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

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most prevalent multidrug-resistant pathogens worldwide, exhibiting increasing resistance to the latest antibiotic therapies. Here we show that the triple β-lactam combination meropenem-piperacillin-tazobactam (ME/PI/TZ) acts synergistically and is bactericidal against MRSA subspecies N315 and 72 other clinical MRSA isolates *in vitro* and clears MRSA N315 infection in a mouse model. ME/PI/TZ suppresses evolution of resistance in MRSA via reciprocal collateral sensitivity of its constituents. We demonstrate that these activities also extend to other carbapenem-penicillin–β-lactamase inhibitor combinations. ME/PI/TZ circumvents the tight regulation of the *mec* and *bla* operons in MRSA, the basis for inducible resistance to β-lactam antibiotics. Furthermore, ME/PI/TZ subverts the function of penicillin-binding protein-2a (PBP2a) via allostery, which we propose as the mechanism for both synergy and collateral sensitivity. Showing *in vivo* activity similar to that of linezolid, ME/PI/TZ demonstrates that combinations of older β-lactam antibiotics could be effective against MRSA infections in humans.

Multidrug-resistant (MDR) pathogens are a growing threat to human health, with many infectious diseases effectively regressing to the pre-antibiotic era[1,2], as exemplified by the dramatic rise of community-acquired MRSA infections. In the 1940s, *S. aureus* infections were treated primarily with first-generation β-lactams (penicillins), which target penicillin-binding proteins (PBPs), critical transpeptidases for cell-wall synthesis[3]. PBP1, PBP2, PBP3 and PBP4 perform these functions in *S. aureus*[3]. Emergence of β-lactamase–producing strains led to the development of β-lactamase–resistant second-generation penicillins, including methicillin. Soon after the introduction of methicillin, in 1959, the first MRSA strains were reported[4]. These strains acquired a highly regulated collection of genes from a non–*S. aureus* source that produced inducible resistance to β-lactam antibiotics[3]. One of these genes, *mecA*, encodes PBP2a. PBP2a performs the critical transpeptidase reaction that crosslinks the cell wall, even under challenge by β-lactam antibiotics, when other PBPs are inhibited[5,6]. The mechanistic basis for this outcome is complex and involves a closed conformation for the active site, whose function is regulated by allostery[7,8]. The emergence of MRSA has virtually eliminated the use of β-lactams as therapeutic options against *S. aureus*. The recently developed β-lactam agent ceftaroline, which shows activity in treatment of MRSA infections, does so by binding the allosteric site of PBP2a, which triggers opening of the active site for inactivation by the drug[8,9]; however, resistance to ceftaroline[10] and other antibiotics used to treat MRSA, including linezolid, vancomycin and daptomycin, has been reported[11–13].

Use of multidrug combination therapy targeting orthogonal cellular processes has been successful in treating *Mycobacterium tuberculosis*, *Helicobacter pylori* and other infections[14,15]. However, resistance is increasing against these therapies[16,17]. We have identified a new potential therapy against MRSA consisting of a combination of clinically approved drugs from three distinct generations and subclasses of β-lactam compounds, all targeting cell-wall synthesis: meropenem, piperacillin and tazobactam (ME/PI/TZ). This therapy uses elements from three strategies: (i) the use of semisynthetic antibiotic derivatives that target multiple nodes in the same cellular system[10,18], (ii) combining antibiotics to increase drug potency through synergy[19,20] and (iii) use of collateral sensitivity between constituents of the combination to suppress resistance evolution[21,22]. Each of these methods has been successfully employed against the major MDR Gram-negative and Gram-positive human pathogens[23,24]. However, used individually, these strategies have often been thwarted by the evolution of new resistance in MDR pathogens, leading to diminishing options for treating their infections[4,12,18].

We hypothesize that ME/PI/TZ operates through inhibition of PBP1 by meropenem, targeting of PBP2 by piperacillin, protection of piperacillin from the PC1 class A β-lactamases by tazobactam[5,25–28] and allosteric opening of the active site of PBP2a by meropenem for inhibition by another molecule of meropenem or piperacillin[9]. This culminates in a synergistic response by simultaneous perturbation of multiple components of the cell-wall synthesis machinery in MRSA. We find that exposure of MRSA N315 to the components of ME/PI/TZ reveals reciprocal collateral sensitivities within this highly synergistic triple combination that suppress the evolution of resistance, in contrast to some synergistic combination therapies that instead accelerate it[20,29]. This effect is consistent with recent work showing that collateral sensitivity slows evolution of resistance in a nonpathogenic laboratory strain of *Escherichia coli*[21,30]. Our results support renewed clinical use of older β-lactam antibiotics against MRSA when used in judiciously conceived synergistic combinations of collaterally sensitive components, opening a new treatment paradigm with existing drugs that are already approved for human use.

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RESULTS

Three β-lactams synergistically kill MRSA *in vitro*

On the basis of its high level of resistance against 23 diverse antibiotics (**Supplementary Results** and **Supplementary Table 1**), we selected MRSA N315 (ref. 31) from a group of fully genome-sequenced MDR strains of MRSA for this study. MRSA N315 contains the staphylococcal chromosome cassette *mec* (SCC*mec*) type II encoding the *mec* methicillin-resistance operon[32], as well as penicillinase plasmid pN315 containing the *bla* operon, which encodes a PC1 β-lactamase[33]. From a focused combinatorial screen of these 23 antibiotic compounds, including representatives from every major drug class (**Supplementary Table 1**), the combination of ME/PI/TZ showed highly synergistic, bactericidal activity against MRSA N315 *in vitro*, as assessed by fractional inhibitory concentration index (FICI) of 0.11 (refs. 34,35) (**Supplementary Table 2a**). For any number of drugs in combination, FICI < 1 indicates synergy; FICI = 1 indicates additivity; and FICI > 1 indicates indifference or antagonism[34,35]. Notably, each of these three drugs belongs to a different subclass of β-lactam drugs, though MRSA strains are typically highly resistant to most β-lactams[6]. The general resistance to individual β-lactams results from the inability of these drugs to inhibit the transpeptidase active site of PBP2a, which compensates for β-lactam inhibition of the other transpeptidases in MRSA[6].

ME/PI/TZ showed greater synergy against MRSA N315 than did any of its three constituent double combinations meropenem-piperacillin (ME/PI), meropenem-tazobactam (ME/TZ) and piperacillin-tazobactam (PI/TZ) at clinically relevant concentrations (**Fig. 1** and **Supplementary Table 2b**,**c**). We tested all three β-lactam compounds for final minimal-inhibitory concentrations (MICs) and FICI using a three-dimensional (3D) checkerboard with two-fold dilution series of each compound, from 128 to 2 μg/ml, and drug-free medium. These allowed up to a 64-fold difference in component ratios to be explored for maximal synergy, as well as isolation of results for each compound, all constituent double combinations and the triple combination. Using the 3D checkerboard, we determined the optimal ratio for ME/PI/TZ to be 1:1:1 for minimal drug input and maximal synergy against MRSA N315. The MICs of the meropenem and piperacillin components in the combination against MRSA N315 (2 μg/ml each) were below the clinical susceptibility breakpoints for each of these drugs alone against methicillin-susceptible *S. aureus* (MSSA) (4–8 μg/ml), and tazobactam alone has no susceptibility breakpoint and is given clinically at a 1:8 ratio with piperacillin[36]. The constituent double combinations ME/PI and PI/TZ were also synergistic against MRSA N315, with FICI = 0.44 and 0.22, respectively, whereas ME/TZ was less synergistic, at 0.67. On the basis of the Loewe additivity model of synergy, drugs cannot be synergistic with themselves[30]. Though the β-lactams all target the cell-wall synthesis pathway, our use of the FICI method (Loewe additivity) confirms the nonadditive nature of these interactions. In contrast to the high synergy of ME/PI/TZ seen in MRSA N315, the combination showed no additive activity (FICI = 1.12) in the MSSA reference strain ATCC 29213 (refs. 36,37) (**Supplementary Table 2b**,**c**), and we hypothesize that PBP2a is required for synergy to occur.

