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NOTE

Screening of NCI-DTP library to identify new drug candidates for *Borrelia burgdorferi*

Venkata Raveendra Pothineni[1], Dhananjay Wagh[1], Mustafeez Mujtaba Babar[1], Mohammed Inayathullah[1], R Edward Watts[1], Kwang-Min Kim[1], Mansi B Parekh[1], Abhijit Achyut Gurjarpadhye[1], David Solow-Cordero[2], Lobat Tayebi[3] and Jayakumar Rajadas[1]

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Lyme disease is the most rapidly growing tick borne zoonotic disease of the Northern Hemisphere and is among the 10 most commonly reported nationally notifiable diseases in the United States.[1] Clinical presentations include erythema migrans, fever, chills, muscle and joint pain.[2,3] Though these symptoms tend to fade away even without therapeutic intervention, a significant number of untreated patients develop arthritis and persistent myalgia following exposure to *Borrelia burgdorferi*.[4] Furthermore, 10–20% of patients treated for Lyme disease develop symptoms considered typical, or even exaggerated, including muscle, joint pain and generalized fatigue[5,6]. This condition is referred as post-treatment lyme disease syndrome (PTLDS). Though existence of PTLDS is debatable, some researchers consider the presence of persister forms of *B. burgdorferi* and/or the continuous presence of antigenic debris to be the underlying cause of PTLDS.[7–10]

Like other pathogens, *B. burgdorferi* protects itself from the immune system and from drug treatment.[11,12] *B. burgdorferi* evades immune response by antigenic variation of its surface proteins[13–15]. In a recent study, researchers identified *B. burgdorferi* persisters in *in vitro* cultures.[12] They found the killing of *B. burgdorferi* by antibiotics is biphasic, with a small subpopulation of surviving persisters.[12] The surviving antibiotic tolerant cells are not resistant mutants upon regrowth, the population bifurcates into new antibiotic-susceptible and new persister subpopulations.[12] Currently prescribed drugs for treating Lyme disease, including amoxicillin, ceftriaxone and doxycycline were unable to completely eliminate the *B. burgdorferi*.[12,16–18] So, efforts to identify new, potent drug candidates for Lyme disease are on the rise.

Many researchers are performing high-throughput screening of drugs against *B. burgdorferi* persisters to identify molecules that can eliminate complete Borrelial infection.[7,17–20] Screening of chemical compound libraries serves to test a large number of structurally and functionally diverse molecules against pathogenic agents. Among the many chemical libraries currently available, the Developmental Therapeutics Program of the National Cancer Institute, National Institute of Health, provides a unique yet diverse array of chemical compounds for screening purpose. Four sets of compounds within the National Cancer Institute-Developmental Therapeutics Program (NCI-DTP) library (http://dtp.nci.nih.gov/) tend to represent a wide variety of structural and functional diversity.

This study aimed to identify new, effective drugs for Lyme disease. We used a well-established, highly efficient, BacTiter-Glo assay (Promega Corporation, Fitchburg, WI, USA) which can detect as few as 7×10^3 Borrelial cells in BSK-II medium.[21,22] The NCI-DTP compound library of four diverse sets containing 3084 chemical compounds was screened using this assay. We identified 101 unique compounds which inhibited *Borrelia* growth by more than 85% at or below a concentration of 25 μM. From these 101 compounds we selected 12 molecules and studied their MIC and MBC. The lead compounds identified in the current study can be further evaluated for their therapeutic potential in pre-clinical and clinical studies. Moreover, the outcomes of the study could provide a deeper insight into treatment strategies for Lyme disease.

