Supplementary Information

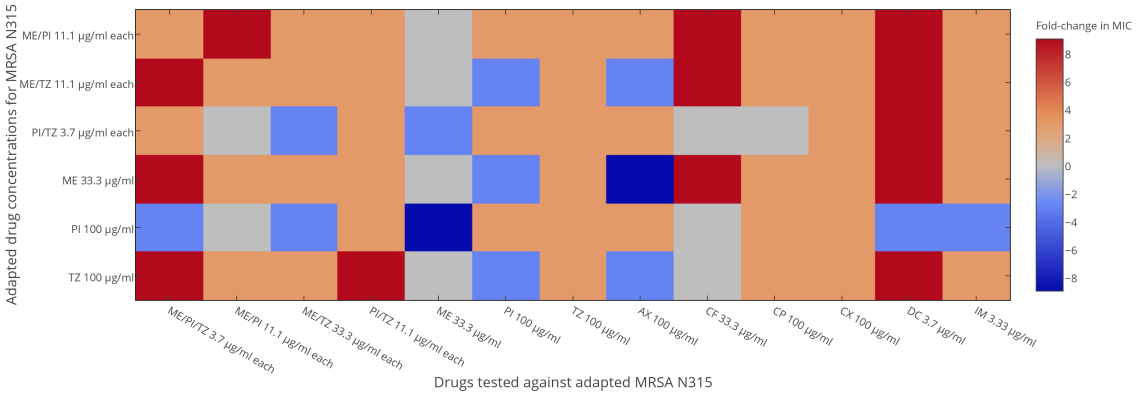
Synergistic, collaterally sensitive β-lactam combinations suppress resistance in MRSA

Patrick R. Gonzales[1], Mitchell W. Pesesky[1], Renee Bouley[2], Anna Ballard[1], Brent A. Biddy[1], Mark A. Suckow[3], William R. Wolter[3], Valerie A. Schroeder[3], Carey-Ann D. Burnham[4,5], Shahriar Mobashery[2], Mayland Chang[2], Gautam Dantas[1,4,6*]

[1]Center for Genome Sciences & Systems Biology, Washington University School of Medicine, St. Louis, Missouri 63108, USA. [2]Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556, USA. [3]Freimann Life Sciences Center and Department of Biological Sciences, University of Notre Dame, Notre Dame, Indiana 46556, USA. [4]Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110, USA. [5]Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri 63110, USA. [6]Department of Biomedical Engineering, Washington University in St. Louis, St. Louis, Missouri 63130, USA. *e-mail: dantas@wustl.edu

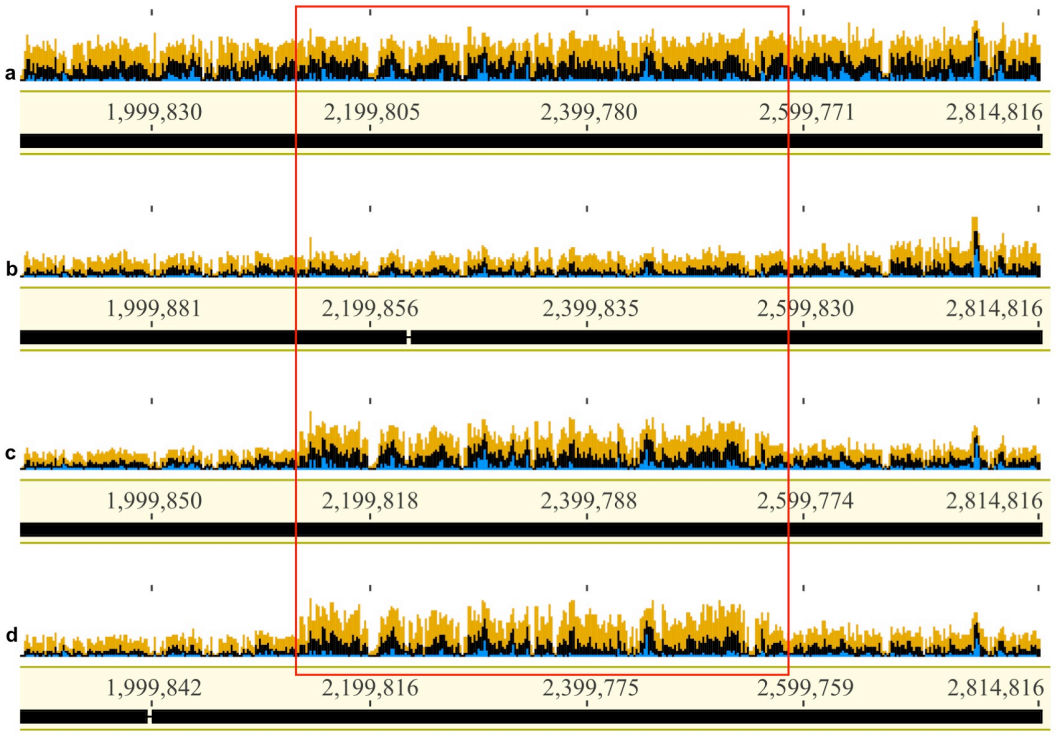
This file includes Supplementary Results (Supplementary Figures/Legends 1 − 3, Supplementary Tables 1 − 7).