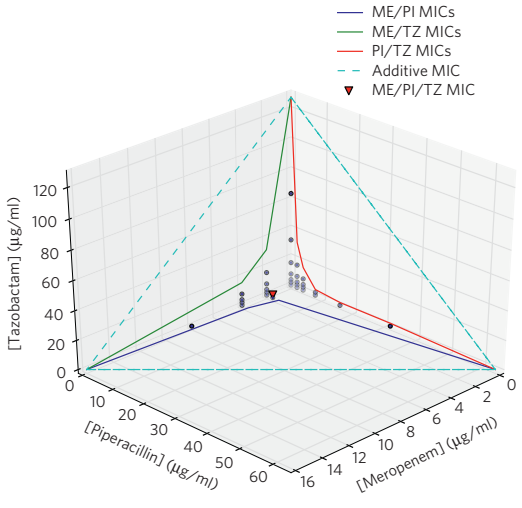

Figure 1 | 3D-checkerboard synergy determination showing isoboles of MIC and *in vitro* growth in single-, double- or triple-drug conditions for ME/PI/TZ. Colored lines and isoboles indicate MICs for two drugs in combination. Dashed lines indicate theoretical concentrations of additive interactions. Points indicate top sub-inhibitory concentrations of meropenem (ME), piperacillin (PI) and tazobactam (TZ) for each condition tested. Red triangle indicates the MIC of the three drugs in combination (each at 2 μg/ml).

We propose that the mechanism of synergy observed for ME/PI/TZ results from allosteric triggering of PBP2a by its constituents, akin to that reported for ceftaroline[8,9]. Indeed, we determined that meropenem binds to the allosteric site of PBP2a with a dissociation constant (K_d) of 270 ± 80 μM (equivalent to 104 ± 31 μg/ml; mean ± s.d.). The mean peak plasma concentration in healthy humans after a bolus intravenous injection of meropenem at the manufacturer's recommended 1-g dose is 112 μg/ml. The concentrations of meropenem achieved clinically are above the K_d; thus at these concentrations meropenem binding to the allosteric site of PBP2a would trigger opening of the active site of PBP2a, enabling access to its transpeptidase active site for acylation and inactivation either by another molecule of meropenem or by other β-lactams in the combination[6,8,38].

We observed that this highly synergistic activity of ME/PI/TZ in MRSA N315 is recapitulated in a panel of 72 clinical MRSA isolates representing multiple SCC*mec* types (**Supplementary Table 3a**,**b**). The MIC of the combination against the clinical isolates ranged from 0.4 to 33.3 μg/ml for each component, with a mean of 9.7 μg/ml and an MIC_{50} and MIC_{90} of 3.7 μg/ml and 33.3 μg/ml, respectively (**Supplementary Table 4a**).

Class-specificity of β-lactam synergy against MRSA

We determined that the observed synergy was not limited to the antibiotics assayed but could be generalized to their respective β-lactam classes by testing MRSA N315 and representative clinical MRSA isolates against other carbapenem-penicillin–β-lactamase inhibitor combinations. We found that treatment of MRSA N315 with imipenem-piperacillin-clavulanate (IM/PI/CV) showed equal or greater synergism to that of ME/PI/TZ. Meropenem-amoxicillin-tazobactam (ME/AX/TZ) maintained high synergy in MRSA N315 only (FICI = 0.04), showing less synergy in a clinical MRSA isolate (FICI = 0.55) (**Supplementary Table 2b**). MICs for components of these substituted triples were all below the mean peak human plasma concentrations of these compounds *in vivo*[39,40]. Similarly to ME/PI/TZ, IM/PI/CV showed less-than-additive activity against MSSA ATCC 29213 (FICI = 1.14) (**Supplementary Table 2b**,**c**). These results further support the necessity of the presence of PBP2a and its attendant allosterism for synergy, owing to lack of synergy of carbapenem-penicillin–β-lactamase inhibitor combinations in MSSA.

We also tested the effect of replacing the carbapenem component of the combination with either a monobactam or a cephalosporin, both later-generation β-lactam derivatives. In contrast to ME/PI/TZ, the triple combinations aztreonam-piperacillin-tazobactam (AZ/PI/TZ) and cefepime-piperacillin-tazobactam (CP/PI/TZ) (FICI = 0.33 for each) had lower levels of synergy than did PI/TZ alone (FICI = 0.22) (**Supplementary Table 2b**), possibly because aztreonam (a monobactam) binds selectively to Gram-negative PBPs[41] whereas cefepime (a cephalosporin) preferentially targets PBP2 over PBP1 (ref. 6).

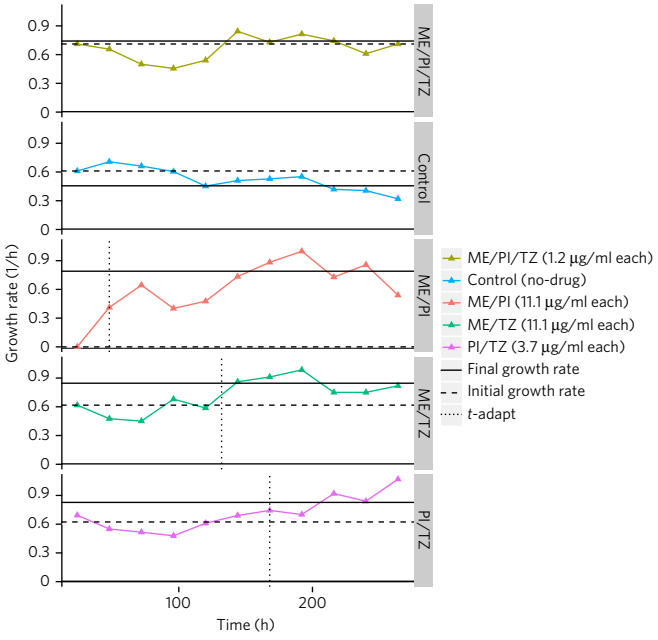

Figure 2 | Change in growth rates of MRSA N315 over time when challenged with antibacterial combinations. Growth rates of MRSA N315 over an 11-d period for each antibacterial combination tested at three-fold dilution below MIC. The difference (Δr) between the growth rate at day 1 (initial growth rate) and the averaged rate over the last 6 d of the assay (final growth rate) was calculated. MRSA N315 was considered to be adapted when $\Delta r > 0.2$. Results are from two replicate experiments. The adaptation time (t-adapt) was calculated as the time at which change in growth rate was half-maximal. Adaptation rate $\alpha = (\Delta r/2) / t\text{-adapt}$ ($1/h^2$) was computed for strains meeting this criterion. ME/PI, $\alpha = 8.23 \times 10^{-3}\ h^{-2}$; ME/TZ, $\alpha = 8.68 \times 10^{-4}\ h^{-2}$; PI/TZ, $\alpha = 4.32 \times 10^{-3}\ h^{-2}$. Only ME/PI/TZ at one three-fold dilution below MIC (1.2 μg/ml each) and no-drug control showed lack of increase in growth rate and were nonadapted.

