Borrelia Persisters and Drugs Targeting Them

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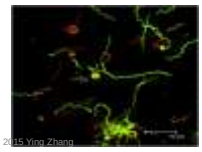


Lyme Disease: Most common vector-borne disease in US

Reported Cases of Lyme Disease - United States, 2013

Lyme disease in United States
300,000 each year

Northeast, mid-Atlantic, upper midwest, and northern California in US

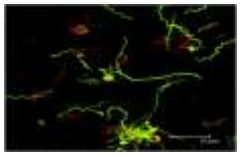
Frequent in central and eastern Europe, most of northern Asia

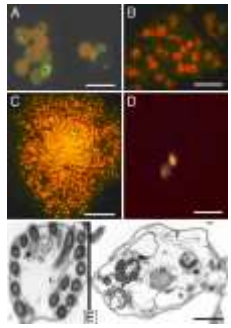
Lyme Disease: Clinical Course/Signs and Symptoms

Systemic disease caused by *B. burgdorferi*
(B. afzelii, B. garinii)

Early localized | Early disseminated | Late disseminated 1/3 | PTLDS

B. burgdorferi Morphology Changes





Øystein Brorson et al. PNAS 2009;106:18656-18661

Images of 3 day log phase (A), 7 day (B) and 10 day (C) stationary phase *B. burgdorferi* cultures

Feng J, Auwaerter PG, Zhang Y (2015) Drug Combinations against Borrelia burgdorferi Persisters In Vitro: Eradication Achieved by Using Daptomycin, Cefoperazone and Doxycycline. PLoS ONE 10(3): e0117207. doi:10.1371/journal.pone.0117207
http://127.0.0.1:8081/plosone/article?id=info:doi/10.1371/journal.pone.0117207

PLOS ONE

Lyme Diagnosis: Under vs Over Diagnosis

- History of tick exposure, in endemic region
- Clinical manifestations
- Lab supportive evidence
  - Serology (2-tier test): ELISA, Western blot
    C6 antibody test (late Lyme, not early disease)
  - Culture (not sensitive)
  - PCR

Lyme Treatment

- Antibiotic therapy: IDSA (2-4 weeks) vs ILADS (4-6 weeks)
  - Doxycycline or Amoxicillin or Cefuroxime (Ceftriaxone, iv)
  - Cure rates: 75-80%
  - Early Lyme
  - Late Lyme
  - Analogy with TB treatment

Persistence Problem in Lyme Disease

- 10-20% (25-50% Europe) Lyme patients after 2-4 week treatment have Persistent and Recurrent Symptoms-

"Post-Treatment Lyme Disease Syndrome" (PTLDS, CDC, 2/24/14) (fatigue, pain, or joint and muscle aches, "brain fog", etc.)

Author	Year	Treatment	Outcome
Klempner	2001	IV Ceftr 4 w+ Doxy 2 month vs placebo	No improvement in fatigue or quality of life
Krupp	2003	IV Ceftr 4 w vs placebo	Improvement in fatigue but not cognition
Fallon	2005	IV Ceftr 10 w vs placebo	Improvement in cognition at 12 w but not 24 w
Cameron	2005	Amoxicillin 12 w vs placebo	Improvement in cognitive and physical functioning

PTLDS: Causes unclear: (A) Host response to antigenic debris; (B) Autoimmune (host genetic factors); (C) Co-infections? (D) Persisters not killed by current antibiotics (bacterial factors)

No FDA-approved treatment for PTLDS

New York Times, 7/8/2013, Jane Brody

Evidence for persisters not killed by current antibiotics

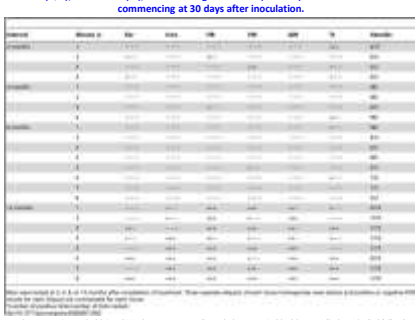
Antibiotic treatment is unable to clear persisting *Borrelia burgdorferi* in mice, dogs and monkeys, but organism **nonculturable** (Barthold S et al., 2010; AAC, 54:643-51; Hodzic et al. 2014, PLoS One 9: e86907; Embers M et al. PLoS One 2012;7:e29914):

