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ORIGINAL ARTICLE

Identification of new compounds with high activity against stationary phase *Borrelia burgdorferi* from the NCI compound collection

Jie Feng, Wanliang Shi, Shuo Zhang and Ying Zhang

Lyme disease is the leading tick-borne disease in the USA. Whereas the majority of Lyme disease patients with early disease can be cured with standard treatment, some patients suffer from chronic fatigue and joint and muscular pain despite treatment, a syndrome called posttreatment Lyme disease syndrome. Although the cause is unclear, ineffective killing of *Borrelia burgdorferi* persisters by current Lyme disease antibiotics is one possible explanation. We took advantage of our recently developed high-throughput viability assay and screened the National Cancer Institute compound library collection consisting of 2526 compounds against stationary phase *B. burgdorferi*. We identified the top 30 new active hits, including the top six anthracycline antibiotics daunomycin 3-oxime, dimethyldaunomycin, daunomycin, NSC299187, NSC363998 and nogalamycin, along with other compounds, including prodigiosin, mitomycin, nanaomycin and dactinomycin, as having excellent activity against *B. burgdorferi* stationary phase culture. The anthracycline or anthraquinone compounds, which are known to have both anti-cancer and antibacterial activities, also had high activity against growing *B. burgdorferi* with low minimum inhibitory concentration. Future studies on the structure–activity relationship and mechanisms of action of anthracyclines/anthraquinones are warranted. In addition, drug combination studies with the anthracycline class of compounds and the current Lyme antibiotics to eradicate *B. burgdorferi* persisters *in vitro* and in animal models are needed to determine if they improve the treatment of Lyme disease.
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INTRODUCTION

Lyme disease is a multisystem disease caused by the spirochetal bacterium *Borrelia burgdorferi* and is the leading tick-borne disease in the USA.[1] The clinical manifestations of Lyme disease are characterized by an erythema migrans rash and an influenza-like illness, with arthritis and neurological disorders as frequent sequelae of the disease. The infection is transmitted to humans by tick vectors that normally feed on rodents, reptiles, birds, and deer.[2] Although the majority of Lyme disease patients with early or early disseminated disease without neurological involvement can be cured with two–four weeks of standard treatment with doxycycline or amoxicillin, approximately 10%–20% of patients treated for Lyme disease have chronic fatigue and joint and muscular pain when assessed six months after treatment, a collection of symptoms called posttreatment Lyme disease syndrome.[3] The question of whether *B. burgdorferi* might persist in some patients after antibiotic therapy and further evade host immune clearance has been raised by some, but it is controversial. In various animal models (mice, dogs, and rhesus macaque monkeys), antibiotic therapy with doxycycline, ceftriaxone, or tigecycline could not eradicate detection of *B. burgdorferi* as shown by xenodiagnosis and polymerase chain reaction, even though viable organisms could not be cultured in conventional culture medium.[4,5,6,7] The findings indicate the continued presence of *B. burgdorferi* in some form and suggest that current Lyme treatment may not be sufficient to eliminate *B. burgdorferi* persisters or that the immune system fails to clear persisting organisms or bacterial debris, which may be underlying causes for those who suffer from non-resolving symptoms of Lyme disease. To date, there is currently no effective antibiotic treatment or preventative strategy for those who suffer from persistent symptoms after Lyme disease.

Consistent with the difficulty to eradicate *B. burgdorferi* in animal models, *B. burgdorferi* develops various morphological variant forms, such as round bodies and microcolonies, that are refractory or resistant to antibiotics and stresses.[8,9,10] For example, it has been demonstrated that whereas the frontline drugs, such as doxycycline and amoxicillin, kill or inhibit the growing spirochetal form of *B. burgdorferi* effectively, they have little activity in killing non-growing persisters that are enriched in the stationary phase or microcolonies or as biofilm-like aggregates of *B. burgdorferi*.[11,12] There is significant interest in the identification of drugs that target *B. burgdorferi* persisters.[8,12]

To identify drugs that can more effectively kill *B. burgdorferi* persisters, we recently developed a new viability assay using SYBR Green I/propidium iodide (PI) dyes,[13] which allowed us to screen an Food and Drug Administration (FDA)-approved drug library against stationary phase *B. burgdorferi* persisters.[12] Using this high-throughput assay, we identified a number of drug candidates, such as daptomycin, clofazimine, cefoperazone, and carbomycin that have excellent activity

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against *in vitro B. burgdorferi* persisters.[12] In our previous study, we found that daptomycin had the highest activity against *B. burgdorferi* persisters among all of the candidate drugs. Although daptomycin could almost eradicate *B. burgdorferi* persisters at 50 μM, this drug concentration is too high for clinical use, and daptomcyin has to be used intravenously, which is not convenient to administer.

To identify new and more effective drugs than daptomycin in killing *B. burgdorferi* persisters, we performed new drug screens on stationary phase *B. burgdorferi* cultures using the chemical repository collection of the National Cancer Institute (NCI) compound library collection. The reason we used *B. burgdorferi* stationary phase culture is because it is known to enrich persisters and contains more than 70%–80% persisters not killed by the current Lyme antibiotics doxycycline or amoxicillin.[12,14] Although *B. burgdorferi* stationary phase culture is comprised of mainly non-growing cells and some growing cells, it can be considered a convenient close proximate for *B. burgdorferi* persisters. In addition, our previous study performed on *B. burgdorferi* stationary phase culture allowed us to identify useful drug candidates, such as daptomycin, clofazimine, and cefoperazone, which are shown to be active against *B. burgdorferi* persisters not killed by doxycycline and amoxicillin. The NCI compound library collection we used has three compound libraries: the diversity set IV compound library (1593 compounds), the mechanistic set II library (816 compounds), and the natural product set III library (117 compounds), altogether 2526 compounds. These compounds were chosen based on structural diversity from more than 250 000 natural products and synthetic compounds.[15] By screening this NCI compound library collection, we identified new compounds active against stationary phase *B. burgdorferi* that were not found in our previous screens.[12] These new active hits could help to develop a new, more effective treatment for Lyme disease.