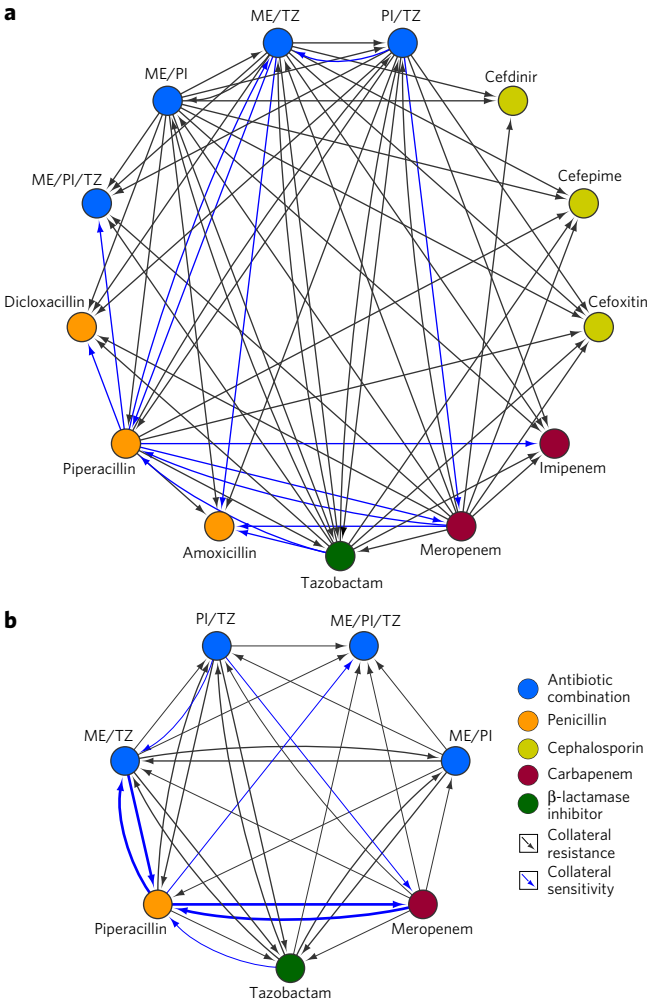

Figure 3 | Collateral sensitivities underlie suppression of adaptation to antibacterial combinations in MRSA N315. (**a**) MRSA N315 interaction network of collateral sensitivities and resistance for ME/PI/TZ, its single and double constituents and other subclasses of β-lactam compounds (cephalosporins, penicillins, carbapenems and β-lactamase inhibitors). Blue arrows indicate collateral sensitivities; black arrows indicate collateral resistance (for example, adaptation to piperacillin sensitizes MRSA N315 to meropenem and imipenem). (**b**) MRSA N315 interaction network of collateral sensitivities and resistance for ME/PI/TZ and its single and double constituents only. Thicker blue arrows indicate reciprocal collateral sensitivities between two nodes (for example, piperacillin and ME/TZ).

We confirmed the targets of the constituents of ME/PI/TZ by reducing the expression of PBP1, PBP2, PBP2a or PBP3 using a xylose-inducible antisense RNA strategy in the MRSA COL strain background[42]. In this system, complementary RNA targeting a PBP-encoding gene reduces its expression and promotes hypersusceptibility to drugs that target its product[43]. When expression of PBP2a was attenuated, the strain behaved as MSSA and was sensitized to all β-lactams tested (**Supplementary Table 5**). When meropenem, piperacillin and tazobactam were tested against the *pbpA* antisense strain, only meropenem showed larger zones of inhibition under xylose induction, confirming PBP1 as a target of meropenem (**Supplementary Table 5**). For the *pbp2* antisense strain both meropenem and piperacillin showed increased effectiveness under xylose induction, demonstrating that each has some activity against PBP2 (**Supplementary Table 5**). We observed no effect with the *pbp3* antisense strain, which is consistent with our hypothesis that ME/PI/TZ activity is focused on disrupting PBP1, PBP2 and PBP2a (**Supplementary Table 5**). All antisense strains except *pbp3* showed sensitization to the triple combination, underscoring the synergy observed.

ME/PI/TZ suppresses resistance evolution in MRSA N315

It is obvious that the development and spread of resistance can dramatically dampen the effectiveness and longevity of any antimicrobial therapy. We used serial passaging in subinhibitory antibiotic concentrations of the triple combination and each of its constituents to demonstrate that ME/PI/TZ suppresses the evolution of resistance in MRSA. To more accurately model a clinical treatment *in vitro* and *in vivo*, we applied these drugs at fixed dosages over extended periods, as occurs in clinical treatment, not at increasing doses over time. During the 11-d experiment, we observed no evolution of resistance in MRSA N315 to ME/PI/TZ. In contrast, resistance evolution occurred against all double combinations (doubles) and single constituents (singles) within 1–8 d, consistent with prior work[20,44] (**Fig. 2**). Viable cells were observed in all conditions above the initially determined MIC for the doubles and singles but not at or above the initial MIC for ME/PI/TZ. We noted increases in growth rates over time in all doubles and singles, whereas the growth rate of MRSA N315 in sub-MIC ME/PI/TZ over time was unchanged throughout the experiment, equivalent to the no-drug control (**Fig. 2**). MRSA N315 exposed to ME/PI showed a three-fold increase in MIC after day 1, indicating that viable cells were present after day 1 but did not grow until further passage and adaptation. Determination of the minimal-bactericidal concentration (MBC) confirmed that the triple combination ME/PI/TZ is bactericidal against

MRSA N315 (**Supplementary Table 4b**). Together, these results demonstrate the suppression of emergence of new resistance against ME/PI/TZ in MRSA N315.

Collateral sensitivities underlie resistance suppression

To determine whether collateral sensitivity was a factor in the suppression of adaptation of ME/PI/TZ, we analyzed the effects of prior exposure of MRSA N315 to a range of β-lactams on susceptibility to the other components (**Fig. 3** and **Supplementary Fig. 1**). We observed strong reciprocal collateral sensitivity between meropenem and piperacillin and between piperacillin and ME/TZ, whereas PI/TZ sensitized MRSA N315 to meropenem, but meropenem did not sensitize N315 to PI/TZ. Prior exposure to tazobactam also conferred collateral sensitivity to piperacillin, but not vice versa. Notably, we found no collateral sensitivity to tazobactam after exposure to any other singles or double combinations. The collateral sensitivity and resistance profiles of amoxicillin and piperacillin were nearly identical, with adaptation to meropenem also sensitizing MRSA N315 to amoxicillin (**Fig. 3** and **Supplementary Fig. 1**). Piperacillin also showed collateral sensitization to imipenem, an even more potent carbapenem against MRSA N315. However, none of the cephalosporins tested for collateral sensitivity by the carbapenem-penicillin–β-lactamase inhibitor combinations or constituents resulted in sensitivity. For this subclass of β-lactams, we noted only increased resistance or indifference. These results confirm that the suppression of resistance by collateral sensitivity observed is specific to the constituent drug classes of ME/PI/TZ.

