High-throughput drug screening on *Borrelia garinii* and *Borrelia afzelii* identified hypocrellin A as an active drug candidate against *Borrelia* species

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Abstract

Lyme disease (LD) is a tick-transmitted infection caused by *Borrelia burgdorferi* sensu lato species which includes *B. burgdorferi, B. afzelii,* and *B. garinii*. The majority of patients with early LD can be cured by standard treatment, yet some still suffer from post-treatment Lyme disease syndrome (PTLDS). The presence of *Borrelia* persisters has been proposed as a contributing factor, which cannnot be completely killed by the currently used antibiotics for Lyme disease. Finding new pharmaceuticals targeting *Borrelia* persisters is crucial in developing more effective treatment. Here, we first confirmed the existence of persisters in cultures of *B. garinii* and *B. afzelii* and then conducted high-throughput screening of a custom drug library against persister-rich stationary-phase cultures of *B. garinii* and *B. afzelii*. Among 2427 compounds screened, hypocrellin A (HA), anthracycline class of drugs, and topical antibiotics along with some other natural compounds were identified to have strong potential in killing persisters of *B. garinii* and *B. afzelii*. HA was the most active anti-*Borrelia* compound, capable of eradicating stationary-phase *Borrelia* persisters, in particular when combined with doxycycline and/or ceftriaxone. Liposoluble antioxidant vitamin E was found to antagonize the activity of HA, indicating HA's target is the cell membrane where HA-triggered reactive oxygen species (ROS) generation took place in the presence of light. HA was found to have distinct bactericidal activity against *Borrelia* species but had poor or no activity against Gram-positive and Gram-negative bacteria. Identification of the above-mentioned drug candidates may help to develop more effective therapies for LD.

Introduction

Lyme disease (LD) is a multi-system disease caused by spirochetal bacteria *Borrelia burgdorferi* sensu lato (*Bb*) species, including *Borrelia burgdorferi*, *Borrelia garinii*, and *Borrelia afzelii* [1]. *Bb* is transmitted by ticks and circulates between ticks and

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vertebrate hosts [2]. In north America, *B. burgdorferi* is the major pathogen of LD, while *B. garinii* and *B. afzelii* are the principal causative agent of LD in Europe and Asia [3,4].

Most patients with early localized or early disseminated LD can be cured by antibiotic treatment [5]. However, 10-20% antibiotic-treated patients develop post-treatment Lyme disease syndrome (PTLDS), manifested as chronic fatigue, joint and muscular pain, and "brain fog" [1,6]. In addition, patients with Lyme arthritis can develop antibiotic refractory arthritis [7]. The cause of PTLDS is complex and remains to be determined. Several hypothesises have been proposed to explain PTLDS, including dysfunctional immune responses [7], metabolic differences between PTLDS and non-PTLDS patients [8], and the presence of antibiotic-tolerant *Borrelia* persisters [9]. The failure of antibiotic therapy in some LD patients raises the question: whether *B. burgdorferi* might persist in patients, which may further evade host immune clearance and continue to cause symptoms.

Bb biofilms have been clinically implicated in human skin infections [10-12], and biofilm-form *B. burgdorferi* is reported to contain drug-tolerant persisters both *in vitro* and *in vivo* [12-15]. The frontline drugs such as doxycycline and amoxicillin could effectively kill or inhibit *B. burgdorferi* in spirochetal form, yet they have little activity against *B. burgdorferi* persisters [16-18]. Moreover, *in vivo* experiments showed that spirochete-form *B. burgdorferi* exposed to mice could be completely eradicated by ceftriaxone, whereas biofilm-like aggregated microcolonies of *B. burgdorferi* could be eliminated neither by ceftriaxone, doxycycline, vancomycin, nor their combinations [13]. Additionally, in a persistent LD model treated with ceftriaxone, although *B. burgdorferi* could not be identified by culture method, low copy numbers of *B. burgdorferi* DNA were detectable at 2-8 months; importantly, "resurgence" in the form of *B. burgdorferi* DNA appeared at 12 months post-treatment [19]. *B. burgdorferi* was also identified by xenodiagnosis in a patient with erythema migrans after antibiotic therapy and in a patient with PTLDS [20]. It is worth noting that *Borrelia* spirochetes could be cultured from genital secretions of patients who had been treated with antibiotic therapy [21]. Recently, *Borrelia* were identified by PCR and immunofluorescent staining in post-mortem brain of a patient who had been diagnosed

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and antibiotic treated for LD and subsequently experienced chronic LD symptoms [22]. These findings indicate that *B. burgdorferi* may form persisters *in vivo* that render the organism insensitive to antibiotics, and the currently used antibiotics for treating LD are insufficient to eradicate *B. burgdorferi* persisters in the infected host.

Although several anti-persister drugs have been identified for *B. burgdorferi* [16], it is important to recognize the difference of *B. burgdorferi* from *B. garinii* and *B. afzelii* in genotype, pathogenicity, and clinical symptoms [23,24], and varied susceptibility to antibiotics [25]. Besides, drugs commonly used to treat LD are generally broad-spectrum antibiotics, which could reduce gut microbiota diversity, cause antibiotic-associated *Clostridium difficile* infections, and select for resistance in non-target bacteria. Therefore, it is valuable to identify new drugs specifically targeting *Borrelia* persisters. The stationary-phase *Borrelia* cultured *in vitro* are widely adopted as a surrogate model for high throughput drug screens [26]. Here, we sought to identify new drugs targeting stationary-phase *B. garinii* and *B. afzelii* by high-throughput screening 2427 custom compounds in a drug library. Several new active compounds, as well as drug combinations, were identified with potent anti-persister activity. In particular, HA stood out in specifically killing *Borrelia* cells by an unusual mechanism of triggering ROS production on the cell membrane. The identified new drug candidates active against *Borrelia* persisters could facilitate the development of anti-persister drugs and offer new therapeutic options to combat persistent *Borrelia* infections.

Materials and Methods

Bacterial strains, media, and culture

B. burgdorferi B31 (ATCC 35210, Bbu), *B. afzelii* (ATCC 51992, Baf), and *B. garinii* (ATCC 51991, Bga) were cultured, respectively, in BSK-H medium (HiMedia Laboratories Pvt. Ltd.), supplemented with 6% rabbit serum (Sigma-Aldrich, USA). Cultures were incubated at 33°C in closed conical tubes. The 7-day-old stationary-phase cultures of *B. burgdorferi* and *B. afzelii* and the 10-day-old stationary-phase cultures of *B. garinii* (1×10^7 spirochetes/mL) were used for drug screening as previously described [13,16]. For the biofilm, log-phase cultures of *Borrelia* (1×10^6

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spirochetes/mL) were transferred into uncoated 96-well plates, with 200 µL/well; after incubation at 33 °C for 10 days, planktonic spirochetes in the supernatants were gently removed and supplemented with fresh BSK-H medium, followed by drug tests against *Borrelia* biofilm. *Escherichia coli* MG1655 and *Staphylococcus aureus* Newman were cultured in Luria-Bertani (LB) medium and tryptic soy broth (TSB) medium, respectively, at 37°C.