Viable but non-culturable (VBNC)

Human study with tick xenodiagnosis showed patient after treatment still had Borrelia bacteria (A Marques et al. Clinical Infectious Diseases, 58:937-945, 2014)

CDC webinar on Lyme persistence (5/22/2014):
http://www.cdc.gov/lyme/resources/May2014_HHS_Lyme_Disease_webinar_mazarin_508.pdf

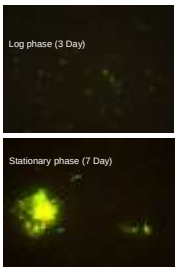
Mouse Study: Analysis of flaB DNA in ear, inoculation site (Inoc), heart base (HB), ventricular muscle (VM), quadriceps muscle (QM), tibiotarsus (Tt), and xenodiagnostic ticks (XenoDx) from mice treated with ceftriaxone commencing at 30 days after inoculation.

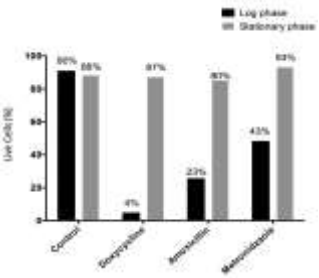


Hodzic E, Imai D, Feng S, Barthold SW (2014) Resurgence of Persisting Non-Cultivable Borrelia burgdorferi following Antibiotic Treatment in Mice. PLoS ONE 9(1): e86907. doi:10.1371/journal.pone.0086907
http://127.0.0.1:8081/plosone/article?id=info:doi/10.1371/journal.pone.0086907

PLOS ONE

Current drugs have little or poor activity for Borrelia persisters!





50 uM drugs 5 day treatment

Feng J, et al. (2014). Emerging Microbes and Infections (www.nature.com/emi), July 2, 2014. 3, e49

Bacterial Persisters

- Bacterial persisters first described by Gladys Hobby in 1942; "persister" given by Joseph Bigger in 1944
- Penicillin kills 99% bacteria, 1%, not growing, not killed by antibiotic, called "persister", still susceptible to antibiotic upon subculture
- Resistance (tolerance) in persisters is phenotypic and distinct from genetic resistance
- Persisters underlie persistent infections (TB, UTI, Lyme)

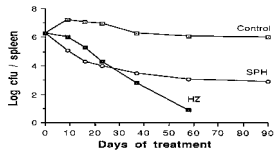
Why Persisters Matter?
Dandelion Phenomenon

Why Persister Drug Matters?

Pyrazinamide: A Remarkable Persister drug

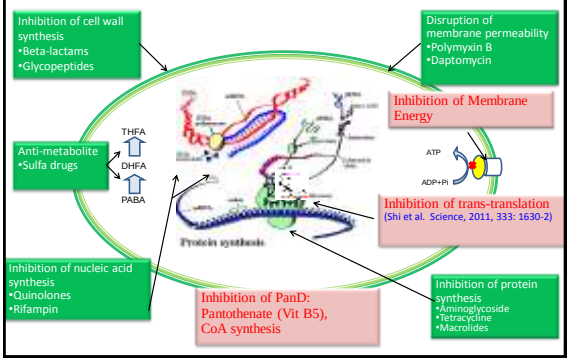
(Zhang Y and Mitchison DA, 2003, Int J Tuberc Lung Dis, 7: 6-21)

PZA - important front-line TB drug, plays a key role in DOTS by shortening therapy (INH, RIF, PZA, EMB), because PZA kills persisters not killed by other TB

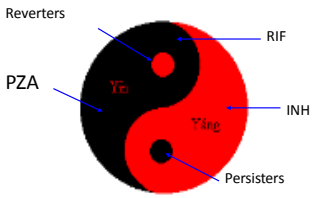


From McCune R M, et al. J Exp Med 1956; 104: 763-802.