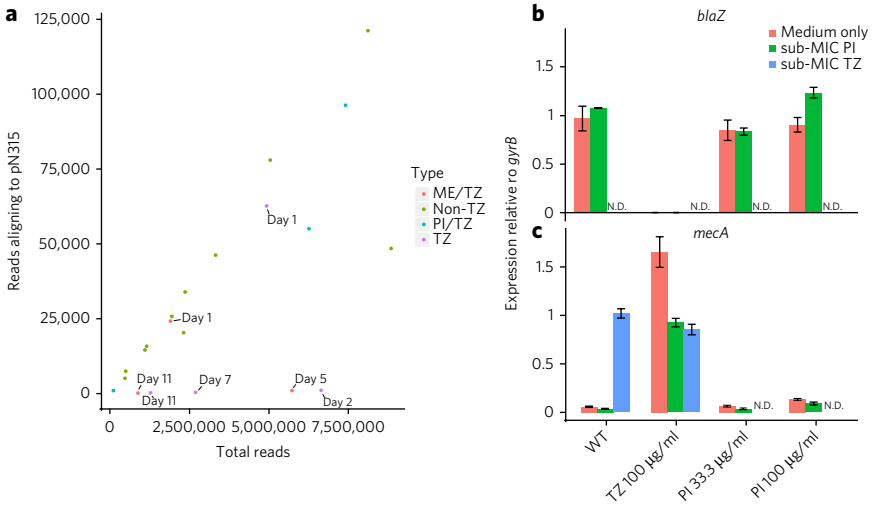

Figure 4 | Genomic evidence for mechanisms of synergy and collateral sensitivity. (**a**) Adaptation of MRSA N315 to ME/TZ or tazobactam alone destabilizes plasmid pN315. Read coverage aligning to pN315 in MRSA N315 adapted to drug combinations containing tazobactam (TZ) or not containing tazobactam (non-TZ), versus total reads per sample. Number of days of adaptation under the given conditions is indicated. (**b**,**c**) qRT-PCR confirms dysregulation of the *bla* and *mec* operons as causative mechanisms of some collateral sensitivities in MRSA N315. Expression of *blaZ* and *mecA* in wild-type (WT) MRSA N315 or adapted strains (N315 adapted to TZ or different concentrations of piperacillin (PI)) grown in medium only or medium and sub-MIC PI or TZ. N.D., not determined. Data are from three replicate experiments; error bars, mean ± s.e.m.

Genomic alterations of adapted MRSA N315

We used whole-genome sequencing to investigate the genomic basis of the sensitivity and resistance phenotypes of wild-type and adapted MRSA N315 strains. We found no mutations in genes encoding PBP or β-lactamase in any of the adapted MRSA N315 isolates. However, absence of read coverage identified that the penicillinase plasmid pN315 was lost in isolates adapted to tazobactam only (100 μg/ml) and ME/TZ (11.1 μg/ml each) (**Fig. 4a**). This plasmid loss occurred much more rapidly than with previously reported techniques for curing plasmids from MRSA, such as high heat and SDS treatment[45]. In PI/TZ-adapted isolates, we observed that approximately 400 kb of the MRSA N315 chromosome (GenBank BA000018.3) was duplicated after analysis of read coverage depth, from approximate genomic positions 2100000 to 2550000. Notably, this interval contains several putative and confirmed genes involved in cell-wall synthesis, including *ddlA*, which encodes D-Ala-D-Ala ligase (**Supplementary Fig. 2**).

The loss of pN315 in MRSA N315 correlates with greater sensitivity to piperacillin and amoxicillin, both penicillins that should be sensitive to the class A β-lactamase (PC1) encoded by *blaZ* on the plasmid. However, the loss of pN315 also resulted in increased resistance to tazobactam and ME/PI/TZ (**Fig. 3**, **Supplementary Fig. 1** and **Supplementary Table 6a**). One possible link between the presence of pN315 and ME/PI/TZ activity is the known regulatory crosstalk between MecI and BlaI repressors and their shared *mec* operon target[46–48]. To test the effect of the loss of pN315 on expression of genes known to be important for ME/PI/TZ activity, we performed quantitative reverse-transcription PCR (qRT-PCR) analysis of the adapted and wild-type MRSA N315 strains (**Fig. 4b**). We observed constitutive expression of the *blaZ*-encoded β-lactamase in pN315 within wild-type MRSA N315 but saw no expression of *blaZ* in clones adapted to tazobactam, which is consistent with loss of pN315 in these clones. We also found that expression of *mecA* was constitutive in the *blaZ*-null MRSA N315 isolate that was adapted to tazobactam at 100 μg/ml, consistent with dysregulation of the *mec* operon via loss of pN315 and the *bla* operon. Finally, we found tazobactam to be a strong inducer of *mecA* in wild-type MRSA N315, at levels similar to the constitutive expression of *mecA* seen in the *blaZ*-null condition.

ME/PI/TZ has synergy against MRSA with evolved resistance

We next examined the role that resistance to components of ME/PI/TZ has on its effectiveness against MRSA (**Supplementary Table 6a**). Previous exposure of MRSA N315 to piperacillin at either 33.3 or 100 μg/ml showed subsequent sensitization of the strain to ME/PI/TZ, from 3.7 to 1.2 μg/ml for each component. However, prior exposure of MRSA N315 to ME/TZ (11.1 μg/ml each) or meropenem only (33.3 μg/ml) showed a nine-fold increase in resistance to ME/PI/TZ (increasing from 3.7 to 33.3 μg/ml for each component). Exposure to tazobactam only gave intermediate gains in resistance to ME/PI/TZ up to day 7 (11.1 μg/ml each), and higher resistance at day 11 (33.3 μg/ml each). Prior exposure to ME/PI or PI/TZ generated only a three-fold increase in MIC (from 3.7 to 11.1 μg/ml) over 11 d.

Despite the elevated MICs to ME/PI/TZ in the isolates adapted to the component drugs, the triple-drug combination still maintained synergy in all adapted isolates (**Supplementary Table 6b**). This is consistent with synergistic drug activity within the range of ME/PI/TZ MICs observed for the 72 clinical MRSA isolates (**Supplementary Table 4**), relative to their single-drug MICs. These results show that even when genomic changes enabling subcomponent resistance can be selected, the overall synergistic activity of the triple-drug combination is maintained. In contrast to recent work with a nonpathogenic *E. coli* strain[30], we observed no change in synergy of the overall drug interaction profile of ME/PI/TZ with increased resistance to any component drug.

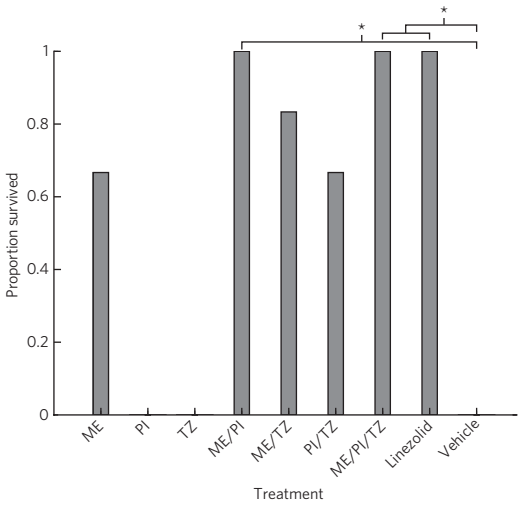

Figure 5 | Efficacy of ME/PI/TZ treatment in a neutropenic mouse peritonitis model of MRSA N315. Survival in neutropenic (cyclophosphamide-treated) mice infected with MRSA N315, assessed after 6 d of treatment with antibiotics or vehicle. $n = 6$ mice per group; *$P = 0.02$, Fisher's exact test with Bonferroni correction.