MATERIALS AND METHODS

Bacterial strain, media, and culture

Borrelia burgdorferi strain B31 (ATCC 35210) was obtained from the American Type Tissue Collection. *B. burgdorferi* was cultured in BSK-H medium (HiMedia Laboratories Pvt. Ltd.) with 6% rabbit serum (Sigma-Aldrich). All culture media were filter sterilized by 0.2 μM filters. Cultures with 1×10^5 spirochetes/mL initial concentrations of inoculum were incubated in 50 mL closed conical tubes (BD Biosciences, California, USA) at 33℃ without antibiotics. Based on our previous study that demonstrated the antibiotic tolerance of the stationary phase cultures,[12] we chose seven-day-old stationary phase *B. burgdorferi* cultures (1×10^7 spirochetes/mL) enriched in persisters for drug screens in 96-well microtiter plates, as previously described.[12]

Microscopy techniques

Specimens were examined on a Zeiss AxioImager M2 microscope equipped with differential interference contrast (DIC) and epifluorescent illumination and were recorded with a Hamamatsu ORCA-R^2 C10600 camera. A cell proliferation assay was performed by direct counting using a bacterial counting chamber (Hausser Scientific Partnership, Horsham, PA, USA) and DIC microscopy. A SYBR Green I/PI assay was performed to assess the viability of *B. burgdorferi*, as previously described.[13] The ratio of live (green) and dead (red) *B. burgdorferi* was calculated by counting the cells using a bacterial counting chamber and epifluorescence microscopy, as previously described.[13] For the aggregated cells, three representative images of each sample were captured for quantitative analysis. Image Pro-Plus software was applied to select the green (including yellow) and red (including orange) areas of different morphological forms to calculate the integrated fluorescence intensity (equal to area × average density or average intensity) of the red and green portions, as previously described.[16]

Antibiotics and the NCI chemical compound library

Antibiotics, including doxycycline, amoxicillin, and daptomycin, were purchased from Sigma and dissolved in appropriate solvents[17] to form stock solutions. All antibiotic stocks were filter sterilized by 0.2 μM filters. Then, the stocks were diluted into 500 μM pre-diluted stocks and stored at −20℃.

The NCI compound library collection, consisting of diversity set V,[18] mechanistic diversity set II,[19] and the natural products set III,[20] was kindly supplied by the National Cancer Institute Developmental Therapeutic Program's Open Compound Repository, NIH. These NCI compound libraries were prepared in 1 mM stock solutions with dimethyl sulfoxide in 96-well plates, leaving the first and the last columns in each plate for controls, which included dimethyl sulfoxide blank controls, doxycycline control, and amoxicillin control. The pre-diluted drug plates were sealed and stored at −20℃.

Screening NCI compound libraries against *B. burgdorferi* stationary phase persisters

To qualitatively determine the effect of the compounds on *B. burgdorferi* persisters, each compound (5 μL) from the pre-diluted stocks was added to a seven-day-old *B. burgdorferi* stationary phase culture (1×10^7 spirochetes/mL) in 96-well microtiter plates. The final volume per well was adjusted to 100 μL to achieve a final drug library concentration of 50 μM. The plates were sealed and placed in a 33℃ incubator for seven days, after which the viability of the bacteria was assessed by SYBR Green I/PI assay, as described in our previous study.[13] With the excitation wavelength at 485 nm, the fluorescence intensities at 535 nm (green emission) and 615 nm (red emission) were measured for each well of the screening plate using a SpectraMax M2 Microplate Reader (Molecular Devices Inc., USA). Some effective candidates were further confirmed by epifluorescence microscopy, as previously described.[13]

Minimum inhibitory concentration (MIC) determination

The standard microdilution method was used to determine the MIC that would inhibit visible growth of *B. burgdorferi* after a 72-h incubation period.[11,21,22] *B. burgdorferi* cells (1×10^5) were inoculated into each well of a 96-well microplate containing 90 μL fresh BSK-H medium per well. Each diluted compound (10 μL) was added to the culture. All experiments were run in triplicate. The 96-well plate was sealed and placed in an incubator at 33℃ for five days. Cell proliferation was assessed using the SYBR Green I/PI assay and a bacterial counting chamber after the incubation, as previously described.[12]

RESULTS

Screening NCI compound library to identify effective drugs active against dormant *B. burgdorferi* persisters

We used the SYBR Green I/PI assay as a high-throughput screening method for rapid viability assessment for *B. burgdorferi* after exposure to the compound libraries.[12] Based on our previous study, some red-colored compounds caused interference in the SYBR Green I/PI assay, which could make the background red and cause false positive results. Thus, in this study, we used microscopic counting rescreen to examine the hit compounds in the SYBR Green I/PI assay.

Table 1 Activity of the top 30 active hits that had good activity (better than current clinical drugs) against stationary phase *B. burgdorferi* [a]

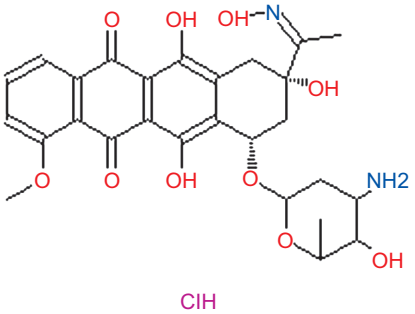
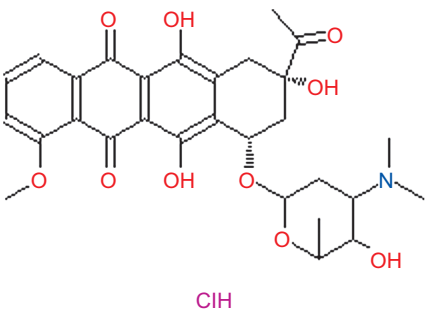
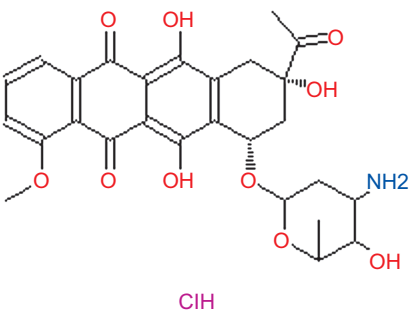
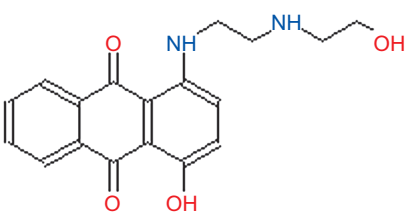
NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
	Control	–	93	95
	Amoxicillin	–	71	77
	Doxycycline	–	68	77
	Daptomycin	–	23	18
143491	Daunomycin 3-oxime hydrochloride		0	6
258812	Dimethyldaunomycin hydrochloride		0	10
82151	Daunorubicin hydrochloride		0	10
299187	9,10-Anthracenedione, 1-hydroxy-4-[[2-[(2-hydroxyethyl) amino]ethyl]amino]-		5	13