Supplementary Results

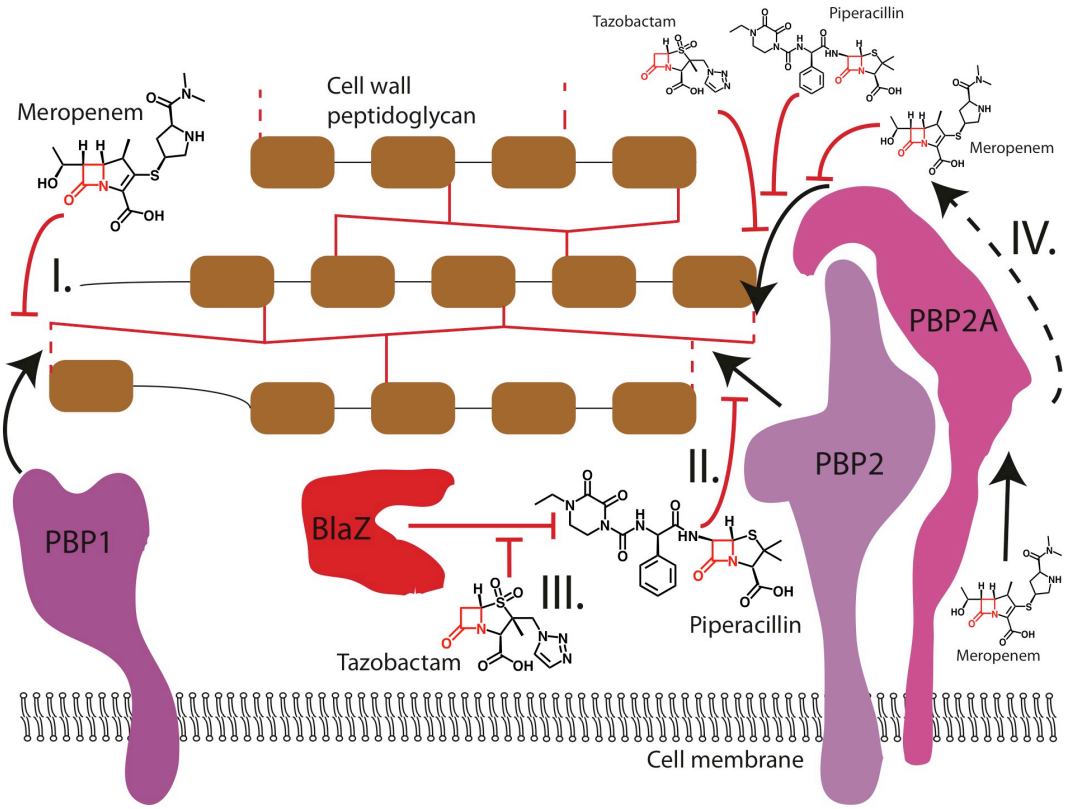


Supplementary Figure 1. Collateral sensitivities underlie suppression of adaptation to β-lactam combinations in MRSA N315. MRSA N315 adapted to single β-lactam drugs, or drugs in combinations after 11-day passage, with the concentrations of drug adaptation, is listed along the Y-axis. Subsequent screening of the adapted MRSA N315 strains against β-lactam combinations or single drugs is shown along the X-axis, along with the wild-type MIC of the drug(s) against MRSA N315. Blue shades indicate collateral sensitization of a MRSA N315 strain to single drugs and/or combinations, after prior adaptation to single and/or double drug combinations. Red shades indicate collateral resistance. Grey shading indicates no change in MIC. Darker shading indicates increased fold-change for the MIC of adapted MRSA N315 to the drug(s) tested after adaptation. For example, adaptation of MRSA N315 to piperacillin (PI) after growth in CAMHB+PI 100 µg/ml yields collateral sensitivity to meropenem (ME), as shown by a 9-fold drop in MIC of ME. Adaptation of MRSA N315 to CAMHB+ME 33.3 µg/ml results in a 3-fold drop in MIC of PI, indicative of reciprocal collateral sensitivity of MRSA N315 to these two β-lactam drugs. MIC values for ME/PI/TZ against MRSA N315 from each compound or combination in column 1 is reflected in Supplementary Table 6, Row 11.

ME = meropenem, PI = piperacillin, TZ = tazobactam, AX = amoxicillin, CF = cefdinir, CP = cefepime, CX = cefoxitin, DC = dicloxacillin, IM = imipenem.