ME/PI/TZ is as effective as linezolid against MRSA *in vivo*

Next we tested whether ME/PI/TZ or its constituents can be effective in treating MRSA infections *in vivo* using a neutropenic mouse model of peritonitis. Blood taken at 11 h after infection from mice treated with ME/PI/TZ, ME/PI (67 mg per kilogram of body weight (mg/kg)) or linezolid (30 mg/kg) yielded no plated colonies and no growth in liquid cultures, indicating clearance of infection (**Fig. 5** and **Supplementary Data Set 1**). All mice ($n = 6$ per group) from each of these treatments survived for 6 d after infection (total duration of the mouse study). We conclude that the efficacy of ME/PI/TZ and ME/PI was similar to linezolid monotherapy on the basis of clearance of MRSA infection and survival of all treated mice compared to vehicle ($P = 0.02$, Fisher's exact test).

In contrast to mice treated with ME/PI/TZ, ME/PI or linezolid, several mice treated with ME/TZ, PI/TZ or meropenem only, and all mice treated with piperacillin or tazobactam only, succumbed to infection, most within 48 h (**Fig. 5** and **Supplementary Data Set 1**). Treatment with these other drug regimens was not significantly different from treatment with vehicle ($P > 0.05$, Fisher's exact test) (**Supplementary Table 7a**), where all mice also succumbed to the infection within 48 h.

We tested MRSA N315 cultures from blood drawn from mice treated with meropenem, piperacillin or vehicle for *in vitro* MICs against ME/PI/TZ and its constituent single drugs to determine whether adaptation occurred during passage *in vivo*. All four isolates of MRSA N315 tested had identical MICs for the triple ME/PI/TZ and all constituent drugs and thus identical synergy (**Supplementary Table 7b**). These data suggest that no adaptation occurred within these strains to overcome the triple ME/PI/TZ tested within the 11-h passage *in vivo*.

DISCUSSION

We have shown that triple antibacterial combinations containing carbapenems, penicillins and β-lactamase inhibitors target multiple nodes in the same cellular system (cell-wall synthesis) and are highly synergistic and bactericidal against diverse MRSA strains *in vitro* at clinically achievable concentrations. This contrasts with recent work showing collateral sensitivity and synergy to arise from combinations of drug classes working against orthogonal cellular targets in nonpathogenic lab strains only[22,30]. Because carbapenems and other drugs at high concentration could have toxic effects, reduced per-drug dosages via synergy mitigate potential toxicities[49]. Our 3D checkerboard testing confirmed the optimal input concentrations for ME/PI/TZ to be given in a 1:1:1 ratio (2 μg/ml each) against MRSA N315, which is below the susceptibility breakpoints for these compounds against MSSA and is an 8- to 64-fold reduction in input concentrations for these formerly inactive drugs against this highly resistant MRSA strain. Our mechanistic analyses support our hypothesis that targeting of PBP1 by meropenem, targeting of PBP2 by piperacillin, protection of piperacillin by tazobactam from β-lactamase cleavage and allosteric opening of the active site of PBP2a by meropenem for inhibition by another molecule of antibiotic in the combination result in synergy by simultaneously perturbing multiple components of the MRSA cell-wall synthesis system (**Supplementary Fig. 3**).

We have also shown that this combination has activity in a neutropenic *in vivo* model of highly lethal MRSA, demonstrating that this triple combination of clinically approved β-lactams can clear infection similarly to substantially more expensive monotherapies such as linezolid. The plasma levels of meropenem observed in mice correlate well with plasma drug levels in healthy humans[50], and meropenem would attain the K_d at these clinically achievable concentrations to trigger allostery for opening of the active site of PBP2a, providing accessibility for inhibition by meropenem and piperacillin[7,8].

Notably, the double combination ME/PI cleared the MRSA N315 infection *in vivo* similarly to ME/PI/TZ and linezolid within 11 h. *In vitro* we observed high synergy scores and reciprocal collateral sensitivity for this combination, which was similar to what we saw for ME/PI/TZ, but ME/PI did not suppress evolution of resistance to the same extent that ME/PI/TZ did. This property may not have been relevant to this aggressive infection model but may be important for the longer treatment times seen in human infections with MRSA. ME/PI/TZ is also likely to be effective at lower total concentrations than ME/PI because of its higher synergy. Longer exposure of the N315 strain to the tazobactam component of ME/PI/TZ *in vivo* may also promote ejection of the pN315 plasmid with concomitant sensitization to the penicillin component, in line with the *in vitro* results for collateral sensitivity and suppression of adaptation. Adequately addressing this question would require testing of potential longer-term *in vivo* resistance evolution under sublethal concentrations of the drugs in follow-up mouse experiments.

Our robust mechanistic *in vitro* results and preliminary *in vivo* results for ME/PI/TZ activity suggest this combination may be made immediately available for use in the clinic, as it includes drugs that have already been approved by the US Food and Drug Administration but that became obsolete as therapies against MRSA decades ago. However, further mechanistic features of the combination that were shown *in vitro* (such as synergy, resistance suppression over longer periods of dosing and collateral sensitivity) will require substantially more *in vivo* testing to support the promising but preliminary activity observed in our highly aggressive neutropenic mouse model.

We note that high resistance to meropenem or tazobactam slightly reduces the effectiveness of ME/PI/TZ, and our analysis cannot account for resistance genes acquired horizontally that could break the relationship among meropenem, piperacillin and tazobactam. Despite these caveats, we believe the ME/PI/TZ combination is an immediately viable anti-MRSA therapeutic and endorse further mechanistic exploration into the putatively superior efficacy of high-order antibiotic combinations that are both synergistic and encoded by collaterally sensitive constituents. Having similar activity to linezolid against MRSA *in vivo*, the potential efficacy of ME/PI/TZ reopens broad prospects for the clinical use of β-lactams against staphylococci. It also suggests that this line of research into repurposing antibiotics in carefully designed synergistic combinations

would address immediate clinical needs, as these agents are already approved for human use. The emergence of resistance to any antibiotic or antibiotic combination is inevitable. Yet, as evidenced in our study, combinations composed of key drug-drug interaction features may help to mitigate the emergence of antibiotic resistance by preserving the usefulness of agents already available in the pharmacological armamentarium.

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METHODS

Methods and any associated references are available in the online version of the paper.