Table 1 (Continued)

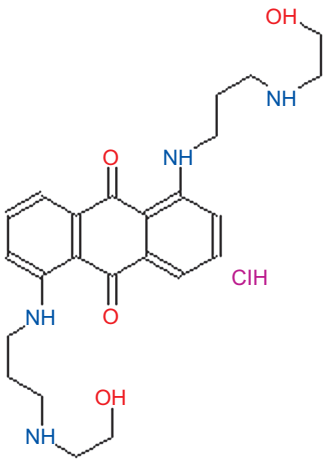
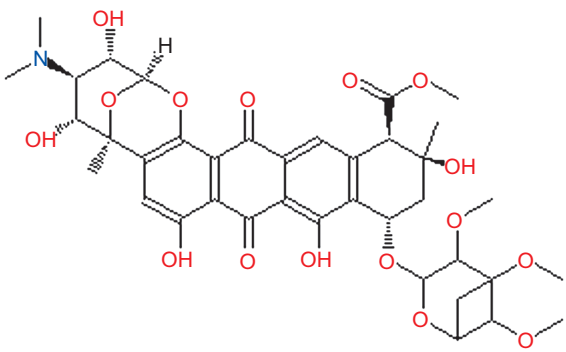
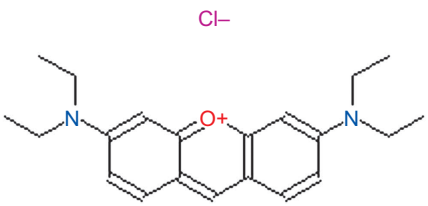
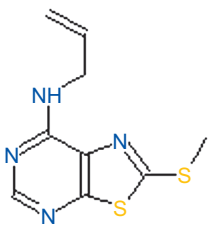
NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
363998	Anthracene-9,10-dione, 1,5-bis[3-[[(2-hydroxyethyl)amino]propyl]amino]-9,10-dihydro-, dihydrochloride		0	13
70845	Nogalamycin		0	15
44690	Pyronin B		0	19
343783	N-Allyl-2-(methylthio)[1,3]thiazolo[5,4-d]pyrimidin-7-amine		67	20

Table 1 (Continued)

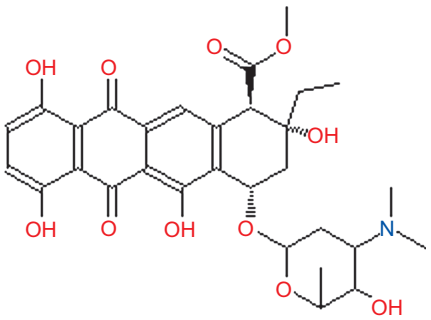
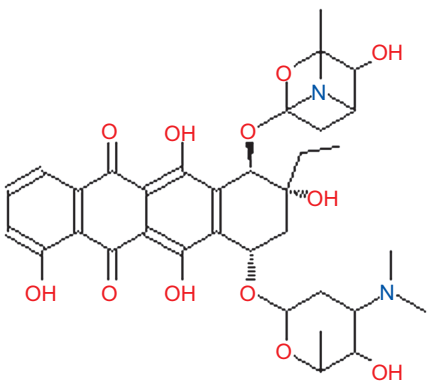
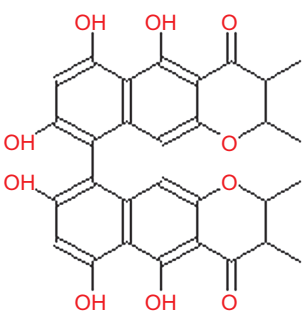
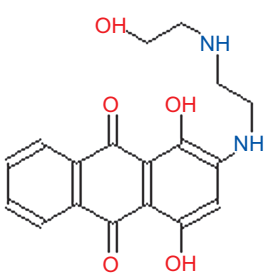
NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
267229	Pyrromycin		0	21
136044	Rhodomycin A		0	22
345647	Chaetochromin		8	22
316157	9,10-Anthracenedione, 1,4-dihydroxy-2-[[2-[(2-hydroxyethyl)amino]ethyl]amino]-		0	23

Table 1 (Continued)

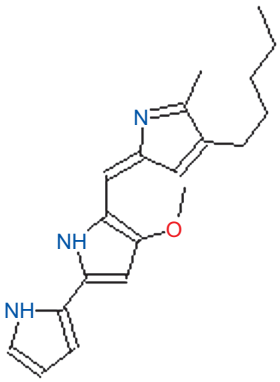
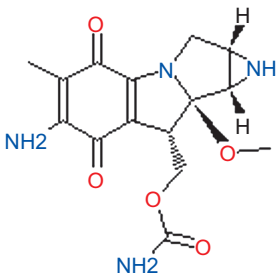
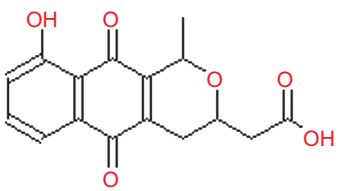
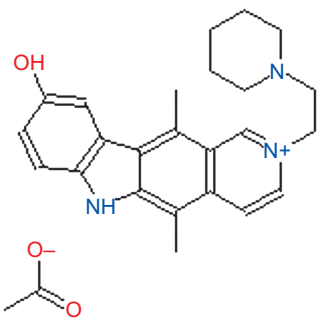
NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
47147	Prodigiosin		0	24
26980	Mitomycin		20	25
267461	Nanaomycin		34	26
311153	9-Hydroxy-2-(2-piperidinylethyl)ellipticinium acetate		45	26

Table 1 (Continued)