We have developed a one-step, straightforward, highly sensitive BacTiter-Glo (Promega Corporation) Assay to screen drugs in high-throughput format. We optimized a BacTiter-Glo (Promega Corporation) Assay in high-throughput screening format as reported in our previous papers.[21,22] The BacTiter-Glo Assay (Promega Corporation) assesses bacterial viability by measuring ATP in the sample. This sensitive assay can reliably detect as few as 10 *Borrelia* cells in phosphate buffered saline or 7×10^3 *Borrelia* cells in BSK-II medium[21,22]. By using this BacTiter-Glo (Promega Corporation) Assay we have screened NCI-DTP library containing 3084 chemical compounds.[22] The NCI-DTP compound library we have screened contains a total of 3084 chemical compounds from four highly divergent sets viz, Structural diversity set (1974 compounds),

[1]Biomaterials and Advanced Drug Delivery & Stanford Cardiovascular Pharmacology Division, Cardiovascular Institute, Stanford University, School of Medicine, Palo Alto, CA, USA; [2]Chemical & Systems Biology, Stanford University School of Medicine, Stanford, CA, USA and [3]Department of Developmental Sciences, Marquette University School of Dentistry, Milwaukee, WI, USA
Correspondence: Dr J Rajadas, Biomaterials and Advanced Drug Delivery, Stanford University, 1050 Arastradero Road, Building A, Room A148, Palo Alto, CA 94304, USA.
E-mail: jayraja@stanford.edu
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Mechanistic diversity set (827 compounds), Natural Products set (230 compounds) and Challenge set (53 compounds). The NCI-DTP library was obtained from High-Throughput Bioscience Center (HTBC), Stanford University. The NCI-DTP library stocks were maintained in dimethyl sulfoxide (DMSO) solutions at 10 mM compound concentrations.

Screening of NCI-DTP library was performed in 384-well plate format according to the procedure we have reported earlier.[22] The NCI-DTP library screening was performed on stationary phase Borrelial cultures grown in BSK-II medium for 7–10 days. The NCI-DTP library compounds from the 10 μM DMSO stocks were pinned into 384-well plates and then 10^6 ml^{-1} *B. burgdorferi* stationary phase cultures with BSK-II medium was added. Each compound was tested at seven different concentrations ranging from 25 to 0.45 μM in a seven-point titration (that is, 25, 12.5, 7.25, 3.625, 1.81, 0.9, 0.45 μM). Then, these 384-well culture plates were incubated at 33 °C for 96 h in a 5% CO_2 incubator. After 96 h of incubation, the BacTiter-Glo (Promega Corporation) reagent was added to the plates and