Mechanisms of Action of Antibiotics

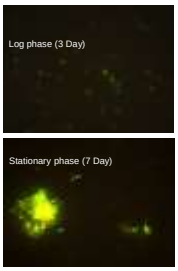


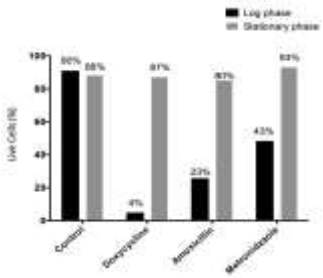
Principle of TB Treatment: Drug Combination



(Y. Zhang, Clin Pharmacol Ther. 2007; 82:595-600)

Current drugs have little or poor activity for Borrelia persisters!





50 uM drugs 5 day treatment

Feng J, et al. (2014). Emerging Microbes and Infections (www.nature.com/emi), July 2, 2014. 3, e49

Problems and Solutions

- ➢ No antibiotics effective against *B. burgdorferi* persisters
- ➢ No high-throughput viability assay of *B. burgdorferi*

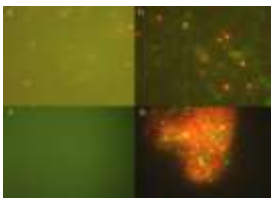
- ✓ Develop a rapid viability assay of *B. burgdorferi*
- ✓ Screen FDA drug library against *B. burgdorferi* persisters
- ✓ Test drug combinations against *B. burgdorferi* persisters

Part I. Development of High Throughput Assay SYBR Green /PI Viability Assay

Feng et al. (2014). PLoS One, November 03, 2014•DOI: 10.1371/journal.pone.0111809

	MTT	XTT	FDA	LIVE/DEAD Ratio G/R (Wash) 1	SYBR Green/PI	Microscope counting
Detection limit (100 uL)	$>10^8$	ND	10^7	5×10^5	10^5	2.5×10^4
Error	ND	ND	30%~40%	20%~30%	<10%	10%~20%
Time (for 96-well plate)	3~5 h	2.5 h	1 h	50 min	20 min	~48 h

1. BSK-H medium was removed in the washing steps prior to the LIVE/DEAD assay.

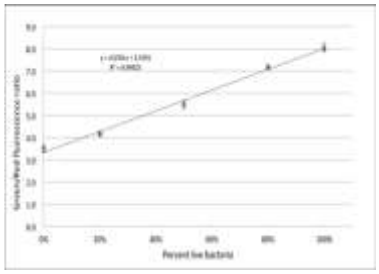


B. burgdorferi B31 strain observed with fluorescent microscopy LIVE/DEAD **BacLight stain (A)**, **SYBR Green/PI stain (B)** and **FDA stain (C)**. *B. burgdorferi* **biofilm** stained by **SYBR Green/PI (D)**.

Green cells= Live; Red cells = Dead

Feng et al. (2014). PLoS One, November 03, 2014•DOI: 10.1371/journal.pone.0111809

Relationship between percentage of live *B. burgdorferi* and Green/Red fluorescence ratio of SYBR Green/PI assay



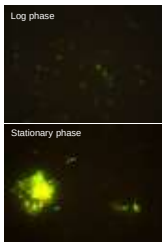
Feng et al. (2014). PLoS One, November 03, 2014•DOI: 10.1371/journal.pone.0111809

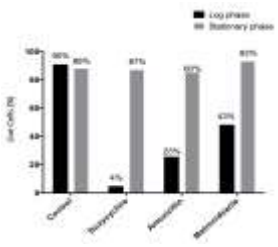
Part II. Screening FDA Drug Library for Effective Drugs against *B. burgdorferi* Persisters

Feng J, et al. (2014). Emerging Microbes and Infections (www.nature.com/emi), July 2, 2014. 3, e49

Stationary phase *B. burgdorferi* as a persister model for drug screen

Current drugs have little or no activity for Borrelia persisters!