MIC determination by microdilution test

The standard microdilution method was used to determine the minimum inhibitory concentration (MIC) of *B. burgdorferi, B. afzelii* and *B. garinii*, respectively, as previously described [27]. All experiments were run in triplicates.

Microscopy techniques

Cell proliferation was evaluated by directly counting cells with a hemocytometer under a dark-field microscope. The SYBR Green I/PI assay was performed to check the viability of cells as described previously [28]. Specimens or 96-well culture plates were examined with an Olympus IX71 inverted fluorescence microscope. For the aggregated cells, representative images of each sample were captured for quantitative analysis. ImageJ with Fuji plugins was applied to calculate the integrated fluorescence intensity of different morphological forms as previously described [29,30]. The lengths of the scale bars were set at 50 µm.

Killing experiments

The log-phase or stationary-phase cultures of *B. afzelii* and *B. garinii* were added with antibiotics, followed by incubation at 33°C for 9 days. Aliquots were sampled, and then serially diluted for counting live/dead spirochetes under a fluorescence microscope after SYBR Green I/PI staining as described previously [28].

Antibiotics and the drug library

Antibiotics or compounds were prepared as stock solutions in appropriate solvents [31], filter-sterilized by 0.22 µm pore-size filters, and stored at -20°C. The custom compound library (TargetMol, Boston, USA, Table S1) is a collection of 2427 custom compounds among which 2342 drugs are approved by the US-FDA, CFDA, and EMA. All the compounds were prepared as 10 mM DMSO-dissolved stocks in 96-well plates, and

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stored at −80°C.

Drug screening against stationary-phase cells of B. garinii and B. afzelii

Drug screening was performed in 96-well microtiter plates with 50 μM drugs to avoid missing promising candidates. Plates were sealed and incubated at 33°C for 7 days, with dim light (10 lux) available. The live/dead ratio of cells after drug exposure was then assessed by the SYBR Green I/PI assay as described previously [28]. Cultures were further checked and analyzed by an inverted fluorescence microscope.

Subculture test for antibiotic-treated Borrelia

The stationary-phase cultures of *B. burgdorferi*, *B. afzelii*, or *B. garinii* treated with 10 μg/mL drugs or drug combinations were subcultured as previously described [29]. Briefly, cells were spun down, rinsed, and resuspended in fresh BSK-H medium, followed by incubation at 33 °C for 10 days. Cell proliferation was then evaluated as the above description.

Antagonism of HA's anti-Borrelia activity by antioxidants

Water-dissolved antioxidant vitamin C and DMSO-dissolved antioxidant vitamin E were added, respectively, to *B. burgdorferi* culture subject to HA treatment. The viability of *B. burgdorferi* cells in varied conditions was analyzed by fluorescence microscope after the SYBR Green I/PI staining.

Statistical analysis

All data were provided as means ± SD. Data were analyzed and plotted using GraphPad Prism Version 8 (GraphPad Software, USA). The differences of residual bacteria were analyzed by two-tailed *t*-tests, the significance level was set at $P < 0.01$.

Results

SYBR Green I/PI assay was applicable in assessing viability of B. garinii and B. afzelii.

The SYBR Green I/PI method, which was proved to be excellent for assessing *B. burgdorferi* viability [28], was evaluated for viability assessment of *B. garinii* and *B. afzelii*. The results showed that the percentages of viable *B. garinii* and *B. afzelii* correlated well with the ratio of green to red fluorescence in a linear relationship (Figure

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S1), strongly demonstrating the feasibility of the SYBR Green I/PI method for assessing the viability of *B. garinii* and *B. afzelii* for drug exposure studies.