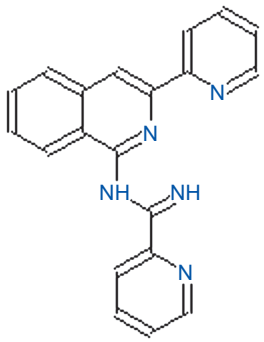
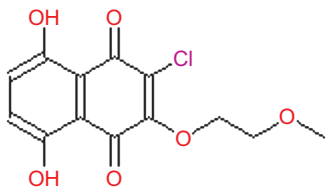
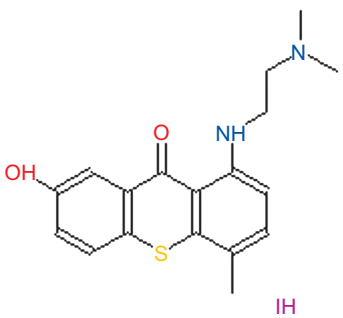
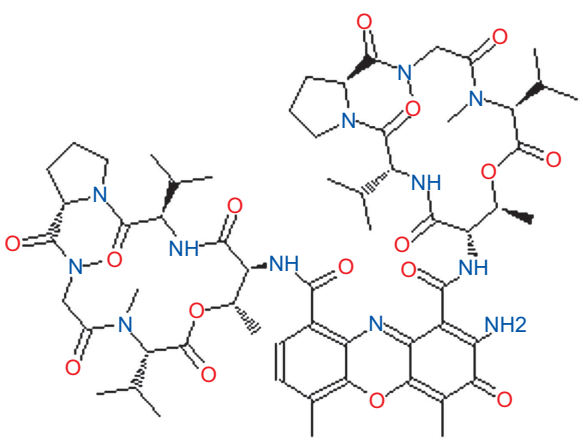
NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
637578	N-[3-(2-Pyridyl)isoquinolin-1-yl]-2-pyridinecarboxamidine		46	26
659997	Naphthalene-1,4-dione, 2-chloro-5,8-dihydroxy-3-(2-methoxyethoxy)-		1	28
317003	9H-Thioxanthen-9-one, 1-[[2-(dimethylamino)ethyl]amino]-7-hydroxy-4-methyl-, monohydriodide		29	30
3053	Dactinomycin		37	30

Table 1 (Continued)

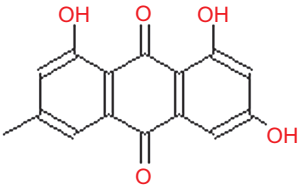
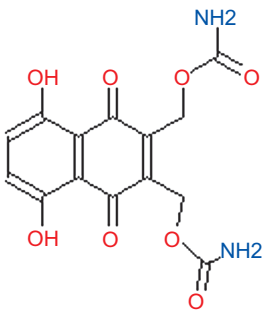
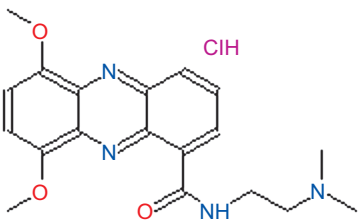
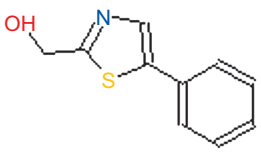
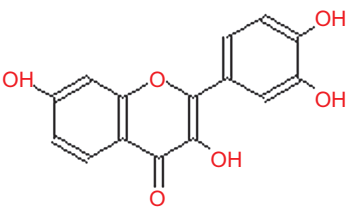
NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
408120	Emodin		18	31
224124	(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalene-2,3-diyl)dimethanediyl dicarbamate		32	31
678917	1-Phenazinecarboxamide, N-[2-(dimethylamino)ethyl]- 6,9-dimethoxy-, monohydrochloride		44	35
118832	(5-phenyl-1,3-thiazol-2-yl)methanol		30	38
407010	3,3′,4′,7-Tetrahydroxyflavone		39	38

Table 1 (Continued)

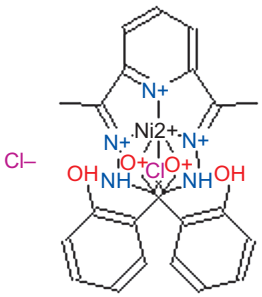
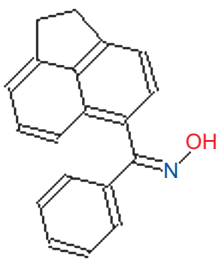
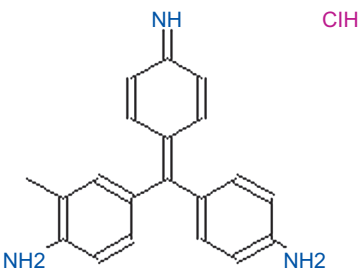
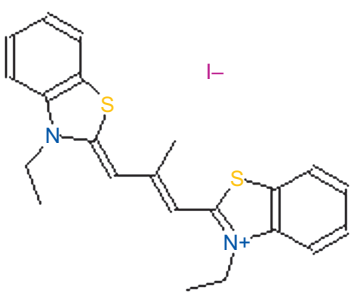
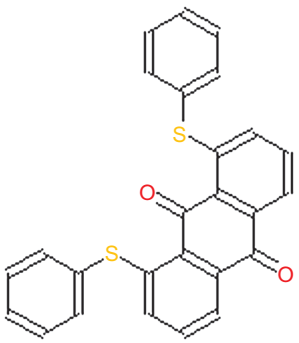
NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
617570	Benzoic acid, 2-hydroxy-, (2,6-pyridinediyldiethylidyne) dihydrazide, nickel complex		44	38
137399	1-(1,2-Dihydro-5-acenaphthylenyl)-N-hydroxy-1-phenylmethanimine		51	41
93739	2-Methyl-4,4′-[(4-imino-2,5-cyclohexadien-1-ylidene)methylene]dianiline hydrochloride		0	43
96932	3,3′-Diethyl-9-methylthiacarbocyanine iodide		0	46

Table 1 (Continued)

NSC[b]	Compounds (50 μM)	Chemical structure	Residual viable cells[c] (%)	Residual viable cells[d] (%)
156516	1,8-Di(phenylthio)anthraquinone		46	50

[a] Seven-day-old stationary phase *B. burgdorferi* culture was treated with drugs or compounds (50 μM) for seven days, at which point the viability of the bacteria was determined, as previously described.[12]

[b] The NSC number is a numeric identifier for substances submitted to the NCI.