Morphological variants of *B. burgdorferi* in stationary phase have different antibiotic susceptibilities

FDA-approved drugs active against stationary phase Borrelia (7 day old)

Drugs (50 μM)	Residual viable cells[a]	Residual viable cells[b]	Ratio of Green/Red fluoresce		
			Primary screening	Rescreening	p-value[d]
Control	93%	94%	8.67	8.59	-
Amoxicillin	76%	76%	7.98	7.82	1.000000
Doxycycline	75%	67%	7.62	7.58	0.233596
Penicillin G	75%	68%	7.41	7.92	0.699416
Tetracycline	54%	50%	7.59	7.18	0.102366
Ceftriaxone	50%	44%	6.74	6.78	0.000182
Cefuroxime	49%	43%	6.59	6.67	0.000317
Clarithromycin	70%	65%	7.70	7.59	0.038775
Azithromycin	77%	80%	8.33	7.92	0.071492
Daptomycin	35%	28%	6.10	6.09	0.000008
Clofazimine	45%	32%	6.56	6.02	0.000599
Cefoperazone	37%	34%	6.54	6.23	0.000126
Carbomycin	41%	37%	6.37	6.32	0.001045
Vancomycin	48%	38%	6.65	6.37	0.000152
Cephalothin	49%	40%	6.74	6.55	0.000133
Amodiaquin	-	45%	6.79	6.85	0.000946
Streptomycin	-	45%	6.72	6.76	0.000175
Ticarcillin	-	46%	6.82	6.93	0.000163
Cefdinir	-	48%	6.88	6.29	0.049107
Ceforanide	-	48%	6.89	6.33	0.043847
Bismuth	-	48%	6.94	6.92	0.000082
Ceftizoxime	-	49%	6.94	7.03	0.000223
Ceftibuten	51%	49%	6.81	7.27	0.004888
Amphotericin B	-	50%	7.14	6.87	0.000783
Quinine hydrobromide	-	50%	7.00	6.88	0.000124
Colistin	50%	54%	7.15	7.23	0.000319
Sulfameter	-	54%	7.13	6.98	0.009635
Tigecycline	58%	51%	6.98	6.96	0.001557

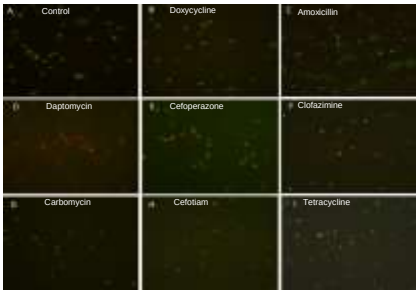
Treatment of Lyme Disease at Different Phases

None of 39 patients given tetracycline developed major late complications (meningoencephalitis, myocarditis, or recurrent attacks of arthritis) compared with 3 of 40 penicillin-treated patients and 4 of 29 given erythromycin. However, with all three antibiotics nearly half of the patients had minor late symptoms such as headache, musculoskeletal pain, and lethargy. These complications correlated with the initial severity of illness.
For early Lyme disease, Tetracycline >Penicillin>Erythromycin
Steere A et al. Ann Intern Med. 1983;99(1):22-26

For clinically active late Lyme disease, Ceftriaxone > Penicillin
Dattwyler, Volkman, Halperin, Luft, Lancet, 1988, 331: 1191–1194

For chronic Lyme, Tetracycline > Doxycycline
Donta ST. Tetracycline therapy of chronic Lyme disease. CID1997;25:52-6.

