline (PBS) at the same volume for *S. aureus* or 10 times concentrated for *B. burgdorferi*. The fluorescence (485 nm excitation, 528 nm emission) and optical density at 600 nm (OD_{600}) of samples (100 μl) were measured by a plate reader. All the fluorescent measurements were normalized by OD_{600} reading.

Microscopy and image acquisition. Late-exponential-phase ($\sim 10^7$ CFU) or stationary-phase *B. burgdorferi* B31 cells were treated with or without antibiotics for 4 h and then incubated with NADA at 0.1 mM for 20 h. Cells were washed 3 times and resuspended in PBS and then observed with a Zeiss confocal microscope system LSM 710 under a $\times 64$ objective. The single-cell fluorescence intensity was calculated by whole fluorescent intensity, with background subtracted, and normalized for cell number using ImageJ.

B. burgdorferi mouse infection model. All animal experiments were conducted according to protocols that were approved by the Institute of Animal Care and Use Committee (IACUC) at Northeastern University (approval no. 16-0619). Wild-type (WT) female C3H mice (Charles River Laboratories) and severe combined immunodeficient (SCID) female and male C3H mice (Jackson Laboratories) were infected with 10^5 *B. burgdorferi* N40 cells by subcutaneous injection, with three animals per treatment group. Infection was established for 3 weeks, and then animals were dosed twice a day for 5 days with saline, ceftriaxone (156 mg/kg), or vancomycin (110 mg/kg) by subcutaneous injection, or doxycycline (50 mg/kg) by oral gavage. The doses used in mice were matched to the pharmacokinetic profile of humans given 1 g ceftriaxone every 12 h (Sai Life Sciences Ltd., India), 100 mg doxycycline every 12 h, or 1 g vancomycin every 12 h (48, 49). Ear punches were collected from each animal every day throughout the duration of treatment and cultured in BSK-II medium. Cultures were monitored for growth daily by dark-field microscopy for 11 days. Animals were sacrificed 2 days after the completion of antibiotic treatment, and skin (whole ear), heart, spleen, and bladder tissues were collected. Skin was used for culture and DNA extraction. All other tissues were used for DNA extraction. The experiment was performed twice for each mouse strain. We saw the eradication of infection after 24 h of vancomycin or ceftriaxone treatment, so we compared the efficacies of ceftriaxone and vancomycin after single- or double-dose treatment. Infection was established in WT and SCID mice for 3 weeks, and then both were treated with vancomycin and ceftriaxone for one or two doses, with 12 h between doses. Mice were sacrificed the next day, and skin, heart, ankle, spleen, and bladder tissues were collected. Skin tissue was used for culture and DNA extraction. All other tissues were used for DNA extraction.

Real-time PCR of B. burgdorferi genes. The mouse and *Borrelia* DNA from infected mouse tissue was extracted using the DNeasy blood and tissue kit (Qiagen). Real-time PCR was performed with primers targeting the *flaB* gene (5′-GCAGCTAATGTTGCAAATCTTTTC-3′ and 5′-GCAGGTGCTGGCTGTTGA-3′) (18), using SYBR green Supermix (Bio-Rad). The *B. burgdorferi* N40 copy number was normalized by extracted tissue DNA amount. Both two-way ANOVA (ceftriaxone groups versus vancomycin groups) and *t* tests (ceftriaxone versus vancomycin, 1 dose and 2 doses separately) were performed to compare the efficacies of ceftriaxone and vancomycin. The burden of every tissue (ear culture, spleen, bladder, and heart DNA analysis) was calculated independently for both two-way ANOVA and *t* tests, *P* values were generated, and significant differences between groups were determined by a *P* value of ≤ 0.05.

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