Table 1 Structure and activity of novel Top 12 hits against *B. burgdorferi*

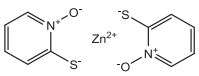
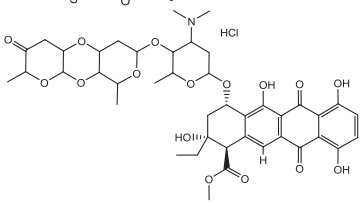
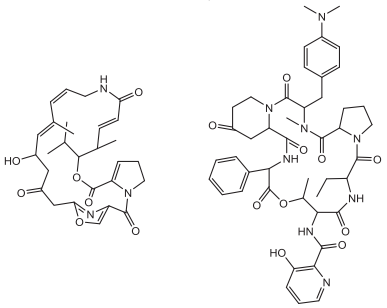
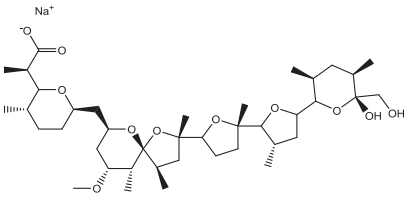
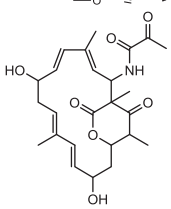
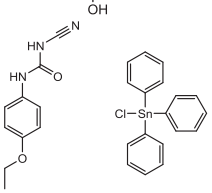
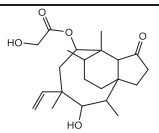
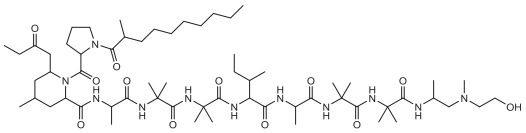
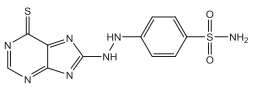
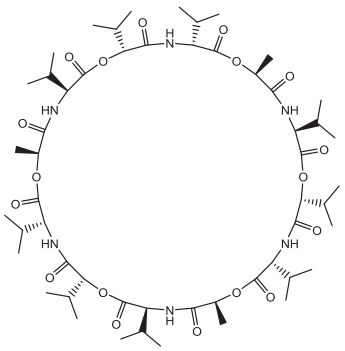
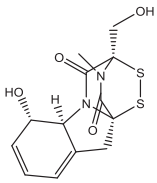
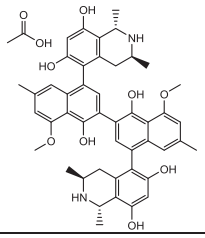
NSC#	Compound Name	Chemical Structure	% inhibition	MIC	MBC
	Control (no drug)	-	0		
	Doxycycline	-	94.14		
	Ceftriaxone sodium	-	95.26		
68093	Zinc pyrithione		97.8	0.31	5
243023	Cinerubin B Hydrochloride		98	0.31	1.25
125176	Mikamycin B		97.9	0.31	1.25
292567	Nigericin		98	0.31	15
145118	Lankacidin C		99.2	0.625	15
353527	Chloro (triphenyl) stannane; 1-cyano-3-(4-ethoxyphenyl)urea		97.1	1.25	6

Table 1 Continued.

NSC#	Compound Name	Chemical Structure	% inhibition	MIC	MBC
121145	Pleuromutilin; Drosophilin B; BC 757		95.32	1.25	>40
301460	Trichopolyn -B		95.3	20	>40
75140	4-[2-(6-sulfanylidenepurin-8-yl)hydrazinyl]benzene sulfonamide		94.61	2.5	>80
122023	Valinomycin		97.9	2.5	40
102866	Gliotoxin		97.7	2.5	20
661755	Michellamine B diacetic acid salt		97.8	3	40

luminescence was measured in relative luminescence units on a Flex Station 3 Microplate Reader (Molecular Devices LLC, Sunnyvale, CA, USA). The data was analyzed and interpreted by MDL Assay Explorer (Elsevier MDL, San Ramon, CA, USA). Compounds were designated as hits if the luciferase signal was decreased significantly in comparison with the control compounds.[22]

From the primary screening, we have identified 101 hit molecules which inhibited bacterial growth $\geqslant$85% compared with control. Out of these 101 hit molecules, 88% are FDA approved compounds. We selected top 12 molecules which were shown in Table 1, based on their ability to inhibit $>$95% of *Borrelia* growth in primary screening. This level of inhibition is higher than the currently prescribed drugs, doxycycline (94.14%) and ceftriaxone sodium (95.26%) which is shown in Table 1. We also confirmed the activity of these 12 compounds (Table 1) by a secondary screening with BacTiter-Glo (Promega Corporation) Assay. In addition, we provided data on 31 novel compounds belonging to different classes of molecules, which inhibited more than 95% of Borrelial viability (Supplementary Table S1).

After the primary screening the hits were validated again by secondary screening with BacTiter-Glo (Promega Corporation) cell viability assay.[21] The BacTiter-Glo (Promega Corporation) cell viability assay was performed as per manufacturer's instructions.[21] All the compounds tested in secondary screening were graciously provided by National Cancer Institute Development Therapeutics Program, National Institute of Health, USA. The compounds were tested in 96-well plates at different drug concentrations ranging from 250 to 0.31 μM. The efficacy of some compounds that were confirmed by secondary screening using BacTiter-Glo (Promega Corporation) Assay is shown in Figure 1. The compounds chloro-triphenyl-stannane showed *Borrelia* viability at 1.25 μM, cinerubin B at 0.31 μM and mikamycin B at 0.31 μM (Figure 1a). In Figure 1b, trichopolyn-B