Some FDA-approved drugs active against stationary phase Borrelia persisters



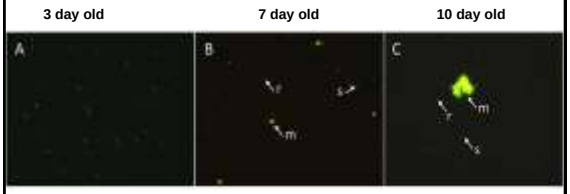
MIC values and anti-persister activity of some antibiotics for *B. burgdorferi*

Antibiotics	MIC (μg/ml)	C_{max} literature data (μg/ml)	Activity against persisters
Doxycycline	≤ 0.25	3.6-4.6	-
Amoxicillin	≤ 0.25	1.5-13.8	-
Metronidazole	25	12.5-19.4	+/-
Daptomycin	12.5-25	57.8-93.9	++++
Clofazimine	6.25	0.47-0.7	+++
Cefoperazone	≤ 0.25	111-375	+++

Part III. Drug Combinations that Eradicate *B. burgdorferi* Persisters

Feng et al. (2015). PLoS One, Mar 25;10(3):e0117207.

B. burgdorferi develops into different morphologies as culture ages



Varying degrees of susceptibility of different forms of *Bb* to commonly used Lyme antibiotics

	Percentage of different forms of *B. burgdorferi* [a]			Percentage of residual viable cells[b,c]		
	Spirochete	Round body	Microcolony	Doxycycline	Amoxicillin	Ceftriaxone
3 day log phase culture[d]	96%	4%	0%	8%	23%	6%
7 day stationary phase culture	38%	23%	39%	71%	80%	47%
10 day stationary phase culture	20%	16%	64%	80%	83%	70%

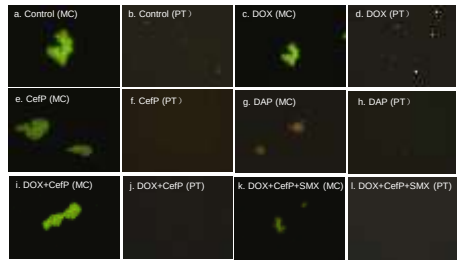
Feng et al. (2015). PLoS One, Mar 25;10(3):e0117207.

Effect of drug combinations on stationary phase *Bb* culture (10 day old-microcolonies)

Live Cell %	C	MCZ	PMB	SMX	DAP	CFZ	CAR	VAN	Nisin	CAB	OFL	CLAR	TIG	HCQ
C	87%	65%	77%	82%	52%	73%	65%	64%	81%	67%	81%	63%	67%	71%
Dox	72%	53%	64%	71%	32%	43%	59%	63%	65%	64%	60%	60%	59%	68%
AMO	75%	51%	75%	68%	41%	56%	63%	-	-	-	-	-	-	-
Ceftriaxone	68%	48%	64%	64%	41%	57%	63%	-	-	-	-	-	-	-
Cefoperazone	64%	45%	64%	65%	41%	46%	60%	64%	62%	63%	62%	63%	51%	53%
Dox+CefP	59%	39%	59%	37%	19%	43%	55%	50%	56%	59%	56%	55%	51%	48%
DAP	48%	37%	35%	**27%**	35%	**20%**	**26%**	**23%**	30%	**20%**	**27%**	32%	**23%**	31%
DAP+Dox	34%	34%	33%	**24%**	32%	**20%**	**23%**	**25%**	**24%**	**16%**	**20%**	31%	**21%**	**25%**

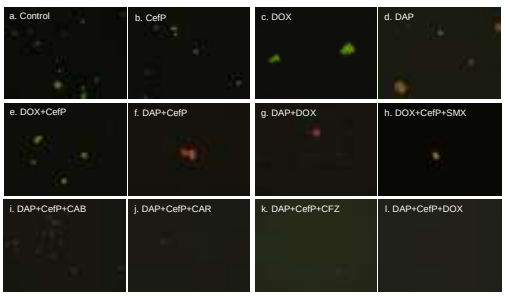
- The most effective drug combinations (10 μg/ml, 7 days) reflected by residual viable cell <30% are shown in bold type.
- The best drug combinations without daptomycin are underlined.
- Abbreviations: Dox, doxycycline; AMO, amoxicillin; CefP, cefoperazone; CFZ, clofazimine; MCZ, miconazole; B; DAP, daptomycin; SMX, sulfamethoxazole; CAB, carbenicillin; CAR, carbomycin; VAN, vancomycin; OFL, ofloxacin; CLAR, clarithromycin; TIG, tigecycline; HCQ, hydroxychloroquine; RIF, rifampin; "-" indicates not determined; and C, control.