Supplementary Figure 2. Genomic duplication in MRSA N315 adapted to piperacillin/tazobactam. Histogram showing the total read coverage across the genome of N315 adapted to **a**, meropenem/tazobactam for five days, **b**, tazobactam alone for two days, **c**, piperacillin/tazobactam for six days, and **d**, piperacillin/tazobactam for 11 days. Average per-base read coverage across the entire genome and only in the region indicated by the red box are, respectively: a) 116.6 reads/bp and 126.6 reads/bp; b) 124.5 reads/bp and 128.9 reads/bp; c) 157.8 reads/bp and 302.2 reads/bp; and d) 120.1 reads/bp and 230.6 reads/bp. Clones in **a** and **b** were chosen to be representative of all non-piperacillin/tazobactam adaptations.



Supplementary Figure 3. Proposed mechanism of synergy of meropenem/piperacillin/tazobactam (ME/PI/TZ) against MRSA. Our data support the proposed synergistic mode of action against cell-wall synthesis in MRSA involving: I.) suppression of transpeptidation by PBP1 at the division septum by carbapenems, II.) suppression of transpeptidation by PBP2 by penams (penicillins), III.) suppression of β-lactamase activity against penams by β-lactamase inhibitors, and IV.) allosteric opening of the active site of PBP2a by meropenem, allowing inhibition by meropenem or by other β-lactams.

Supplementary Table 1. 23 antibacterial compounds used to formulate combinations in this study. Compounds are grouped by target mechanism of action. *Compound not formally classified as an antibiotic drug, but has known antibacterial properties.