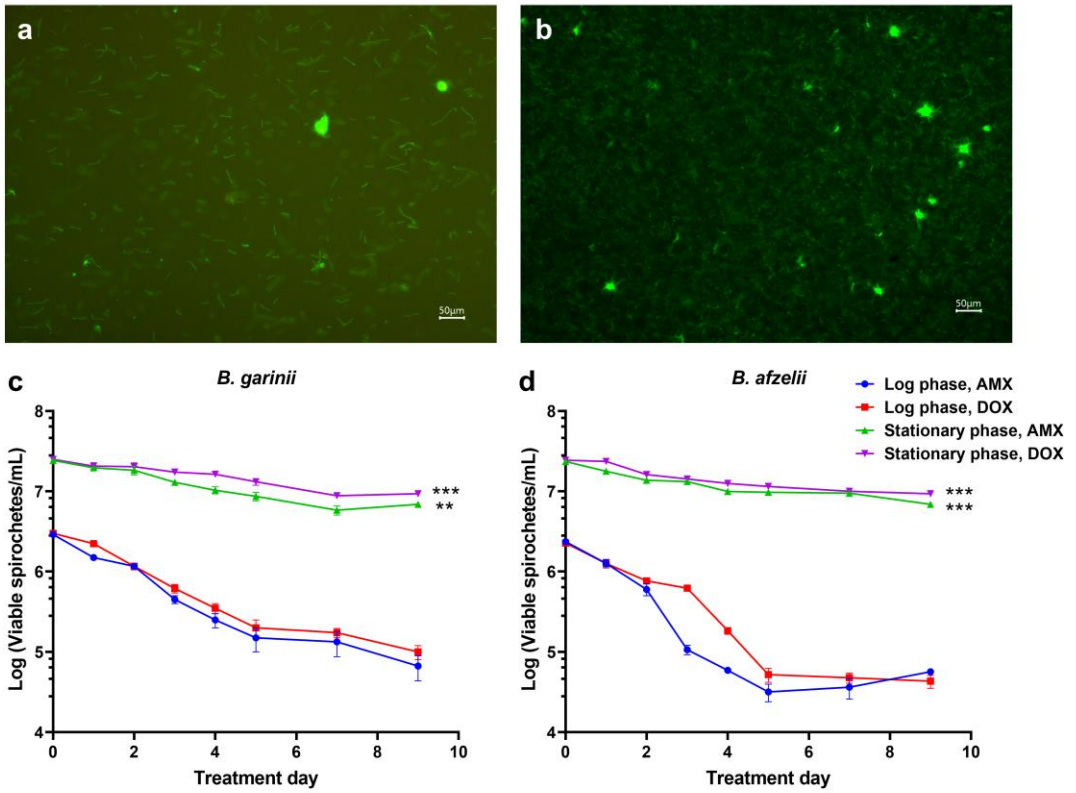

Figure. 1. Fluorescence microscope images and time-dependent killing curves of *B. garinii* and *B. afzelii*. Cells of *B. afzelii* (a) and *B. garinii* (b) were in spirochetal form, round body form (letters "r") and aggregated microcolony form (letters "m") in stationary-phase culture. Cultures of *B. garinii* (c) and *B. afzelii* (d) in the log-phase and stationary phase were treated with amoxicillin (30 μg/mL) and doxycycline (30 μg/mL). ** $P < 0.001$, *** $P < 0.0001$ vs. the log-phase culture, n = 3 for each group.

B. garinii and B. afzelii could form drug-tolerant persisters.

Cells of *B. garinii* and *B. afzelii*, particularly at the stationary phase, displayed varied morphologies, including spirochetes, round bodies, and aggregated microcolonies (Figure 1a, 1b) [13]. Doxycycline and amoxicillin, in the concentration of 100-fold MIC, killed most log-phase cells in 5 days, followed by a slow phase of death in the next 4 days (Figure 1c, 1d). The above biphasic pattern of the killing curve, which is the hallmark of antibiotic persistence, indicated the presence of drug-tolerant persisters in

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the culture. Furthermore, stationary-phase *B. garinii* and *B. afzelii* could survive the antibiotic treatment much better (40 and 50% viable cells, respectively) than that at the log-phase (less than 3% viable cells) after 9 days of drug exposure (Figure 1c, 1d). The possibility that the surviving cells of *B. garinii* and *B. afzelii* were drug-resistant mutants was excluded by the fact that the MIC of doxycycline and amoxicillin remained unchanged for the sub-cultured cells (data not shown). Similar as *B. burgdorferi,* biofilm-like aggregates or microcolonies of *B. garinii* and *B. afzelii* showed more drug tolerance than their spirochetal form.

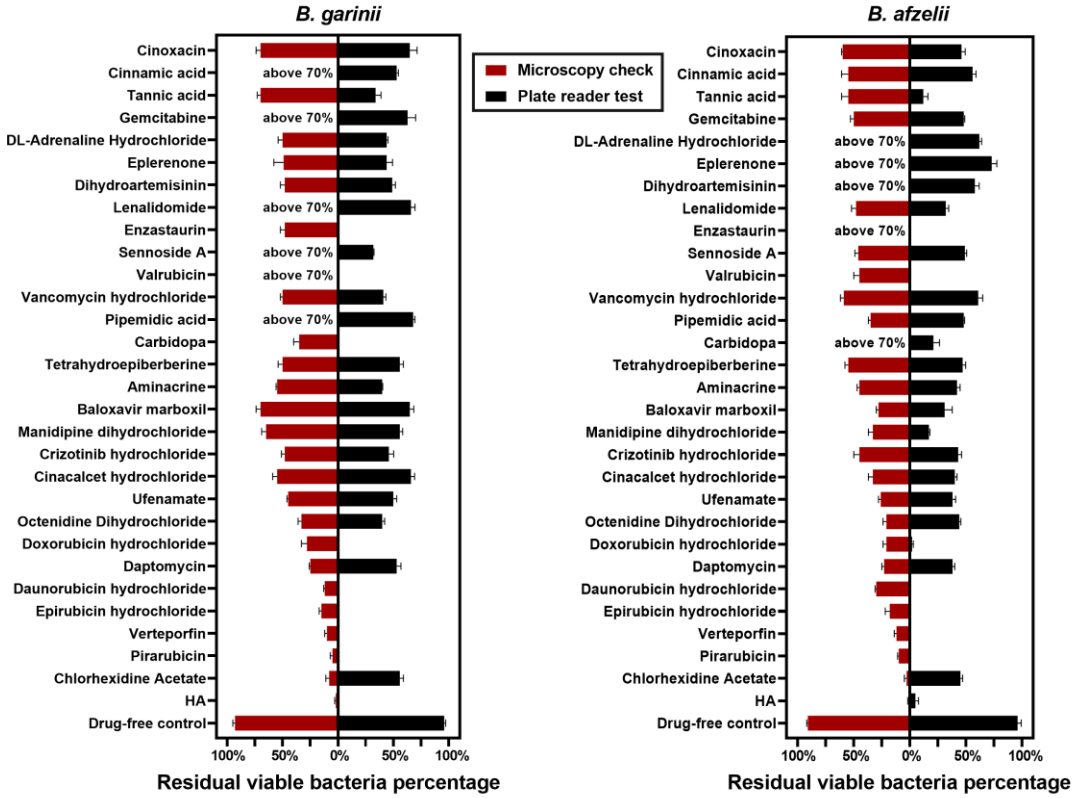

Figure 2. The activity of top active hits against stationary-phase *B. garinii* or *B. afzelii*.

Stationary-phase cultures of *B. garinii* (10-day-old) and *B. afzelii* (7-day-old) were treated with antibiotics or compounds (50 μM) for 7 days, n = 3 for each drug.

A custom compound library was screened to identify effective compounds against persisters of B. garinii and B. afzelii.

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Most antibiotics, including the clinically used antibiotics such as doxycycline, amoxicillin, ampicillin, ceftriaxone, and penicillin G, showed poor activity against stationary-phase cells of *B. garinii and B. afzelii* (Table S1). Of 2427 compounds in the custom compound library, 852 for *B. garinii* and 731 for *B. afzelii* were identified in the primary screen that showed higher efficacy against persisters than commonly used doxycycline, amoxicillin, and ceftriaxone. Cell viability checking under a fluorescence microscope confirmed the top 30 active hits, which generally led to less than or close to 50% residual viable cells after treatment (Figure 2). HA was identified as the most active compound, which could completely eradicate stationary-phase cells of *B. garinii* and *B. afzelii*, including round bodies and biofilm-like aggregates or microcolonies (Figure 2, Figure 3). Anthraquinone compounds of pirarubicin, epirubicin, daunorubicin, and doxorubicin, showed strong activity. The effective killing was also observed for verteporfin and daptomycin (DAP). Two natural compounds of tetrahydroepiberberine and dihydroartemisinin, as well as cinnamic acid, an analog of natural product cinnamaldehyde, were quite active. In addition, two quinolones of pipemidic acid and cinoxacin were identified to have moderate activity. Finally, disinfectants or topical drugs including chlorhexidine, octenidine, ufenamate, and aminacrine, as well as 10 non-antibiotic drugs displayed anti-persister activity against *B. garinii* and *B. afzelii* at various levels.