[c] Residual viable *B. burgdorferi* was calculated according to the regression equation and the ratio of Green/Red fluorescence obtained by the SYBR Green I/PI assay, as previously described.[12]

[d] Residual viable *B. burgdorferi* was assayed by epifluorescence microscope counting, as previously described.[12]

To identify effective chemical compounds that have activity against *B. burgdorferi* persisters, we used the stationary phase *B. burgdorferi* as a persister model, as it is known to be enriched in persisters.[12,14] to screen the NCI compound libraries. Meanwhile, the currently used Lyme disease antibiotics doxycycline and amoxicillin were included as control drugs. Consistent with our previous results,[12] the currently used Lyme antibiotics had poor activity against the stationary phase *B. burgdorferi* persisters, and the bacteria treated with the two antibiotics still had 75% and 76% viable cells remaining, respectively, compared with 93% viable cells in the drug-free control (Table 1).

Of the 2526 compounds in the NCI compound library collection tested, 237 were found to have higher activity against *B. burgdorferi* persisters than doxycycline and amoxicillin in the primary screen. The 237 candidates were rescreened by microscope counting with the SYBR Green I/PI viability assay. After the rescreening by microscopy, we confirmed the top 30 active hits that had less than 50% residual viable cells after treatment (Table 1, Figure 1). Among the 30 active hits, 22 compounds were found in mechanistic set II, nine compounds in diversity set IV, and three compounds in natural product set III. Nanaomycin and dactinomycin were in both mechanistic set II and natural product set III, whereas NSC311153 and NSC637578 were in both mechanistic set II and diversity set IV. These compounds are all aromatic compounds. We identified several clinically used drugs that had excellent activity against stationary phase *B. burgdorferi*. The activity of some drugs against stationary phase *B. burgdorferi* was significantly higher than that of the frontline antibiotics doxycycline or amoxicillin, and even more active than daptomycin, the best antibiotic against *B. burgdorferi* persisters in our previous study (Table 1, Figure 1). We found that six anthraquinone antibiotics and compounds, daunomycin 3-oxime, dimethyldaunomycin, daunomycin, NSC299187, NSC363998, and nogalamycin showed the highest activities (residual viable cells from 6% to 15%) against stationary phase *B. burgdorferi*. These six compounds showed higher activity than daptomycin (18% residual viable cells). In addition, another five anthraquinone compounds, pyrromycin, rhodomycin A, NCS316157, emodin, and NSC156516 also had good activity against stationary phase *B. burgdorferi* (residual viable cells 21%–50%). In addition to the six anthraquinones, pyronin B, a xanthene compound, had good activity (residual viable cells 19%) against stationary phase *B. burgdorferi*. We found seven nitrogen-containing aromatic compounds, including NSC343783 (residual viable cells 20%) and prodigiosin (24% residual viable cells), NSC637578, NSC 678917, NSC118832, NSC617570, and NSC96932, to be among the 30 most active compounds. Moreover, chaetochromin, a bis-naphtho-γ-pyrone compound, showed good activity, with 22% residual viable cells. Mitomycin, an aziridine-containing benzoquinone antitumor drug, showed reasonably good activity with 25% residual viable cells. We also found three 1,4-naphthoquinones, nanaomycin (residual viable cells 26%), NCS659997 and NCS224124, had relatively good activity against stationary phase *B. burgdorferi*. A polypeptide antibiotic dactinomycin also had relatively high activity against stationary phase *B. burgdorferi* (residual viable cells 30%). In addition to 11 clinically used drugs (daunomycin 3-oxime, dimethyldaunomycin, daunomycin, nogalamycin, pyrromycin, chaetochromin, prodigiosin, mitomycin, nanaomycin, and dactinomycin), we found 19 non-medicinal compounds that showed good activity against stationary phase *B. burgdorferi* to varying levels (Table 1, Figure 1).

Relationship between MIC values and anti-persister activity

We found some compounds that have good activity against the non-growing stationary phase *B. burgdorferi* (Table 1), but it is necessary to determine the MICs of these compounds against growing *B. burgdorferi* (Table 2). The standard microdilution method was used to determine the MIC, as described in our previous study.[12] We found that three anthracycline antibiotics, daunomycin 3-oxime, daunorubicin,