Compound	Target mechanism in bacteria	Antibiotic Class	MIC in MRSA N315 (µg/ml)
Sulfamethoxazole	folic acid pathway	Sulfonamide	100
Trimethoprim	folic acid pathway	Pyrimidine derivative	6.2
Levofloxacin	DNA synthesis	Fluoroquinolone	0.4
Bleomycin	DNA synthesis	Glycopeptide	>500
Gemfibrozil	lipid synthesis	*Fibrate (hyperlipidemia agent)	>200
Sulfometuron	amino acid biosynthesis	*Broad-spectrum urea herbicide	>200
Disulfiram	osmotic stress response	*Thiuram disulfide (anti-alcohol therapeutic)	11.1
Tigecycline	protein synthesis	Tetracycline	0.4
Mupirocin	protein synthesis	Pseudomonic acid	0.4
Linezolid	protein synthesis	Oxazolidinone	3.7
Azithromycin	protein synthesis	Macrolide	>200
Clindamycin	protein synthesis	Lincosamide	>500
Chloramphenicol	protein synthesis	Amphenicol	11.1
Tobramycin	protein synthesis	Aminoglycoside	>500
Rifampin	transcription	Rifamycin	0.4
Vancomycin	cell wall synthesis	Glycopeptide	0.4
Piperacillin	cell wall synthesis	β-lactam/Penicillin (Penam)/Broad-spectrum	64
Aztreonam	cell wall synthesis	β-lactam/Monobactam/Gram-negative specific	>500
Cefepime	cell wall synthesis	β-lactam/Cephalosporin 4th generation (Cephem)/Broad-spectrum	100
Meropenem	cell wall synthesis	β-lactam/Carbapenem/Ultra-broad-spectrum	16
Tazobactam	cell wall synthesis	β-lactamase inhibitor (Penam)	128
D-Cycloserine	cell wall synthesis	Analogue of the amino acid D-alanine	56
Colistin	cell membrane lysis	Polymyxin	500

Supplementary Table 2. Fractional Inhibitory Concentration Index (FICI) profiling of combinations. a, Interpretive criteria for FICI scoring. **b**, FICI profiles of various triple combinations of carbapenems/penicillins/β-lactamase inhibitors against MRSA and MSSA strains. **c,** MIC profiles of same combinations (μg/ml). Constituent double combinations are shown for comparison.

a

FICI	Interpretation
≤0.5	Synergy
>0.5 to <1.0	Partial synergy
1.0	Additivity
>1.0 to <4.0	Indifference
≥4.0	Antagonism

b

Combination in strain	ME/PI/TZ	CP/PI/TZ	AZ/PI/TZ	ME/AX/TZ	ME/AX/CV	IM/PI/CV	ME/PI	ME/TZ	PI/TZ	IM/PI	IM/CV	PI/CV
MRSA N315 SCC*mec* type II	0.11	0.33	0.33	0.04	0.41	0.06	0.44	0.67	0.22	0.15	0.67	0.44
MRSA #181 SCC*mec* type II	0.28	ND	ND	0.55	ND	0.11	ND	ND	ND	ND	ND	ND
MSSA ATCC 29213	1.12	ND	ND	ND	ND	1.14	2.97	8.61	0.36	1.11	1.04	0.43

c

Combination in strain	ME/PI/TZ	CP/PI/TZ	AZ/PI/TZ	ME/AX/TZ	ME/AX/CV	IM/PI/CV	ME/PI	ME/TZ	PI/TZ	IM/PI	IM/CV	PI/CV
MRSA N315 SCC*mec* type II	2 each	11.1 each	11.1 each	0.4 each	3.7 each	0.12/1.2/1.2	2/4	8/2	16/2	0.37/3.7	1.11/11.1	11.1 each
MRSA #181 SCC*mec* type II	11.1 each	ND	ND	11.1 each	ND	0.37/3.7/3.7	ND	ND	ND	ND	ND	ND
MSSA ATCC 29213	0.27 each	ND	ND	ND	ND	0.04/0.4/0.4	0.4 each	1.2 each	1.2 each	0.04/0.4	0.04/0.4	1.2 each

Supplementary Table 3. Compiled FICI data for ME/PI/TZ against MRSA N315 and 72 clinical MRSA isolates. a, **b**, 72 clinical MRSA isolates (with SCC*mec* type, if known) and FICI scores for ME/PI/TZ against 72 clinical MRSA isolates.