shows Borrelial inhibition at 20 μM, whereas zinc pyrithione shows inhibition at 0.31 μM. The vehicle DMSO (Control) did not show any significant effect on the *Borrelia* growth. The positive control doxycycline (94.14%) showed viability at 5 μM. Except trichopolyn-B, all other drugs showed better viability than prescribed drug doxycycline. On the basis of bacterial inhibition observed at low concentrations from the secondary screening, the MIC and MBC values were determined for selected potential candidates.

After confirming the bacterial inhibition by secondary screening, MIC and MBC of the drugs were evaluated to determine the amount of drug needed to kill bacteria. The standard microdilution methods were used to study MIC and MBC of the drugs.[16,22] For the determination of MIC, 10^6 ml^{-1} of *B. burgdorferi* was cultured in BSK-II medium with different concentrations (0.3–160 μM) of test compounds for 72 h at 33 °C. The evaluated MIC and MBC for top 12 hit compounds confirmed after secondary screening were shown in Table 1. The MIC values of cinerubicin B hydrochloride, mikamycin B, zinc pyrithione and nigericin is 0.31 μM. The compound lankacidin C inhibited *Borrelia* below ⩾1 μM which are shown in Table 1. For the drugs NSC 121145, NSC 75140, chloro-triphenyl-stannane, valinomycin, gliotoxin and michellamine B the MIC values are ⩾3 μM. The MIC value of trichopolyn-B showed 20 μM which is higher compared with other drugs tested. The MBC was determined by sub-culturing 20 μl of the *Borrelia* cultures grown at different drug concentrations in fresh BSK-II medium for 21 days. The MBC was determined when no motile spirochete was observed microscopically in the subculture.[16,23] Among the selected 12 compounds, cinerubicin B hydrochloride and mikamycin B had the lowest MBC of 1.25 μM. The MBC values for, zinc pyrithione and chloro-triphenyl-stannane are ⩾6 μM. For the remaining other compounds MBC values are higher than 6 μM. The MBC value of trichopolyn-BT, NSC 121145 and NSC 75140 are very high which is more than 40 μM (Table 1).

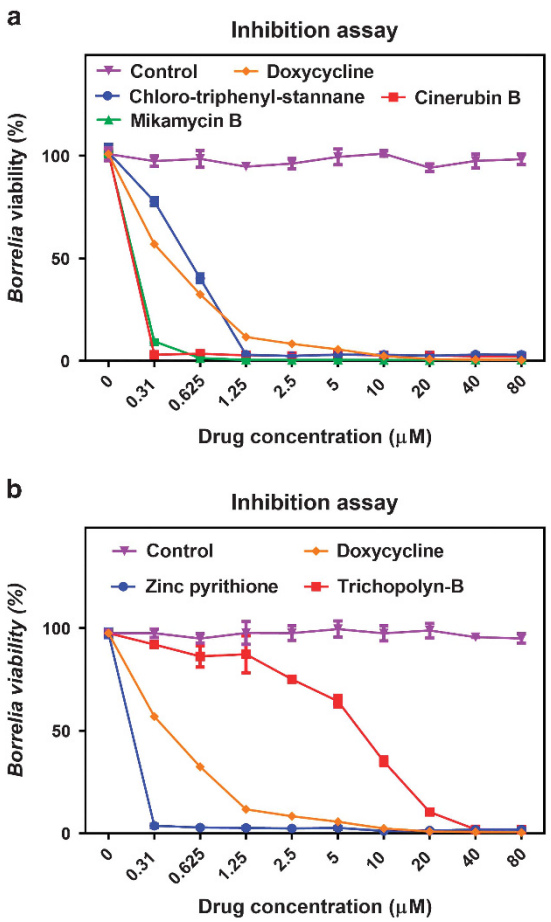


Figure 1 Bac-titer Glo Inhibition assay of drugs on CA8 strain. Effect of drugs on *Borrelia* cell viability was studied with drugs (**a**) Chloro-triphenyl-stannane, Cinerubin B and Mikamycin B. (**b**) Trichopolyn-B and Zinc pyrithione. The control has no drugs. The results represent mean ± s.d.