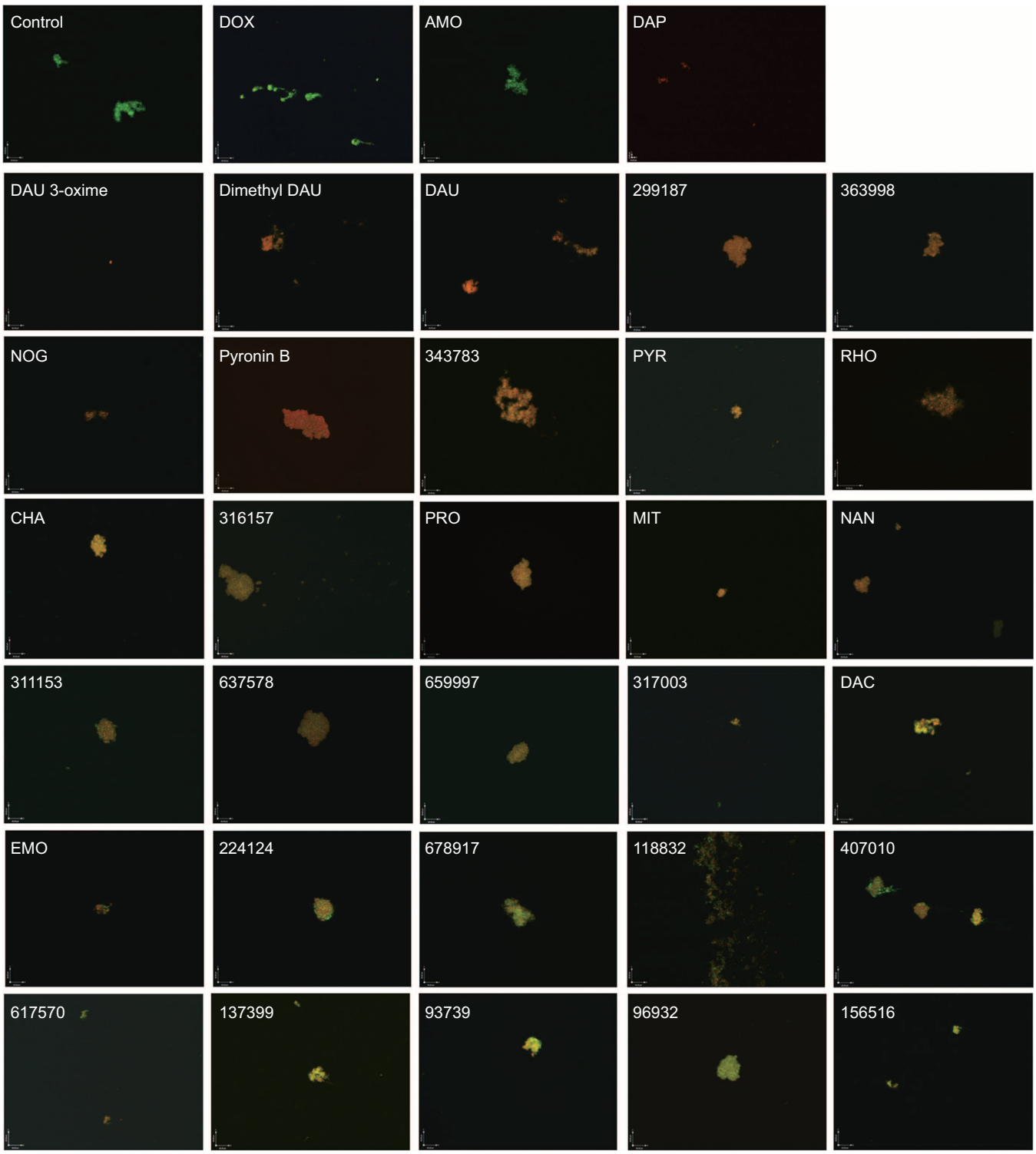


Figure 1 Representative images at ×100 magnification of stationary phase *B. burgdorferi* treated with different compounds (50 μM) followed by staining with the SYBR Green I/PI assay. Abbreviations: DOX, doxycycline; AMO, amoxicillin; DAP, daptomycin; DAU, daunomycin; NOG, nogalamycin; PYR, pyrromycin; RHO, Rhodomycin A; CHA, chaetochromin; PRO, prodigiosin; MIT, mitomycin; NAN, nanaomycin; DAC, dactinomycin; EMO, emodin.

and pyrromycin, in addition to having good activity against stationary phase *B. burgdorferi* were also highly active against log phase growing *B. burgdorferi* with low MICs ($\leqslant$0.36, $\leqslant$0.36, 0.36–0.72 μg/mL, respectively). Another anthraquinone compound, NSC299187, had a relatively high MIC (3.26–6.52 μg/mL) although it had excellent activity against stationary phase *B. burgdorferi* (residual viable cells 13%). We noted that prodigiosin (nitrogen-containing aromatic rings compound), mitomycin (aziridine-containing benzoquinone), nanaomycin (1,4-naphthoquinone), and dactinomycin (polypeptide antibiotic) had good activity against replicating *B. burgdorferi* with low

Table 2 Comparison of the MIC values and the activities of some compounds against stationary phase *B. burgdorferi*

Antibiotics	MIC (μg/mL)	Activity against stationary phase *B. burgdorferi*[a] (%)
Doxycycline	⩽0.25	77
Amoxicillin	⩽0.25	77
Daptomycin	12.5–25	18
Daunomycin 3-oxime	⩽0.36	6
Daunorubicin	⩽0.35	10
NSC299187	3.26–6.52	13
Pyronin B	1.8–3.6	19
Pyrromycin	0.37–0.73	21
Chaetochromin	2.74–5.47	22
Prodigiosin	⩽0.2	24
Mitomycin	⩽0.21	25
Nanaomycin	0.76–1.57	26
Dactinomycin	⩽0.78	30

[a] Shown as the residual viable cell percentage from epifluorescence microscope counting data.

MICs (⩽0.2, ⩽0.21, 0.76–1.57, ⩽0.78 μg/mL, respectively). On the other hand, pyronin B and chaetochromin were less potent against growing *B. burgdorferi*, with relatively high MICs (1.8-3.6, 2.74–5.47 μg/mL, respectively), but they had excellent activity against stationary phase *B. burgdorferi*.

Comparison of anti-persister activity at low drug concentrations

Although we obtained many highly effective hits from the NCI compound library with a 50 μM compound screen, this drug concentration is likely too high for *in vivo* experiments. Daptomycin at 50 μM showed strong activity against stationary phase *B. burgdorferi* in our previous study,[12] but it could not kill the microcolony form of *B. burgdorferi* persisters at lower concentration, such as 10 μg/mL.[14] To further compare the activity of the hit compounds and daptomycin, we tested the activity against stationary phase *B. burgdorferi* at a 20 μM drug concentration (approximately 10 μg/mL for most compounds and 32 μg/mL for daptomycin). Most of the residual viable percentages of stationary phase *B. burgdorferi* increased with the decrease of drug concentration (Table 3, Figure 2), but five anthracyclines: dimethyldaunomycin, NCS363998, nogalamycin, pyrromycin, and Rhodomycin A, at 20 μM still showed as strong an activity against stationary phase *B. burgdorferi* as at 50 μM (Table 3, Figure 2). Other non-anthracycline compounds showed relatively weaker activity than daptomycin at 20 μM.

DISCUSSION

We recently identified a number of drug candidates that have excellent activity against non-replicating *B. burgdorferi* stationary phase culture enriched with persisters from an FDA-approved drug library.[12] In this study, we attempted to identify new drug candidates or compounds that have high activity against *B. burgdorferi* stationary phase culture using the NCI compound library collection. From the 2526 compounds in three NCI compound libraries, 237 compounds were found to have higher activity against *B. burgdorferi*
stationary phase culture than doxycycline or amoxicillin, from which the top 30 active hits were confirmed by microscopy rescreening. The use of the mechanistic compound library helped to identify the anthraquinone (anthracycline) class of drugs, which has high activity against stationary phase *B. burgdorferi*. More than one-third of the 30 most active compounds possess an anthraquinone (also called anthracenedione or dioxoanthracene) structure. The top six active compounds, daunomycin, daunomycin 3-oxime, dimethyldaunomycin,

Table 3 Comparison of the activity of some hit compounds at 20 μM and 50 μM against stationary phase *B. burgdorferi*[a]