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References

1. Walsh, T.R., Weeks, J., Livermore, D.M. & Toleman, M.A. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect. Dis.* (2011).
2. Davies, J. & Davies, D. Origins and evolution of antibiotic resistance. *Microbiol. Mol. Biol. Rev.* **74**, 417–433 (2010).
3. Fuda, C.C.S., Fisher, J.F. & Mobashery, S. β-lactam resistance in *Staphylococcus aureus*: the adaptive resistance of a plastic genome. *Cell. Mol. Life Sci.* **62**, 2617–2633 (2005).
4. Chambers, H.F. & Deleo, F.R. Waves of resistance: *Staphylococcus aureus* in the antibiotic era. *Nat. Rev. Microbiol.* **7**, 629–641 (2009).
5. Malouin, F. & Bryan, L.E. Modification of penicillin-binding proteins of β-lactam resistance. *Antimicrob. Agents Chemother.* **30**, 1–5 (1986).
6. Fuda, C., Suvorov, M., Vakulenko, S.B. & Mobashery, S. The basis for resistance to β-lactam antibiotics by penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus*. *J. Biol. Chem.* **279**, 40802–40806 (2004).
7. Fuda, C. *et al.* Activation for catalysis of penicillin-binding protein 2a from methicillin-resistant *Staphylococcus aureus* by bacterial cell wall. *J. Am. Chem. Soc.* **127**, 2056–2057 (2005).
8. Otero, L.H., Rojas-Altuve, A., Llarrull, L.I., Carrasco-López, C. & Kumarasiri, M. How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function. *Proc. Natl. Acad. Sci. USA* **110**, 16808–16813 (2013).
9. Villegas-Estrada, A., Lee, M., Hesek, D., Vakulenko, S.B. & Mobashery, S. Co-opting the cell wall in fighting methicillin-resistant *Staphylococcus aureus*: potent inhibition of PBP 2a by two anti-MRSA β-lactam antibiotics. *J. Am. Chem. Soc.* **130**, 9212–9213 (2008).
10. Long, S.W. *et al.* PBP2a mutations causing high-level ceftaroline resistance in clinical methicillin-resistant *Staphylococcus aureus* isolates. *Antimicrob. Agents Chemother.* **58**, 6668–6674 (2014).
11. Gu, B., Kelesidis, T., Tsiodras, S., Hindler, J. & Humphries, R.M. The emerging problem of linezolid-resistant *Staphylococcus*. *J. Antimicrob. Chemother.* **68**, 4–11 (2013).
12. van Hal, S.J., Paterson, D.L. & Gosbell, I.B. Emergence of daptomycin resistance following vancomycin-unresponsive *Staphylococcus aureus* bacteraemia in a daptomycin-naïve patient-a review of the literature. *Eur. J. Clin. Microbiol. Infect. Dis.* **30**, 603–610 (2011).
13. Arias, C.A. & Murray, B.E. Antibiotic-resistant bugs in the 21st century—a clinical super-challenge. *N. Engl. J. Med.* **360**, 439–443 (2009).
14. Bhusal, Y., Shiohira, C.M. & Yamane, N. Determination of *in vitro* synergy when three antimicrobial agents are combined against *Mycobacterium tuberculosis*. *Int. J. Antimicrob. Agents* **26**, 292–297 (2005).
15. Sjölund, M., Wreiber, K., Andersson, D.I., Blaser, M. & Engstrand, L. Long-term persistence of resistant enterococcus species after antibiotics to eradicate *Helicobacter pylori*. *Ann. Intern. Med.* **139**, 483–487 (2003).
16. Tupin, A. *et al.* Resistance to rifampicin: at the crossroads between ecological, genomic and medical concerns. *Int. J. Antimicrob. Agents* **35**, 519–523 (2010).
17. Comas, I. *et al.* Whole-genome sequencing of rifampicin-resistant *Mycobacterium tuberculosis* strains identifies compensatory mutations in RNA polymerase genes. *Nat. Genet.* **44**, 106–110 (2012).
18. Fischbach, M.A. & Walsh, C.T. Antibiotics for emerging pathogens. *Science* **325**, 1089–1093 (2009).
19. Zimmermann, G.R., Lehár, J. & Keith, C.T. Multi-target therapeutics: when the whole is greater than the sum of the parts. *Drug Discov. Today* **12**, 34–42 (2007).
20. Hegreness, M., Shoresh, N., Damian, D., Hartl, D.L. & Kishony, R. Accelerated evolution of resistance in multidrug environments. *Proc. Natl. Acad. Sci. USA* **105**, 13977–13981 (2008).
21. Lázár, V. *et al.* Bacterial evolution of antibiotic hypersensitivity. *Mol. Syst. Biol.* **9**, 700 (2013).
22. Imamovic, L. & Sommer, M.O.A. Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci. Transl. Med.* **5**, 204ra132 (2013).
23. Boucher, H.W. *et al.* Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin. Infect. Dis.* **48**, 1–12 (2009).
24. Rice, L.B. Antimicrobial resistance in Gram-positive bacteria. *Am. J. Infect. Control* **34**, S11–S19 (2006).
25. Waxman, D.J. & Strominger, J.L. Penicillin-binding proteins and the mechanism of action of β-lactam antibiotics. *Annu. Rev. Biochem.* **52**, 825–869 (1983).
26. Lee, S.H. *et al.* Antagonism of chemical genetic interaction networks resensitize MRSA to β-lactam antibiotics. *Chem. Biol.* **18**, 1379–1389 (2011).
27. Koga, T. *et al.* Affinity of Tomopenem (CS-023) for penicillin-binding proteins in *Staphylococcus aureus, Escherichia coli*, and *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother.* **53**, 1238–1241 (2009).
28. Yang, Y., Bhachech, N. & Bush, K. Biochemical comparison of imipenem, meropenem and biapenem: permeability, binding to penicillin-binding proteins, and stability to hydrolysis by β-lactamases. *J. Antimicrob. Chemother.* **35**, 75–84 (1995).
29. Campbell, E.M. & Chao, L. A population model evaluating the consequences of the evolution of double-resistance and tradeoffs on the benefits of two-drug antibiotic treatments. *PLoS ONE* **9**, e86971 (2014).
30. Munck, C., Gumpert, H.K., Wallin, A.I.N., Wang, H.H. & Sommer, M.O.A. Prediction of resistance development against drug combinations by collateral responses to component drugs. *Sci. Transl. Med.* **6**, 262ra156 (2014).
31. Kuroda, M. *et al.* Whole genome sequencing of methicillin-resistant *Staphylococcus aureus*. *Lancet* **357**, 1225–1240 (2001).
32. Goldstein, F. *et al.* Identification and phenotypic characterization of a β-lactam-dependent, methicillin-resistant *Staphylococcus aureus* strain. *Antimicrob. Agents Chemother.* **51**, 2514–2522 (2007).
33. Arêde, P., Ministro, J. & Oliveira, D.C. Redefining the role of the β-lactamase locus in methicillin-resistant *Staphylococcus aureus*: β-lactamase regulators disrupt the MecI-mediated strong repression on *mecA* and optimize the phenotypic expression of resistance in strains with constitutive *mecA*. *Antimicrob. Agents Chemother.* **57**, 3037–3045 (2013).
34. Berenbaum, M.C. What is synergy? *Pharmacol. Rev.* **41**, 93–141 (1989).
35. Saiman, L. Clinical utility of synergy testing for multidrug-resistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis: 'the motion for'. *Paediatr. Respir. Rev.* **8**, 249–255 (2007).
36. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing (document no. M100-S19) (CLSI, Wayne, Pennsylvania, 2009).
37. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically: approved standard (CLSI document M07-A8) (CLSI, Wayne, Pennsylvania, 2009).
38. Fishovitz, J. *et al.* Disruption of allosteric response as an unprecedented mechanism of resistance to antibiotics. *J. Am. Chem. Soc.* **136**, 9814–9817 (2014).
39. Somani, P., Freimer, E.H., Gross, M.L. & Higgins, J.T. Pharmacokinetics of imipenem-cilastatin in patients with renal insufficiency undergoing continuous ambulatory peritoneal dialysis. *Antimicrob. Agents Chemother.* **32**, 530–534 (1988).
40. Kinzig, M., Brismar, B. & Nord, C.E. Pharmacokinetics and tissue penetration of tazobactam and piperacillin in patients undergoing colorectal surgery. *Antimicrob. Agents Chemother.* **36**, 1997–2004 (1992).
41. Stutman, H.R., Welch, D.F., Scribner, R.K. & Marks, M.I. *In vitro* antimicrobial activity of aztreonam alone and in combination against bacterial isolates from pediatric patients. *Antimicrob. Agents Chemother.* **25**, 212–215 (1984).
42. Lee, S.H. *et al.* Antagonism of chemical genetic interaction networks resensitize MRSA to β-lactam antibiotics. *Chem. Biol.* **18**, 1379–1389 (2011).
43. Bouley, R. *et al.* Discovery of antibiotic (*E*)-3-(3-carboxyphenyl)-2-(4-cyanostyryl)quinazolin-4(3*H*)-one. *J. Am. Chem. Soc.* **137**, 1738–1741 (2015).
44. Ankomah, P., Johnson, P.J.T. & Levin, B.R. The pharmaco-, population and evolutionary dynamics of multi-drug therapy: experiments with *S. aureus* and *E. coli* and computer simulations. *PLoS Pathog.* **9**, e1003300 (2013).
45. Sonstein, S.A. & Baldwin, J.N. Loss of the penicillinase plasmid after treatment of *Staphylococcus aureus* with sodium dodecyl sulfate. *J. Bacteriol.* **109**, 262–265 (1972).
46. Hackbarth, C.J. & Chambers, H.F. *blaI* and *blaR1* regulate β-lactamase and PBP 2a production in methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* **37**, 1144–1149 (1993).