a

Clinical MRSA isolate (SCC*mec* type)	FICI score
4	0.22
7	0.22
13	0.15
15	0.5
22	0.22
25	0.22
27	0.67
31	0.15
35 (II)	0.17
37	0.15
39 (II)	0.67
41 (II)	0.09
45	0.22
48	0.15
53	0.22
59	0.07
64 (II)	0.44
66	0.22
70	0.67
72	0.15
73 (IV)	0.34
74	0.34
75	0.34
77 (II)	0.67
85	0.5
89	0.07
90	0.22
95	0.22
99	0.44
101	0.22
103	0.37
104 (II)	0.15
109	0.17
118	0.07
121	0.22
122	0.22

b

Clinical MRSA isolate (SCC*mec* type) (Continued)	FICI score (Continued)
124	0.28
131 (II)	0.37
132	0.22
140	0.22
144	0.15
146	0.22
150	0.67
152	0.22
155	0.39
161	0.37
163	0.37
164	0.17
165	0.15
167	0.22
168	0.22
169	0.17
171	0.17
172	0.67
175	0.15
177	0.22
181 (II)	0.28
182	0.5
189	0.15
190	0.34
193 (II)	0.34
194	0.15
195	0.15
197	0.15
200	0.15
201	0.44
204	0.39
205	0.22
206	0.22
213	0.44
217	0.15
219	0.15

Supplementary Table 4. Compiled MIC and MBC data for ME/PI/TZ against MRSA isolates. a, Distribution of MIC resistance profiles of studied MRSA isolates against ME/PI/TZ. **b,** Confirmation of minimum bactericidal concentration (MBC) for ME/PI/TZ in MRSA N315. MRSA N315 was grown overnight at 37 °C in CAMHB media + indicated concentrations of ME/PI/TZ per well, in triplicate. * indicates liquid MIC of MRSA N315. 100 µl aliquots of 1:100 dilutions of 50 µl MRSA N315 culture from wells containing ME/PI/TZ at indicated concentrations were plated in duplicate onto MHA plates and incubated for 24 h. No colony growth at or two dilutions above the MIC confirmed bactericidal activity.

a

MIC of ME/PI/TZ components (µg/ml)	# of MRSA isolates	% of total
33.3/33.3/33.3	9	12.3
11.1/11.1/11.1	27	36.9
3.7/3.7/3.7	27	36.9
1.2/1.2/1.2	8	10.9
0.4/0.4/0.4	2	2.7
Total	73	–

b

Plate Concentrations	Colonies Plate A	Colonies Plate B
ME/PI/TZ 2/2/2	Punctate lawn	Too Many to Count
ME/PI/TZ 4/4/4*	40	0
ME/PI/TZ 8/8/8	2	0
ME/PI/TZ 16/16/16	0	0
ME/PI/TZ 32/32/32	8	0

*MIC = 4/4/4 ng/µl

Supplementary Table 5. Xylose induction of hypersusceptibility to β-lactams in MRSA COL antisense (AS) strains confirms mechanism of action against critical PBPs. Targeted repression of critical PBPs in a MRSA COL background showed increased susceptibility for β-lactams in the ME/PI/TZ combination when under xylose induction, resulting in differential zones of inhibition (ZOI) size (+++, more than twofold increase in zone diameter with xylose induction. ++, twofold increase in zone diameter. +, less than twofold increase. −, no change in zone diameter.). TZ showed no inhibition of any growth on any plate ± xylose. Targeted repression of PBP2a showed increased susceptibility for meropenem, piperacillin, and all combinations. Targeted repression of PBP1 showed increased susceptibility to meropenem, piperacillin, and for the ME/PI, ME/TZ, and ME/PI/TZ combinations. Targeted repression of PBP2 showed increased susceptibility to meropenem, piperacillin, and for all combinations. Targeted repression of PBP3 showed a slight increase in susceptibility for the ME/PI combination; no change in susceptibility was observed for any of the single drugs, or other combinations.