		Residual viable cells (%)		
NSC number	Compounds	20 μM[b]	20 μM[c]	50 μM[c]
258812	Control	93	94	95
	Amoxicillin	77	77	77
	Doxycycline	76	77	77
	Daptomycin	32	25	18
	Dimethyldaunomycin hydrochloride	0	10	10
363998	Anthracene-9,10-dione, 1,5-bis[3-[[(2-hydroxyethyl)amino] propyl]amino]-9,10-dihydro-, dihydrochloride	22	14	13
70845	Nogalamycin	3	15	15
267229	Pyrromycin	6	20	21
136044	Rhodomycin A	5	21	21
345647	Chaetochromin	31	33	22
47147	Prodigiosin	0	45	24
267461	Nanaomycin	39	45	26
659997	Naphthalene-1,4-dione, 2-chloro-5,8-dihydroxy-3-(2-methoxyethoxy)-	40	50	28
224124	(5,8-dihydroxy-1,4-dioxo-1,4-dihydronaphthalene-2,3-diyl)dimethanediyl dicarbamate	54	77	31
617570	Benzoic acid, 2-hydroxy-, (2,6-pyridinediyldiethylidyne) dihydrazide, nickel complex	66	50	38
93739	2-Methyl-4,4′-[(4-imino-2,5-cyclohexadien-1-ylidene)methylene]dianiline hydrochloride	0	77	43

[a] Seven-day-old stationary phase *B. burgdorferi* culture was treated with drugs for seven days.
[b] Residual viable *B. burgdorferi* was calculated according to the regression equation and the ratio of Green/Red fluorescence obtained by the SYBR Green I/PI assay.
[c] Residual viable *B. burgdorferi* was assayed by epifluorescence microscope counting.

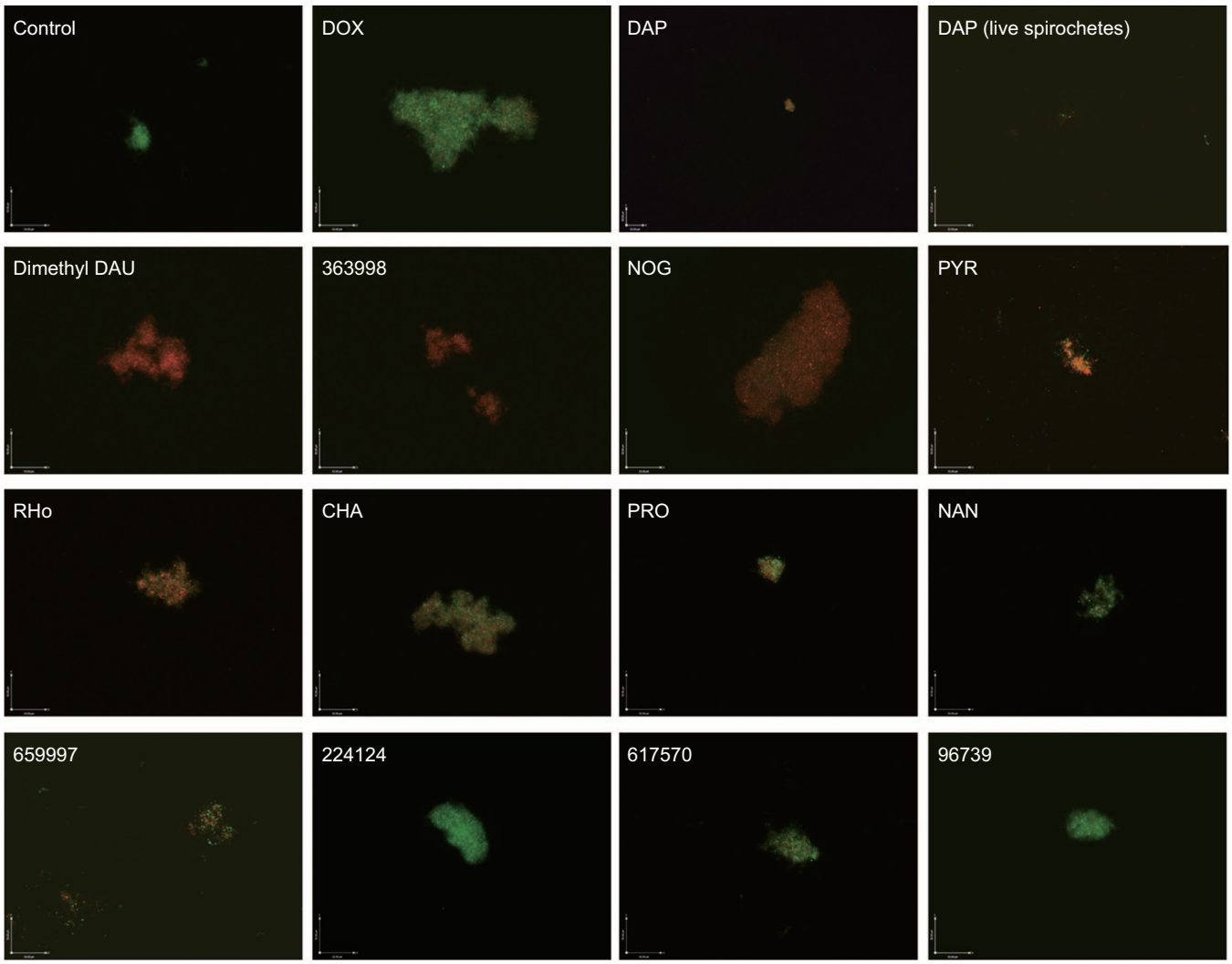


Figure 2 Representative images at ×100 magnification of stationary phase *B. burgdorferi* strain B31 treated with different compounds (20 μM) followed by staining with the SYBR Green I/PI assay. Abbreviations: DOX, doxycycline; DAP, daptomycin; DAU, daunomycin; NOG, nogalamycin; PYR, pyrromycin; RHO, Rhodomycin A; CHA, chaetochromin; PRO, prodigiosin; NAN, nanaomycin.

NSC299187, NSC363998, and nogalamycin, are all anthraquinone derivatives, characterized by three aromatic rings linked together with a benzoquinone in the center. Previously, we noted the anti-persister activity of the anthracycline antibiotic doxorubicin,[12] but we mistakenly excluded it from the active drugs because it has a red color and interfered with the SYBR Green I/PI staining. However, careful examination by microscopy confirmed the activity of red-colored anthraquinone drugs, including doxorubicin. Not all red-colored anthraquinone compounds have good activity against stationary phase *B. burgdorferi*. For example, NCS156516 had weak activity and still had 50% residual viable (green) cells (Figure 1). Thus, confirmation by careful microscopic examination is needed to assess compounds that have red color and have activity against stationary phase *B. burgdorferi* using a low concentration of compounds and subculture studies.