47. Lowy, F.D. Antimicrobial resistance: the example of *Staphylococcus aureus*. *J. Clin. Invest.* **111**, 1265–1273 (2003).
48. Blázquez, B. *et al.* Regulation of the expression of the β-lactam antibiotic-resistance determinants in methicillin-resistant *Staphylococcus aureus* (MRSA). *Biochemistry* **53**, 1548–1550 (2014).
49. Craig, W.A. The pharmacology of meropenem, a new carbapenem antibiotic. *Clin. Infect. Dis.* **24** (suppl. 2) S266–S275 (1997).
50. DeRyke, C.A., Banevicius, M.A., Fan, H.W. & Nicolau, D.P. Bactericidal activities of meropenem and ertapenem against extended-spectrum-β-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in a neutropenic mouse thigh model. *Antimicrob. Agents Chemother.* **51**, 1481–1486 (2007).

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Author contributions

P.R.G. designed the study, performed *in vitro* experiments, analyzed results and wrote the paper. M.W.P. and C.-A.D.B. analyzed results and wrote the paper. R.B. performed *in vivo* experiments, analyzed results and wrote the paper. A.B. and B.A.B. performed *in vitro* experiments and analyzed results. M.A.S., W.R.W. and V.A.S. performed *in vivo* experiments. S.M. and M.C. designed *in vivo* experiments, analyzed results and wrote the paper. G.D. designed the study, analyzed results and wrote the paper.

Competing financial interests

The authors declare competing financial interests: details are available in the online version of the paper.

Additional information

Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to G.D. (dantas@wustl.edu).

ONLINE METHODS

Microbiological studies. MRSA N315 was a gift from S. Gill (University of Rochester). *S. aureus* ATCC 29213 was acquired from the American Type Culture Collection. 72 de-identified clinical MRSA isolates were selected at random from the clinical isolate strain bank at Barnes-Jewish Hospital (St. Louis). Minimal inhibitory concentration (MIC) assays for inhibition of growth were performed following the recommendations of the Clinical and Laboratory Standards Institute (CLSI)[37]. Briefly, 23 antibacterial compounds (**Supplementary Table 1**) were selected on the basis of coverage of all major drug classes, including three compounds not classified as antibiotics for human use but with known antibacterial properties. Compounds were dissolved in DMSO to a stock concentration of 50 mg/ml, with the following exceptions: sulfometuron (20 mg/ml in DMSO); tobramycin, D-cycloserine and colistin (50 mg/ml in H_2O and filtered at 0.2 µm). The 23 compounds were formulated into all 253 possible unique pairwise combinations at fixed ratios and at 100× concentrations in solvent. To increase the range of concentrations assayed for possible synergistic or antagonistic drug interactions (>2,000-fold), the drug stocks were arrayed at 20-µl volumes into threefold dilution series down eight rows in 96-well (Costar) master drug plates, using a BioMek FX robotic liquid handler (Beckman Coulter, Inc.). Drugs were then mixed 1:100 into 96-well plates containing 200 µl/well of cation-adjusted Mueller-Hinton medium (CAMHB). All drug-susceptibility assay wells were inoculated with ~1 µl of mid-log-phase MRSA N315 bacterial culture at 0.5 McFarland standard (~2×10^8 colony-forming units (c.f.u.) per ml) using a sterile 96-pin replicator (Scinomix), the plates sealed with Breathe-Easy membranes (Sigma-Aldrich Co.) and placed into a sealable plastic bag containing a moist paper towel and grown at 37 °C without shaking for 24 h. Endpoint growth at 37 °C after 24 h was determined by optical density at 600 nm (OD_{600}) ≥ 0.1 using a Synergy H1 reader (BioTek, Inc.).

Synergy of antibiotic combinations was determined using the fractional inhibitory concentration index (FICI) method[51,52]. By this method, the MIC of the antibiotic compound in combination is divided by the MIC of the compound alone, yielding the fractional contribution of each drug component in the combination. Quotients for all compounds in a combination are summed and drug interactions scored using the formula

$$\mathrm{FICI} = (\mathrm{MIC\,A}_{\mathrm{comb\,A/B/C}})/\mathrm{MIC}_{\mathrm{agent\,A}} + (\mathrm{MIC\,B}_{\mathrm{comb\,A/B/C}})/\mathrm{MIC}_{\mathrm{agent\,B}} + (\mathrm{MIC\,C}_{\mathrm{comb\,A/B/C}})/\mathrm{MIC}_{\mathrm{agent\,C}}$$

Select pairwise combinations against MRSA were then combined with each of the 21 remaining single drugs to make triple combinations, formulated and tested in identical fashion to the double combinations. Synergy of combinations was confirmed via triplicate measurements of drug conditions at the MIC. On the basis of its high synergy against MRSA N315 in the sparse screening, ME/PI/TZ and its constituents were selected for further characterization. Final susceptibility testing of ME/PI/TZ and its components was performed in triplicate in 96-well plates with a 3D checkerboard assay. Master plates of each single drug were made at 300× concentrations in solvent, combined into 8 final master plates at 100× (with each of the master plates comprising a standard 2D checkerboard of meropenem and piperacillin at increasing twofold concentrations across wells) and containing a fixed two-fold dilution concentration of tazobactam in all wells. These master plates were then mixed 1:100 into deep-well 96-well plates (USA Scientific, Inc.) containing 600 µl/well of CAMHB and divided into triplicate plates resulting in eight two-fold dilutions for each drug component, from 128 µg/ml to 0 µg/ml in CAMHB, at 200 µl/well. In this manner, varying concentrations of all three drugs were tested against each other, generating MIC determinations of triple-, double- and single-drug MICs. Plates were inoculated with MRSA N315 as above using a sterile 96-pin replicator, sealed with Breathe-Easy membranes, placed into sealable plastic bags containing a moist paper towel, and grown at 37 °C without shaking for 24 h. Endpoint growth at 37 °C after 24 h was determined by OD_{600} ≥ 0.1 using a Synergy H1 reader.