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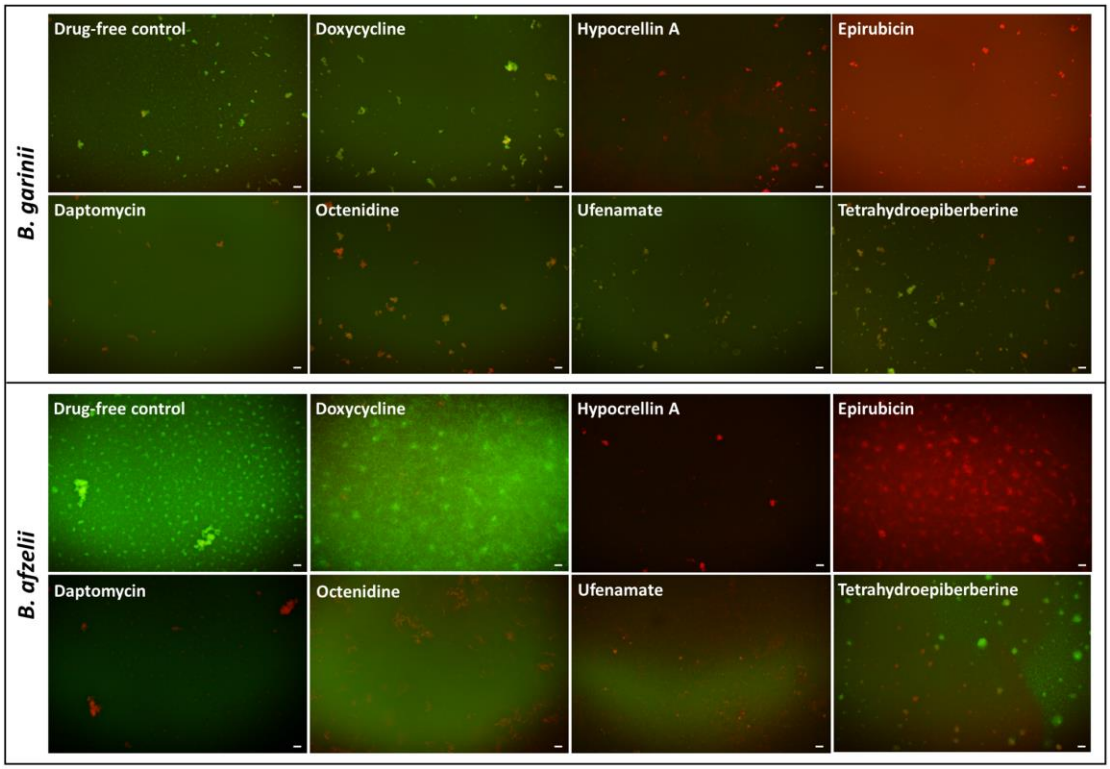

Figure. 3. Representative images of stationary-phase cells of *B. garinii* and *B. afzelii* after drug exposure. Cells were treated with different compounds (50 μM) followed by staining with SYBR Green I/PI.

HA was confirmed to have great potential in killing Borrelia cells and biofilm.

It is important to recognize that drugs that are effective on non-growing persisters do not always kill growing cells efficiently, as proved by daptomycin against *B. burgdorferi* in this study (Table 1) and in our previous study [16]. Drugs from the primary screening and some homologs, showed essentially non-biased MIC and anti-persister activities for *Borrelia*. Compared with daptomycin, HA had a lower MIC for *Borrelia*, indicating its impressive activity against growing *Borrelia*. Meanwhile, it could efficiently kill stationary-phase *Borrelia* cells, with residual viable cells less than 30% (Table 1, Figure S2).

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239 **Table 1. MICs and anti-persister activities of compounds for *B. burgdorferi*, *B. garinii*, and *B. afzelii*.**

Drugs	Plasma concentration[a] (μg/mL)	MIC (μg/mL)			Activity against persisters[b]		
		Bbu	Bga	Baf	Bbu	Bga	Baf
					20 μg/mL	20 μg/mL	20 μg/mL
Ampicillin	0.02-20	0.31-0.16	0.63-0.31	0.31-0.16	64 ± 0.1%	67 ± 0.1%	58 ± 0.6%
Doxycycline	1-10	0.31-0.16	0.16-0.08	0.31-0.16	71 ± 1.3%	66 ± 2.2%	62 ± 1.3%
Ceftriaxone	15-75	0.16-0.08	0.16-0.08	0.16-0.08	71 ± 3.4%	66 ± 0.1%	67 ± 2.8%
Vancomycin	5-12	0.63-0.31	0.31-0.16	0.31-0.16	71 ± 0.4%	70 ± 1.9%	68 ± 1.3%
Ofloxacin	2.5-5.5	5-2.5	2.5-1.25	2.5-1.25	82 ± 0.1%	75 ± 0.3%	74 ± 0.1%
Daptomycin	0-133	50-25	50-25	50-25	54 ± 0.01%	48 ± 5.3%	50 ± 3.7%
HA	0.42-5.43[c]	1.25-0.63	0.63-0.31	1.25-0.63	20 ± 0.4%	25 ± 0.6%	28 ± 4.1%
Octenidine	ND	1.25-0.63	1.25-0.63	1.25-0.63	58 ± 0.8%	54 ± 1.6%	52 ± 2.1%
Tetrahydroepiberberine	ND	≥ 20	≥ 20	20-10	66 ± 6.4%	72 ± 0.2%	72 ± 2.3%
Berberine	ND	≥ 20	≥ 20	≥ 20	73 ± 1.0%	77 ± 0.1%	82 ± 1.6%
Dihydroartemisinin	ND	≥ 20	≥ 20	20-10	73 ± 1.4%	74 ± 0.8%	78 ± 0.5%
Artemisinin	0-0.792	20-10	20-10	20-10	75 ± 2.2%	74 ± 1.1%	84 ± 0.3%
Pipemidic acid	0.49-4.27	10-5	20-10	20-10	72 ± 1.4%	77 ± 0.6%	81 ± 1.0%
Cinoxacin	15	≥ 20	≥ 20	20-10	70 ± 1.2%	75 ± 0.6%	77 ± 1.1%

240 The antibiotics shown in bold are reference antibiotics.

241 a. The values are derived from the literature [32-36].