The top six anthraquinone compounds with residual viable cells ranging from 6% to 15% were more active than daptomycin, which had 18% residual viable cells when using the seven-day-old stationary phase culture (Table 1). Meanwhile, these compounds also had very good activity (low MIC) against growing *B. burgdorferi* (Table 2). Further drug combination and subculture studies are needed to confirm whether the top six anthraquinone compounds are indeed more active than daptomycin against *B. burgdorferi* persisters *in vitro* and *in vivo*.

Anthraquinones are a class of naturally occurring phenolic compounds isolated from *Streptomyces* and have diverse medical uses, including anti-cancer, antimalarial, and laxative. Anthracycline antibiotics, such as daunomycin, nogalamycin, pyrromycin, and rhodomycin A, are used in chemotherapy for some cancers, especially for several types of leukemia.[23] Anthracycline drugs have antibacterial activity against *S. aureus*, and the MICs of daunomycin and doxorubicin are 8–32 μg/mL and 0.12–0.5 μg/mL, respectively.[24] Daunomycin did not show bactericidal activity for gram-negative bacteria *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, and *E. coli*.[18] Our study is the first to demonstrate the activity of this class of anthraquinone compounds against both growing and non-growing forms of *B. burgdorferi*. However, the mechanisms of action of this class of anthraquinone compounds against *B. burgdorferi* are unclear and remain to be determined.

Anthracycline antibiotics inhibit DNA and RNA synthesis by inserting base pairs into the DNA/RNA strands.[25] Anthracycline antibiotics stabilize the topoisomerase II complex and prevent dissociation of

topoisomerase II from its nucleic acid substrate, leading to DNA damage and blocking DNA transcription and replication as well as producing reactive oxygen species, which could damage mitochondria and lead to cardiotoxicity as the main side effect.[26,27] The sugar moiety of daunomycin plays a critical role in determining its anticancer activity.[24] In this study, we found anthracycline antibiotics with sugar structures and anthraquinone compounds (NCS299187 and NCS363998) without sugar structures both have good activity against stationary phase *B. burgdorferi*. These findings suggest that the mechanism of action of anthraquinone drugs may not be identical for its anti-cancer activity and its activity in *B. burgdorferi*. Future studies are needed to identify the mechanism of action of anthracycline antibiotics against *B. burgdorferi*, to address the structure–activity relationship of this class of compounds and to explore the possibility of utilizing the strong activity of this class of anthracycline compounds without untoward toxicity to host cells.

In addition to the anthracycline antibiotics, we found that some 1,4-naphthoquinones, such as nanaomycin, NCS659997 and NCS224124, showed high activity against stationary phase *B. burgdorferi*. 1,4-naphthoquinone has an analogous molecular skeleton to anthraquinone. Nanaomycin may interfere with the function of the bacterial cell membrane,[28] and such a mode of action may be responsible for its activity against stationary phase *B. burgdorferi*.

We found that chaetochromin, a bis-naphtho-γ-pyrone produced by several species of chaetomium, also showed high activity against stationary phase *B. burgdorferi*. Bis-naphtho-γ-pyrones have a broad range of biological activities, such as inhibition of adenosine triphosphate synthesis in mitochondria, cell proliferation inhibition, triacylglycerol synthesis inhibition, and antimicrobial activity.[29] Bis-naphtho-γ-pyrones are active against various bacteria, such as *S. aureus*, *E. coli*, and *M. tuberculosis*, with MIC values ranging from 2 μg/mL to 50 μg/mL.[29] Inhibition of adenosine triphosphate synthesis can explain the activity of bis-naphtho-γ-pyrone against stationary phase *B. burgdorferi*. Cephalochromin has been shown to inhibit fatty acid biosynthesis.[30] It is possible that fatty acid synthesis plays a role in *B. burgdorferi* persister formation, and future studies are needed to confirm this possibility.

In this study, we found that some antibiotic compounds, such as prodigiosin, mitomycin, and dactinomycin, had partial activity against stationary phase *B. burgdorferi*, although their activities (24%–30% residual viable cells) are not as strong as daptomycin (18% residual viable cells) (Table 1). Prodigiosin is a secondary metabolite of *Serratia marcescens* and has antibacterial, antifungal, antiprotozoal, antimalarial, immunosuppressive, and anticancer activities.[31] Mitomycin shows its activity as a DNA cross-linker through its aziridine functional group and cross-links the complementary strands of the DNA double helix to cause the death of a bacterial cell.[32,33] The activity of mitomycin against stationary phase *B. burgdorferi* may be due to its DNA cross-linking activity. Dactinomycin is a polypeptide antitumor antibiotic isolated from the soil bacteria *Streptomyces*,[34] and it binds DNA and interferes with DNA replication.[34] and also inhibits RNA transcription.[35]

Persisters are heterogeneous and include persisters in the stationary phase, which can grow after antibiotic exposure and those that survive antibiotic treatment *in vivo* but cannot grow (viable but non-culturable) in a continuum.[36] Thus, the use of stationary phase cultures as a surrogate of persisters in this study has limitations because they cannot represent the viable but non-culturable persisters that have been found *in vivo* in different animal models after antibiotic treatment.[5,6,7] Nevertheless, the discovery of the drug candidates with activity against *B. burgdorferi* stationary phase cultures from this study and our previous study[12,14] offers the opportunity to assess whether such drugs are useful for eradicating persistent infection *in vivo* in animal models and in patients in future studies.

In summary, we identified the anthracycline class of compounds including daunomycin, daunomycin 3-oxime, dimethyldaunomycin, NSC299187, NSC363998, and nogalamycin along with some other compounds, including prodigiosin, mitomycin, nanaomycin, and dactinomycin, as having excellent activity against both the non-growing stationary phase and growing *B. burgdorferi* cultures. The structure–activity relationship and mechanisms of action of the anthracycline/anthraquinone class of compounds against *B. burgdorferi* persisters should be addressed in future studies. In addition, drug combination studies with the anthracycline class of compounds and the current Lyme antibiotics are required to assess whether they improve treatment of Lyme disease in animal models and in patients.

ACKNOWLEDGEMENTS

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