In this study cinerubin B and the mikamycin B are the drugs which inhibited and killed *Borrelia* at very low concentrations. The cinerubin B is an anthracycline antibiotic inhibited Borrelial growth by 98% and gave a MIC value of 0.31 μM, thereby making it one of the most potent drug molecules studied. Mikamycin B, a macrolide antibiotic, was similarly potent, inhibiting growth by 98% and having a MIC value of 0.31 μM. These drugs target the DNA replication and translation machinery, respectively.[24]

In parallel to our work, Feng *et al.*[17] have also screened a NCI compound collection containing 2526 compounds against stationary phase *B. burgdorferi*. Out of their reported 30 active hits, 11 compounds (Table 2) match with our 101 hit compounds of our primary screen. These 11 compounds in Table 2 are different from the 12 compounds (Table 1) we selected in this study. The 11 compounds (Table 2) which are already reported by *Feng et al.*[17] are NSC267229, NSC267461, NSC345647, NSC637578, NSC82151, NSC143491, NSC70845, NSC258812, NSC311153, NSC3053 and NSC136044. All these compounds showed more than 90% inhibition of *Borrelia* growth in our screening as shown in Table 2. We have screened 558 more chemical compounds compared with the study published by *Feng et al.* Of those, 381 compounds were from the Structural Diversity Set, 11 from the Mechanistic Diversity Set, 113 from the Natural Products Set and we are the first to report screening the 53 compounds from the Challenge Set. Finding the same compounds validates both screens. The 12 selected compounds in our study have not been reported previously. We identified novel compounds using our highly sensitive BacTiter-Glo (Promega Corporation) Assay.

This study identified compounds with diverse structural features and functional activity. Reports on the antimicrobial activity of these compounds against other bacteria, viruses and fungi are available which can help in determining and establishing the therapeutic

Table 2 Percentage inhibition *Borrelia* growth of our compounds identified from our screening similar to reported list of compounds earlier

NCI-DTP	*% inhibition*	*Compound Name*
—	0	Control (no drug)
—	94.14	Doxycycline
—	95.26	Ceftriaxone sodium
NSC267229	91	Pyrromycin
NSC267461	92.3	Nanomycin A
NSC345647	93.9	Chaetochromin
NSC637578	96.7	N-[3-(2-Pyridyl)isoquinolin-1-yl]-2-pyridine-carboxamidine
NSC82151	96.8	Daunorubicin hydrochloride
NSC143491	97.3	Daunomycin 3-oxime hydrochloride
NSC70845	97.8	Nogalamycin
NSC258812	98.4	Dimethyldaunomycin hydrochloride
NSC311153	98.5	9-Hydroxy-2-(2-piperidinylethyl)ellipticinium acetate
NSC3053	99.1	Actinomycin D
NSC136044	99.2	Rhodomycin A

potential of these agents. Moreover, these compounds could serve as leads for the design of new derivatives and serve to guide medicinal chemistry efforts. In addition, the scope of targets exploited by these agents is very broad. Though the exact mode of action is not known in *Borrelia,* for many of these compounds, their mechanism is well-established in other microbes. They act by targeting cellular membrane, cellular organelles, metabolic processes and genetic machinery among other means.[12,23,24] The current study, therefore, provides a means to identify novel therapeutic combinations for the treatment of the disease. Since the candidate molecules have been identified by a highly sensitive and efficient three-step screening method against both actively dividing and stationary phases of the spirochete, the identified molecules could be directly advanced for further *in vivo* analysis. In conclusion, the current report could inform the design and development of newer, improved therapies against Lyme disease.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)