242 b. Shown as residual viable cell percentage calculated from cell counting under a fluorescence microscope.

243 c. Mouse plasma concentration.

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Borrelia biofilm displayed tightly aggregated structures (Figure S3) and strong tolerance to antibiotics such as doxycycline and amoxicillin (Figure 4), which is consistent with the previous studies [11-15]. Daptomycin effectively killed biofilm-form *Borrelia* in this study (Figure 4). However, HA nearly killed all *Borrelia* biofilm as revealed by the barely observed green color after SYBR Green I/PI staining (Figure 4a). Of note, HA also largely dispersed the aggregated biofilm, making the biofilm structure less compact.

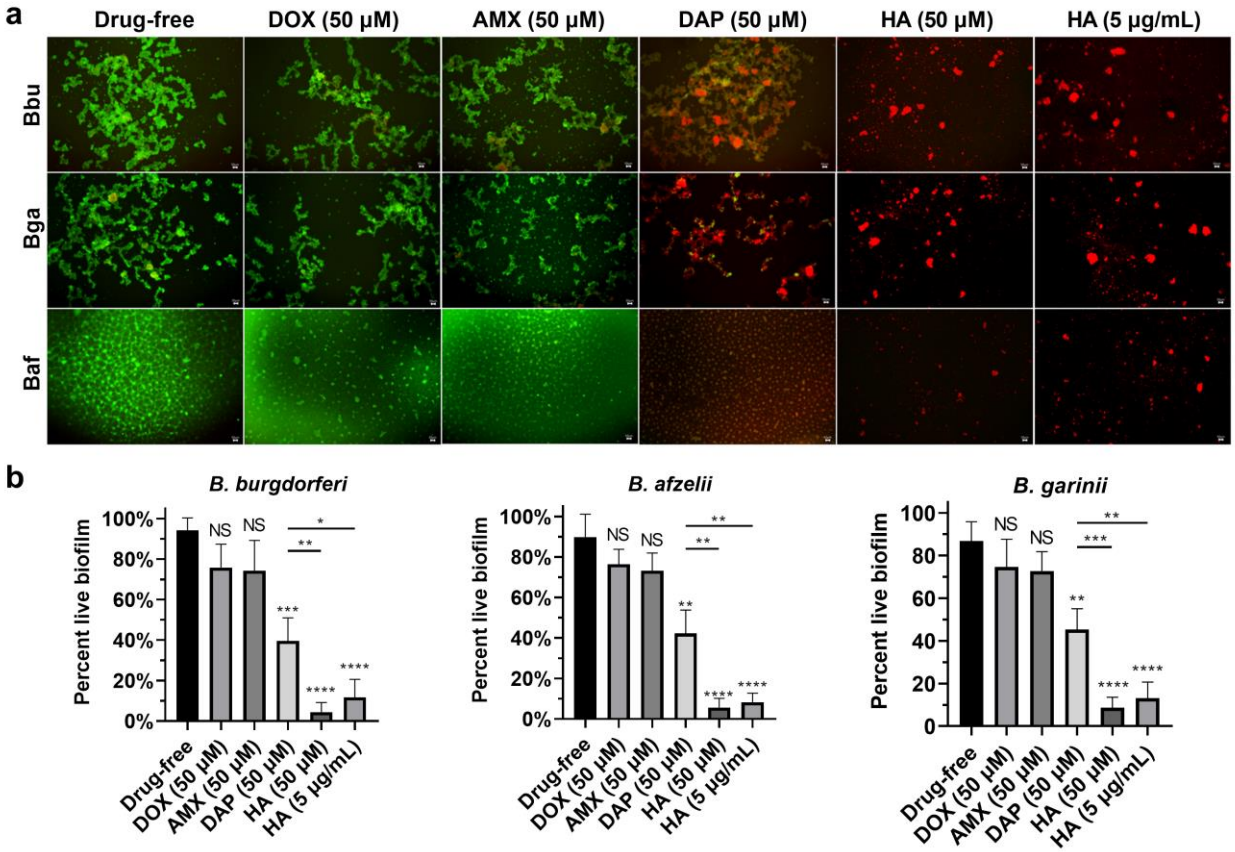

Figure. 4. HA eradicated *Borrelia* biofilm. The 10-day-old biofilms of *B. burgdorferi*, *B. garinii*, and *B. afzelii* in 96-well plates were treated with indicated drugs for 10 days. Plates were then stained by SYBR Green I/PI, followed by fluorescence microscopy (a) and plate reader assay (b). * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$, **** $P < 0.00001$, n = 5 for each group.

Subculture tests confirmed the activity of HA and its drug combination against stationary-phase Borrelia cells.

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Due to HA's red color, the possibility of underestimating live cells necessitates subculture test to verify HA's capability in eradicating persisters of *Borrelia*. In the subculture of HA-treated *Borrelia* cells, no growth of *B. burgdorferi* and *B. garinii* was observed, indicating HA could eliminate *B. burgdorferi* and *B. garinii* thoroughly. Unfortunately, regrowth of *B. afzelii* was detected in subculture (Table S2).

The drug combination is an efficient strategy in killing persisters or biofilm bacteria [13,37]. Here, doxycycline and ceftriaxone, prescribed for LD treatment, were assigned to combine with HA, individually or together, to treat stationary-phase *Borrelia* cells at a concentration of 10 μg/mL. No viable *Borrelia* cells including round bodies, biofilm-like aggregates or microcolonies were detected by fluorescence microscope after 10-day exposure to HA-involved drug combinations (Figure S4), and the complete eradication was further confirmed by no regrowth in subculture tests (Table S2). In contrast, the combination of ceftriaxone and doxycycline without HA displayed a weak anti-*Borrelia* activity (Figure S4, Table S2). Meanwhile, the drug combination of daptomycin, ceftriaxone, and doxycycline eliminated *B. burgdorferi* persisters, which was in line with our previous studies [13,37]. This drug combination was also powerful enough to thoroughly eradicate all live *B. garinii* cells, whereas it was not enough for *B. afzelii*, with cells regrowing in the subculture.

HA specifically eradicated Borrelia persisters by photodynamic antibacterial activity

To investigate the anti-*Borrelia* mechanism of HA, we first compared the activity of HA against stationary-phase *B. burgdorferi* cells with or without light supply. HA in darkness could barely eliminate *Borrelia* cells (Figure 5a), whereas it killed most of cells after 7 days and completely eradicated cells after 10 days in the presence of light. Strikingly, the outstanding efficacy of HA against *Borrelia* was extremely amplified in moderate-light condition (860 lux), as it only took 3 hours to eradicate *B. burgdorferi* cells. The above results indicate that the *Borrelia*-targeting activity of HA strongly depends on its photodynamic action.