Aggregated forms are more resistant than planktonic forms



B. burgdorferi (10 day old) aggregated **micro-colony (MC)** form was more resistant to different antibiotics or their combinations than **planktonic form (round body and spirochetal form) (PT)** as observed by fluorescence microscopy at 400 × magnification. Abbreviation: Dox, doxycycline; CefP, cefoperazone; DAP, daptomycin; SMX, sulfamethoxazole.

Effect of drug combinations on aggregated form of *B. burgdorferi*



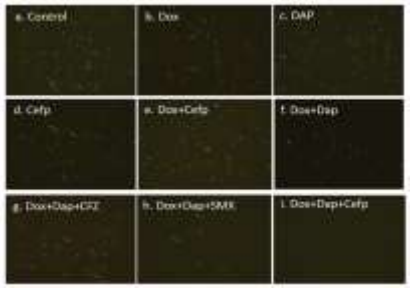
Susceptibility of *B. burgdorferi* microcolony form to antibiotics and antibiotic combinations was assessed by fluorescence microscopy at 200 × magnification. Abbreviation: Dox, doxycycline; CefP, cefoperazone; CFZ, clofazimine; MCZ, miconazole; DAP, daptomycin; SMX, sulfamethoxazole; CAB, carbenicillin; CAR, carbomycin.

Subculture to assess viability of drug-treated stationary phase *B. burgdorferi*

Drugs[a]	Residual viable cells[c]	After 7 day subculture		After 15 day subculture	
		G/R ratio[d]	Spirochetes[e]	G/R ratio[d]	Spirochetes[e]
Control	82%	7.28	5×10^6	7.38	6×10^6
Dox	67%	6.23	9×10^5	6.89	4×10^6
Amoxicillin	80%	6.32	1×10^6	7.23	6×10^6
Dox+Dap+CefP	10%	4.93	ND	4.82	ND
Dox+Dap+CFZ	15%	4.86	ND	7.38	6×10^6
Dox+Dap+SMX	18%	5.34	ND	6.41	2×10^6
Dox+Dap	23%	5.91	3×10^5	7.31	6×10^6
Dox+CefP	56%	6.01	7.5×10^5	6.87	4×10^6
Dap	55%	7.01	3.1×10^6	7.11	5×10^6
CefP	61%	6.13	1.5×10^6	6.95	5×10^6

- 7 day old *B. burgdorferi* culture (1×10^7 spirochetes/ml) (500 μl) was treated with 10 μg/ml drugs alone or drug combinations for 7 days, and subcultured in BSK-H medium for 7 days and 15 days.
- Abbreviations: G/R ratio, Green/Red fluorescence ratio; Dox, doxycycline; CefP, cefoperazone; CFZ, clofazimine; Dap, daptomycin; SMX, sulfamethoxazole.

Subculture to assess viability of drug-treated stationary phase *B. burgdorferi*



Subculture (15 days) of 10 day old *B. burgdorferi* stationary phase culture treated with different antibiotics alone or in combinations (10 μg/ml).

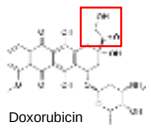
IV. New screens identifies anthracycline antibiotics as having high activity against *B. burgdorferi*

NCI compound library collection consisting of 2526 compounds against stationary phase *B. burgdorferi* persisters.

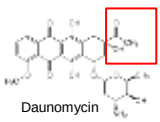
Top 30 new active hits include: anthracycline antibiotics, prodigiosin, mitomycin, nanaomycin and dactinomycin

Feng et al. (2015). Emerging Microbes and Infections, June 3, 2015
http://www.nature.com/emi/journal/v4/n6/full/emi201531a.html

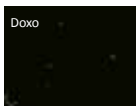
Comparison of activity of Daunomycin and Doxorubicin