Antibiotic	Antisense RNA ± xylose			
	PBP2a	PBP1	PBP2	PBP3
ME	+++	+	++	−
PI	+++	+	+++	−
ME/PI	+++	+++	+++	+
ME/TZ	+++	+++	+++	−
PI/TZ	+++	−	+++	−
ME/PI/TZ	+++	+++	+++	−

Supplementary Table 6. Change in ME/PI/TZ resistance phenotype of MRSA N315 over 11 days after repeated exposure to constituents of ME/PI/TZ. a, Wild-type MIC of ME/PI/TZ against MRSA N315 is 3.7 μg/ml each. Isolates were selected on days when an increase in MIC or growth rate was noted. Antibacterial concentrations listed (in μg/ml) show the adaptation conditions for MRSA N315. Post-adaptation MICs to each component of ME/PI/TZ are shown in selected isolates versus passage day. Outlined isolates from passage-day 11 were used in the adaptation assay shown in Fig. S1. **b**, FICI of MRSA N315 against ME/PI/TZ after adaptation to components *in vitro*.

a

Passage day /Adaptation conditions	ME/PI 11.1 μg/ml each	ME/TZ 11.1 μg/ml each	PI/TZ 3.7 μg/ml each	Meropenem 33.3 μg/ml	Piperacillin 100 μg/ml	Piperacillin 33.3μg/ml	Tazobactam 100 μg/ml
1	no change	11.1/11.1/11.1	1.2/1.2/1.2	11.1/11.1/11.1	no change	1.2/1.2/1.2	11.1/11.1/11.1
2	no change	no change	no change	no change	3.7/3.7/3.7	3.7/3.7/3.7	11.1/11.1/11.1
3	no change	no change	no change	no change	no change	no change	no change
4	no change	no change	no change	no change	no change	no change	no change
5	no change	33.3/33.3/33.3	no change	no change	no change	no change	no change
6	no change	no change	11.1/11.1/11.1	no change	no change	no change	no change
7	11.1/11.1/11.1	no change	no change	33.3/33.3/33.3	no change	no change	11.1/11.1/11.1
8	no change	no change	no change	no change	1.2/1.2/1.2	1.2/1.2/1.2	no change
9	no change	no change	no change	no change	no change	no change	no change
10	no change	no change	no change	no change	no change	no change	no change
11	11.1/11.1/11.1	33.3/33.3/33.3	11.1/11.1/11.1	33.3/33.3/33.3	1.2/1.2/1.2	1.2/1.2/1.2	33.3/33.3/33.3

b

MRSA N315 isolate adapted to:	ME/PI	ME/TZ	PI/TZ	ME	PI	TZ
$FICI_{ME/PI/TZ}$	0.22	0.83	0.22	0.83	0.05	0.83

Supplementary Table 7. a, Statistics of *in vivo* treatments with β-lactams. **b**, *in vitro* MICs and FICI scores for MRSA N315 after passage *in vivo* under indicated drug conditions.

a

Drug condition tested	p-value versus vehicle	After multiple hypothesis correction (Bonferroni)
ME	0.606	4.848 (1)
PI	1	8 (1)
TZ	1	8 (1)
ME/PI	0.0022	0.0176
ME/TZ	0.0152	0.1216
PI/TZ	0.606	4.848 (1)
ME/PI/TZ	0.0022	0.0176
Linezolid	0.0022	0.0176

b

Colonies from mice given: / MIC (μg/ml) for:	ME	PI	Vehicle	Wild-type N315
ME/PI/TZ	3.7/3.7/3.7	3.7/3.7/3.7	3.7/3.7/3.7	3.7/3.7/3.7
ME	33.3	33.3	33.3	33.3
PI	33.3	33.3	33.3	33.3
TZ	100	100	100	100
FICI	0.26	0.26	0.26	0.26

Supplementary Dataset

***In vivo* data for ME/PI/TZ, constituent compounds, and controls in neutropenic mice.** This supplementary dataset comprises the *in vivo* testing of ME/PI/TZ, its constituent compounds, vehicle (−), and linezolid (+) controls, against neutropenic mice, over the 48 h time course of drug administration. Also included are final results of drug treatments in the replicate mice (n = 6 per treatment).