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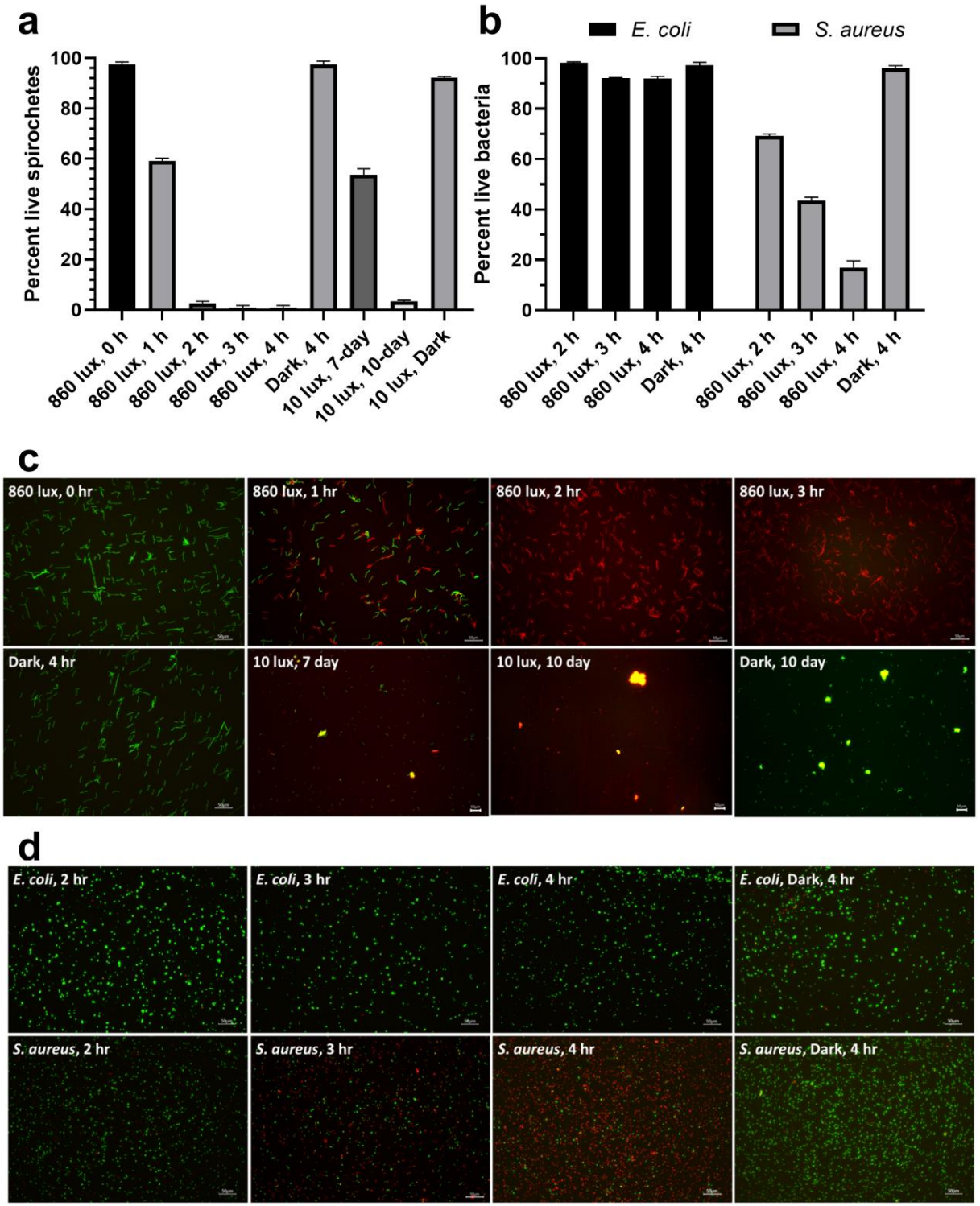

Figure. 5. HA specifically eradicated *Borrelia* persisters in light. Residual live percentage of stationary-phase *B. burgdorferi* (a), *E. coli* and *S. aureus* (b) treated by 5 μg/mL HA, n = 3 for each group. (c) Representative images of stationary-phase *B. burgdorferi* cells treated with 5 μg/mL HA followed by SYBR Green I/PI staining. (d) Representative images of stationary-phase *E. coli* or *S.*

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aureus treated with 5 μg/mL HA followed by SYBR Green I/PI staining.

However, HA showed significantly weaker efficacy in killing *S. aureus* under moderate-light condition, with approximately half the amount of *S. aureus* cells still alive after 4 hours of treatment. Moreover, no killing activity of HA against Gram-negative *E. coli* was detected under the same illumination intensity (Figure 5b). Based on the above results, HA was proved to have a species-specific characteristic of photodynamic action.

HA damaged the Borrelia cell membrane by ROS

Previous studies showed that HA photodynamic activity produces reactive oxygen species (ROS) [38]. Here, no significant change in ROS levels was detected in HA-treated *Borrelia* in light conditions (Figure S5). Because HA is a strongly hydrophobic compound, the principal target of HA-triggered photodynamic anti-*Borrelia* action probably is located specifically on the cell membrane. To confirm this, we chose two water-soluble antioxidants vitamin C and liposoluble vitamin E, to assess their ability to counteract the effects of HA-induced ROS (Figure 6). Vitamin C poorly offset HA's killing effect, whereas vitamin E significantly facilitated the survival of *Borrelia* cells, keeping more than 50% of cells viable with 80 μM vitamin E. The effect of vitamin E was enhanced when supplied vitamin E reached to 160 μM. The anti-ROS effect delivered by liposoluble vitamin E instead of water-soluble vitamin C suggested that HA targets the cell membrane via photodynamic activity.