Doxorubicin



Daunomycin



Cell pellet
Drug=5 ug/ml

Mitomycin C

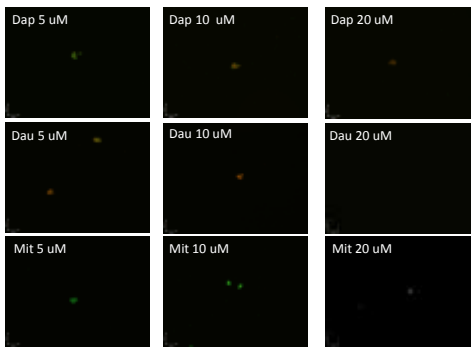
MIC values and activities of new drugs against stationary phase *B. burgdorferi*

Antibiotics	MIC (μg/mL)	Activity against stationary phase *B. burgdorferi* (% viable cells)
Doxycycline	≤ 0.25	77%
Amoxicillin	≤ 0.25	77%
Daptomycin	12.5-25	18%
Daunomycin 3-oxime	≤ 0.36	6%
Daunorubicin	≤ 0.36	10%
Mitomycin C	≤ 0.21	25%

Daunomycin>Daptomycin>Mitomycin C

Feng et al. (2015). Emerging Microbes and Infections, June 3, 2015

Comparison of Daunomycin, Daptomycin, and Mit C



- Using 7 day stationary culture; Treated for 7 day.

Subculture to assess the viability of drug treated Bb

	Live %	Subculture 7 days	Subculture 21 days
Control	79%	6×10^6	2×10^7
Dox+Cefu	67%	1×10^6	1×10^7
Dox+Cefp	67%	9×10^5	1×10^7
Dox+Cefu+Dap	30%	0	0
Dox+CefP+Dap	29%	0	0
Dox+Cefu+MitC	45%	0	1×10^6
Dox+Cefu+Dau	12%	0	0

- 15 day old stationary phase culture
- Low drug concentration 10 ug/ml
- Treated for 7 day

Results of additional 113 active hits from FDA drug library that have higher activity against stationary phase *Bb* than Lyme antibiotics

Many antimicrobial agents (antibiotics, antivirals, antifungals, anthelmintics or antiparasitics) had better activity than the current Lyme antibiotics. These include antibacterials such as rifamycins, thiostrepton, quinolone drugs (sarafloxacin, clinafloxacin, tosufloxacin), and cell wall inhibitors carbenicillin, tazobactam; antifungal agents such as fluconazole, mepartricin, bifonazole, climbazole, oxiconazole, nystatin; antiviral agents zanamivir, nevirapine, tilorone; antimalarial agents artemisinin, methylene blue, and quidaldine blue; antihelmintic and antiparasitic agents toltrazuril, antimonyl tartrate trihydrate, oxantel, closantel, hycanthone, pyrimethamine, and tetramisole.

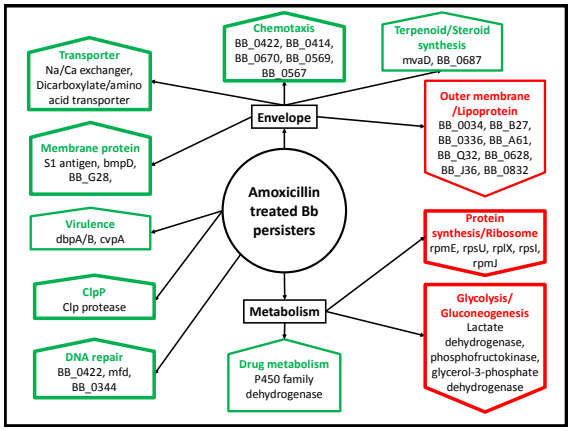
Drugs used for treating other non-infectious conditions including verteporfin, oltipraz, pyroglutamic acid, pidolic acid, and dextrorphan tartrate, that act on glutathione/γ-glutamyl pathway involved in protection against free radical damage, and also antidepressant drug indatraline