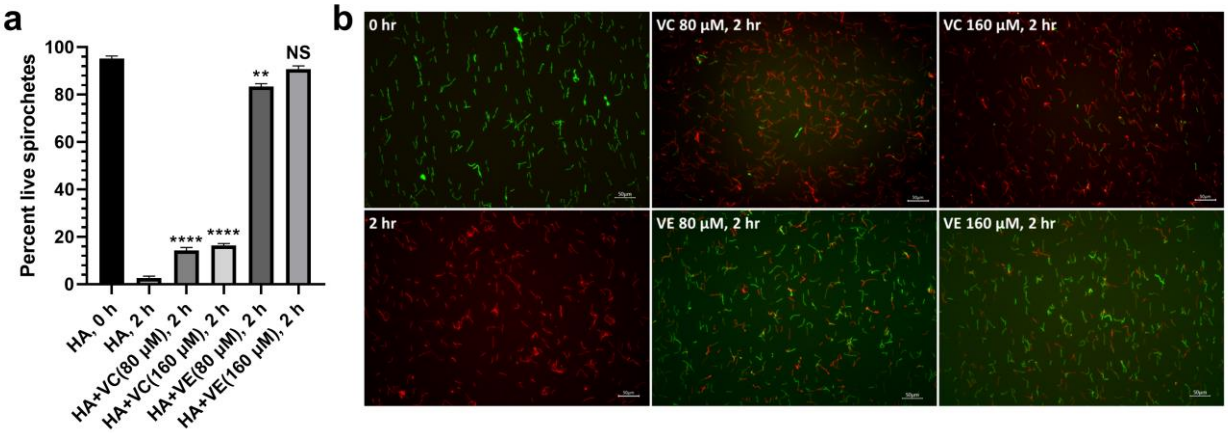

Figure. 6. Liposoluble antioxidants vitamin E and water-soluble vitamin C showed distinct capacity to antagonize the anti-*Borrelia* activity of HA. (a) Residual live percentage of stationary-phase *B. burgdorferi* treated by 5 μg/mL HA, ** $P < 0.001$, **** $P < 0.00001$, n = 3 for

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each group. (b) Representative images of stationary-phase *B. burgdorferi* cells treated with 5 μg/mL HA, with supplementation of indicated antioxidants, followed by SYBR Green I/PI staining. All experiments are under illumination of 860 lux.

Discussion

Persisters are a subpopulation of non-growing or slow-growing bacterial cells which are tolerant to multiple antibiotics but do not have acquired genetic drug resistance [39,40]. It has been well documented that bacterial cells including *B. burgdorferi* show classic biphasic killing curves as a result of the coexistence of antibiotic-susceptible cells and antibiotic-tolerant persisters [41]. However, there is no study about whether *B. garinii* and *B. afzelii* can form persisters before this work. Here, *B. garinii* and *B. afzelii* treated with clinically used antibiotics (doxycycline and amoxicillin) also showed biphasic killing curves (Figure 1c, 1d). Importantly, stationary-phase cells of *B. garinii* and *B. afzelii* showed strong antibiotic-tolerance, but had unchanged MICs, indicating they are not drug-resistant mutants. Instead, it is attributable to the presence of persister subpopulation. This, combined with the presence of abundant cells in round-body form and aggregated-microcolony form (Figure 1a, 1b), confirms that the stationary-phase cultures of *B. garinii* and *B. afzelii* are enriched in persisters. Unfortunately, clinically used antibiotics had weak activity against persister-rich stationary-phase cultures of *B. garinii* and *B. afzelii*, and are unable to kill *Borrelia* persisters even with 50 × MIC (Table S1). Although the relationship between *Borrelia* persisters and chronic LD or PTLDS still needs to be confirmed, seeking effective anti-persister drugs would facilitate the clinical management of LD.

In addition, it is important to note that this study screened an overlapping but different drug library consisting of more compounds than the one we screened in our pioneer study published in this journal in 2014 [16]. By screening 2427 compounds in a custom compound library against stationary-phase cultures of *B. garinii* and *B. afzelii*, the new hits including HA, chlorhexidine, octenidine, ufenamate, and aminacrine were proved to be good candidates for killing persisters of *B. garinii* and *B. afzelii*. They probably could benefit the treatment of acrodermatitis chronica atrophicans (ACA), the most common late and chronic manifestation of Lyme borreliosis resulting predominantly from *B. afzelii* infection [42].

Pipemidic acid and cinoxacin, the first generation of quinolones, displayed anti-persister

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activities against *B. garinii* and *B. afzelii*; whereas 29 2nd, 3rd or 4th generation of fluoroquinolones, did not show comparable activity, although the fluorine atom at C-6 of fluoroquinolone increased gyrase inhibition by 10-fold in the antibacterial process [43]. Accordingly, pipemidic acid and cinoxacin may target *Borrelia* persisters with other unknown mechanisms. In addition, drugs normally applied for noninfectious disease treatment, such as cinacalcet, crizotinib, manidipine, etc., were active against stationary-phase cells of *B. garinii* and *B. afzelii*, and were relatively more effective than commonly used antibiotics for LD therapy. Further study is needed to evaluate their potential to be promising anti-persister compounds.

HA was identified as the most active anti-*Borrelia* drug in this study, no matter for log-phase growing cells or persisters including the most drug-tolerant *Borrelia* biofilm (Figure 4). This activity was further enhanced by combining HA with other antibiotics (Table S2). Since *Borrelia* biofilm likely plays an important role in clinical persistent infection, especially skin infection [10-12], HA is worth to be studied further about its usage and clinical effects in the treatment of chronic LD. HA is one type of pigment isolated from *Hypocrella bambuase*, a parasitic fungus of bamboo *Sinarundinaria*. *Sinarundinaria* has been used for rheumatoid arthritis treatment in traditional Chinese medicine [44,45]. Considering the similarity between rheumatoid arthritis and Lyme arthritis, it is possible that, throughout history, people have unknowingly used HA-containing herbs to treat Lyme arthritis in ancient China. Currently, HA is a CFDA (China Food and Drug Administration) approved topical drug for white lesions of the vulva, keloid, vitiligo, tinea capitis, and lichen amyloidosis in China [38]. Additionally, HA has been proven to have potent antibacterial, anticancer, antifungal, and anti-leishmanial activity [46-48]. The cytotoxicity tests showed that hypocrellins had only weak toxicity on A549 cells and no toxicity on normal human intestinal epithelial cells (HIEC) at 10 μM [48]. Moreover, animal studies have shown that HA was not toxic to the mouse skin below a concentration of 1.0 mg/mL [49]. The biological activity of HA is tightly correlated with its characteristic of eliciting ROS generation in light conditions [38]. Despite HA could efficiently eradicate *B. burgdorferi* cells within a short time (Figure 5a), no significant rise in ROS level was detected intracellularly in the cells. HA-triggered ROS generation also did not take place extracellularly in the medium, as the water-soluble antioxidant of vitamin C failed to offset the killing activity of HA (Figure 6). Considering the liposoluble property of HA, HA-triggered ROS generation could probably take place on cell membrane, which would destruct the membrane and

cause lethal damage to *Borrelia* cells. This suggestion is supported by the fact that liposoluble membrane protective antioxidant of vitamin E [50] substantially increased the fraction of viable cells in HA-treated *Borrelia* cultures (Figure 6). .

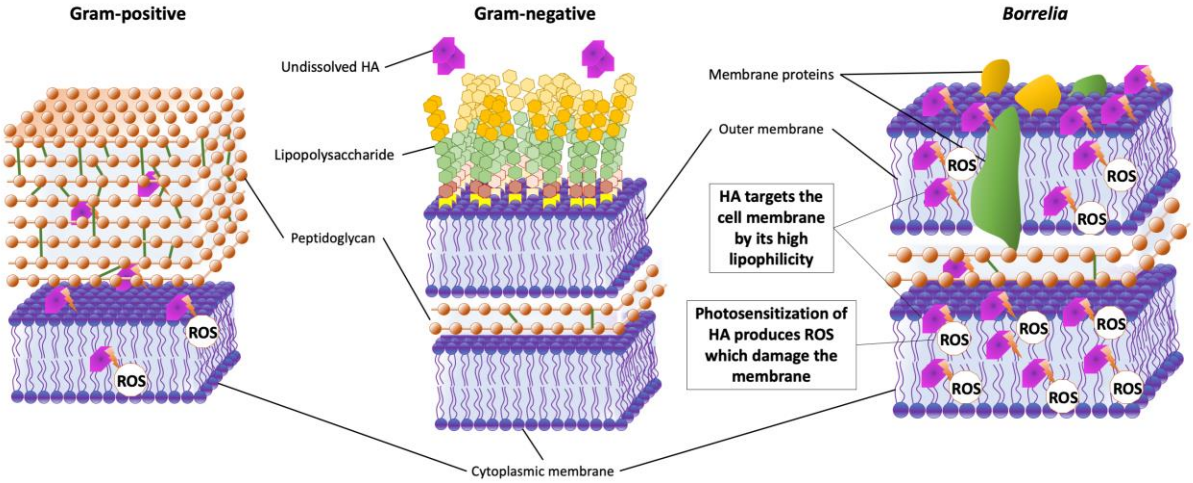

Figure. 7. Schematic diagram showing how HA targets the cell membrane of typical Gram-positive and Gram-negative bacteria as well as *Borrelia*.

In contrast to the *Borrelia*-eradicating capability, HA acted poorly on typical Gram-negative *E. coli* and exhibited a certain degree of killing on Gram-positive *S. aureus* (Figure 5). These results suggest that cell membrane-targeted photokilling activity of HA probably depends on the distinct composition and architecture of bacterial cell membranes. As illustrated in Figure 7, Gram-positive bacteria harbor peptidoglycan cell wall matrix outside of plasma membrane, which could allow penetration of HA, leading to moderate photokilling activity. In contrast, the hydrophilic lipopolysaccharide layer of Gram-negative bacteria would repel the docking of HA on the outer membrane. Of note, *Borrelia* spirochetes, which are often perceived as Gram-negative bacteria owing to their double-membrane envelopes, actually possess membrane proteins and surface lipoproteins instead of a lipopolysaccharide layer on the outer membrane [51]. Without a lipopolysaccharide layer, HA could easily target the cell membrane of *Borrelia* by its high lipophilicity. When the light excited triplet HA returns to the ground state, energy transition produces ROS [38], which would then cause fatal membrane damage. Disturbing bacterial membrane is an important mode of directly killing bacterial persisters for many anti-persister drugs

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394 or compounds, such as daptomycin, clofazimine, 2D-24, etc.; some membrane-targeting anti-
395 persister drugs can also enhance the activity of other drugs by promoting drug penetration [52]. Thus,
396 HA could potentially show anti-persister activity against other bacteria by direct killing or in drug
397 combination in therapeutics. Skin is one of the organs primarily infected by *Borrelia*, especially *B.*
398 *afzelii*, which can form cutaneous biofilm and cause persistent LD in skin [11]. As a CFDA-approved
399 topical drug, HA is a promising candidate for treatment of cutaneous Lyme borreliosis. The
400 characteristic of HA in specifically killing *Borrelia* will help to reduce its side-effects on normal
401 microbiome and improve its clinical application for persistent *Borrelia* skin lesions. Further
402 evaluation of anti-*Borrelia* activities of HA on different *Borrelia* clinical isolates, animal models or
403 LD patients with difficult-to-heal skin lesions would be of interest.

404 In summary, the formation of drug-tolerant persisters of *B. garinii* and *B. afzelii* was confirmed
405 *in vitro* in this study, especially in stationary-phase cultures. Dozens of clinically used antibiotics
406 and compounds including HA, anthracycline drugs, topical antibiotics, and some other natural
407 compounds were found to have good activity against stationary-phase cells of *B. garinii* and *B.*
408 *afzelii*. Among them, HA was the most powerful anti-*Borrelia* agent, which could specifically target
409 the cell membrane of *Borrelia* by triggering ROS generation in light condition. HA should be a
410 promising candidate for clinical topical application in the treatment of LD, particularly cutaneous
411 Lyme borreliosis.

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Supplementary Materials

Table S1: The activities of some representative FDA-approved antibiotics against stationary-phase cultures of *B. garinii* and *B. afzelii*.

Table S2: Subculture tests to evaluate the residual viability of stationary-phase *Borrelia* after drug exposure.

Figure S1: The linear relationship between the viability of *Borrelia* cells and the ratio of Green/Red fluorescence.

Figure S2: Representative images of stationary-phase *Borrelia* cells treated with different compounds (20 μg/mL) followed by staining with SYBR Green I/PI.

Figure S3: Representative images of *Borrelia* biofilms followed by SYBR Green I/PI staining.

Figure S4: Representative images of stationary phase *Borrelia* cells treated with drugs and drug combinations (10 μg/mL) followed by SYBR Green I/PI staining.

Figure S5: Detection of ROS in the *Bb* with DCFH-AD assay.

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Author Contributions

Conceptualization, J.F., T.L. and Y.Z; Methodology, J.F., Y.X. and D.L.; Data Curation, J.S. and J.L.; Writing-Original Draft Preparation, T.L. and J.F.; Writing-Review & Editing, T. L., J.F. and Y.Z.

Data Availability

The data that support the findings of this study are available upon request to the corresponding author, Jie Feng.

Declaration of interest statement

The authors declare no conflict of interest.

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