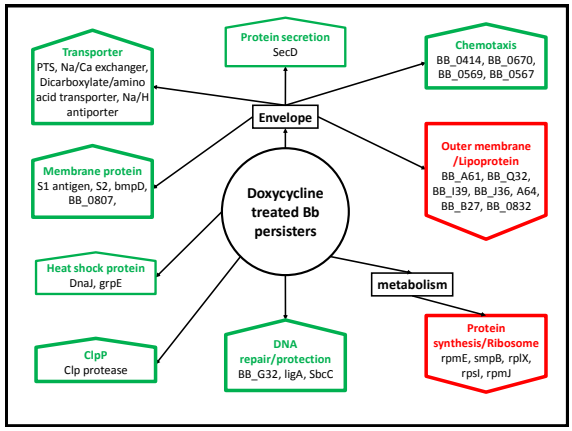
***To be published in MDPI Antibiotics* — Open Access Journal**

Persister Mechanisms in *B. burgdorferi*

Jie Feng, Wanliang Shi, Shuo Zhang and Ying Zhang,
Emerging Microbes & Infections (2015) **4**, e51; doi:10.1038/emi.2015.51
Published online 19 August 2015

- RNA-seq to identify genes preferentially expressed in *Bb* persisters
- Serve as antigens for diagnostic tests for chronic Lyme disease





Summary

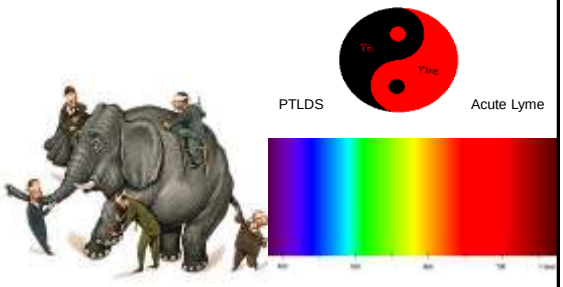
- Borrelia develop persisters (different forms, spirochetal form, round bodies, microcolonies) that are not killed by current Lyme antibiotics
- Screened FDA and NCI drug libraries and identified drug candidates active against Bb persisters
- Drug combos are able to eradicate Bb persisters, even the most resistant forms (microcolonies)
- Persister genes/mechanisms

Ongoing Studies/Future Plans

1. Identify optimal drug combinations against different forms of *Bb* persisters in vitro (oral regimens)
2. Evaluate effective drugs/drug combinations in animal models (Why important? IDSA vs ILADS)
3. Conduct new clinical studies to evaluate drug combos:

TB treatment as a model --- COMBO (Yin and Yang) for more effective treatment of persistent Lyme?

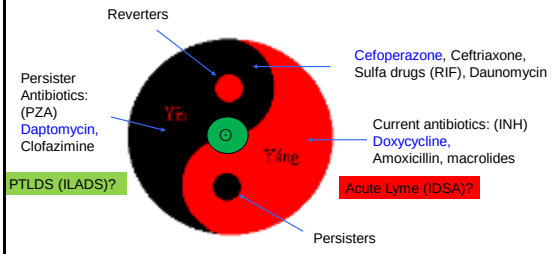
V. Yin-Yang Model of Lyme Disease: Complex, Diagnosis, Treatment



A Unified Yin-Yang Model?

Where Growing Bacteria/Acute Lyme and Persisters/PTLDS meet and Interconvert: Dynamic Continuum

Zhang Y. Emerging Microbes & Infections (2014) **3**, e3; doi:10.1038/emi.2014.3



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Fisher Center

Mechanisms of drugs active against Borrelia persisters

1. Disrupt cell membrane/energy: Daptomycin disrupt cell membrane; Clofazimine
2. Damage DNA: Daunomycin/Doxorubicin, Mitomycin, Clofazimine
3. Inhibit cell wall: Cephalosporins show greater activity than penicillins. Cefoperazone (3rd generation) seems best b-lactam antibiotic against stationary phase *B. burgdorferi*
4. Block DNA synthesis: Sulfa drugs, quinolones active against stationary phase *B. burgdorferi*
5. Block protein synthesis: Macrolides (carbomycin) have higher activity than penicillin or Doxy for *B. burgdorferi* persisters.