Minimal bactericidal concentration (MBC) for ME/PI/TZ in MRSA N315 was determined via duplicate wells of ME/PI/TZ at indicated concentrations in CAMHB inoculated with MRSA N315 in mid-log phase as above using a sterile 96-pin replicator, plates sealed with Breathe-Easy membranes, placed into a sealable plastic bag containing a moist paper towel, and grown at 37 °C without shaking for 24 h. Endpoint growth at 37 °C after 24 h was determined by OD_{600} ≥ 0.1 using a Synergy H1 reader. 100 µl of a 1:100 dilution of 50 µl drawn from duplicate ME/PI/TZ wells was plated on non-selective Mueller-Hinton agar (MHA) plates and incubated overnight for 24 h. No colony growth at or two dilutions above MIC confirmed bactericidal activity, as defined by CLSI[53]. Meropenem (CAS 96036-03-2) and clavulanate (CAS 61177-45-5) were obtained from AK Scientific, Inc. Piperacillin (CAS 59703-84-3), tazobactam (CAS 89786-04-9), imipenem (CAS 74431-23-5), amoxicillin (CAS 26787-78-0), cefdinir (CAS 91832-40-5), cefepime (CAS 123171-59-5), cefoxitin (CAS 33564-30-6), and dicloxacillin (CAS 13412-64-1) were obtained from Sigma-Aldrich Co.

Antibiotic hypersusceptibility assays against MRSA PBPs. Assays were performed as previously described[26]. Briefly, antisense RNA constructs targeting PBPs in MRSA COL (PBP2a, PBP1, PBP2, and PBP3), were cloned into vector pTET15 with a xylose-inducible promoter and selected with 34 µg/ml of chloramphenicol. LB-Miller agar plates ± 50 mM xylose were seeded at 48 °C with 10^7 c.f.u. MRSA COL containing vector and antisense construct, cooled and then exposed to several concentrations of single antibiotics (meropenem, piperacillin, tazobactam), fixed double combinations of the same drugs or ME/PI/TZ. Zones of inhibition (ZOI) were measured in triplicate. Differential ZOI between xylose and no xylose were scored as follows: +++, more than two-fold increase in zone diameter with xylose induction; ++, two-fold increase in zone diameter; +, less than two-fold increase; –, no change in zone diameter.

Adaptation and crossresistance assays. Meropenem, piperacillin, and tazobactam stocks were dissolved in DMSO to a stock concentration of 10 mg/ml. Combinations of doubles ME/PI, ME/TZ and PI/TZ, and the triple ME/PI/TZ were formulated into final stock concentrations of 3.33 mg/ml each (100×) in DMSO. The drug stocks were arrayed at 20-µl volumes into threefold dilution series down eight rows in 96-well (Costar) master drug plates, using a BioMek FX robotic liquid handler (Beckman Coulter, Inc.). Drugs were then mixed 1:100 into deep-well 96-well plates (USA Scientific, Inc.) containing 1,980 µl/well of CAMHB. All combinations were arrayed in duplicate, and all single drugs singly, with no-drug growth controls and medium-only sterility controls in quadruplicate. Highest concentrations of all drug combinations were 33.3 µg/ml for each component and 100 µg/ml for single drugs. From the 2-ml deep 96-well plate, assay reagents were divided into 13 96-well (Costar) plates (150 µl/well), sealed with sterile foil seals (VWR International LLC) and stored at −80 °C. For each day of the adaptation assay, one 96-well (Costar) plate (150 µl/well) was thawed at ~25 °C, shaken for 1 min on a tabletop plate shaker (Thermo Fisher Scientific, Inc.), and briefly centrifuged before removing the sterile foil. On day 0, all drug susceptibility assay wells from plate 1 were inoculated with ~1 µl of mid-log phase MRSA N315 bacterial culture at 0.5 McFarland standard (~2×10^8 c.f.u./ml) using a sterile 96-pin replicator (Scinomix), the plate sealed with Breathe-Easy membrane (Sigma-Aldrich Co.) and grown at 37 °C with constant fast-linear shaking (562 c.p.m.) for 24 h using a PowerWave HT reader (BioTek, Inc.). Growth was determined by OD_{600} every 24 min. At 24 h (end of passage day 1), the plate was removed from the reader, unsealed with sterile technique, and ~1 µl of the well contents transferred to an identical thawed 96-well plate using a sterile 96-pin replicator. Each well of the 24-h growth plate was filled with 150 µl of sterile CAMHB and 30% glycerol, and the plate was stored at −80 °C for later analysis. This process was repeated for a total of 11 d, generating 11 96-well plates of MRSA N315 under adaptation to all combinations and single drugs comprising ME/PI/TZ. To test for cell viability, at the end of the assay on day 11, all wells from the final plate were pinned with a sterile 96-pin replicator and transferred to a 96-well (Costar) plate containing CAMHB only.

From each plate over the 11-d adaptation assay, the absorbance at OD_{600} from each 24-min time point for duplicate wells from each drug combination at each three-fold dilution was averaged, and the natural logarithm (ln) was calculated for each averaged absorbance. The logarithmic growth phase of the cultures was linearized by this method and plotted in Excel. Growth rate r was determined by determining difference in linearized absorbance between start and end of logarithmic growth phase (ΔA), determining time points of those absorbances (Δt), and taking the quotient $\Delta A / \Delta t = r$. Growth rate over time was plotted in Excel. The differences in growth rate (Δr) between day one (initial growth rate) and the averaged rate of the last 6 d of the assay (final growth rate) were calculated. Following Hegreness *et al.*[20], (Δr / 2) was used to determine the adaptation time parameter t-adapt, the time at which change in growth rate was half-maximal. Wells containing cells in drug conditions whose

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differences in growth rates were >0.2 h^{-1} between day 1 and the average of the previous 6 d of growth were considered significantly adapting to conditions and an adaptation rate $\alpha = (\Delta r / 2) / t\text{-adapt}\ (1 / h^2)$ was computed for strains meeting this criterion.

For the antibiotic crossresistance and susceptibility analysis, adapted isolates were chosen from the appropriate wells for each combination or single compound of the day-11 plate. Frozen isolates were streaked out on MHA plates and grown at 37 °C for 24 h to obtain single colonies, then distinct single colonies were picked and re-grown at 37 °C for 24 h in antibiotic-containing medium conditions identical to those in which they grew originally on the day-11 plate. The adapted MRSA N315 cultures in mid-log phase were diluted to 0.5 McFarland standard, then re-inoculated in sterile antibiotic-containing 96-well Costar plates (as with the original the adaptation plates) containing CAMHB and triplicate three-fold dilutions of ME/PI/TZ, its constituent double combinations and single drugs, and the β-lactam drugs amoxicillin (AX), cefdinir (CF), cefepime (CP), cefoxitin (CX), dicloxacillin (DC), and imipenem (IM) using a sterile 96-pin replicator. Plates were then sealed with Breathe-Easy membranes and placed into a sealable plastic bag containing a moist paper towel and grown at 37 °C without shaking for 24 h. Endpoint growth at 37 °C after 24 h was determined by $OD_{600} \geq 0.1$ using a Synergy H1 reader. As MRSA N315 grown with ME/PI/TZ showed no increase in growth rate, no adapted isolates were present for that combination. MICs of the adapted MRSA N315 with these drug combinations or single compounds were compared to the MIC of the drug(s) for wild-type MRSA N315, and the fold differences in MIC were plotted in a heatmap using Plotly.

Expression profiling with qRT-PCR. Wild-type and adapted MRSA N315 isolates were grown in triplicate in 100-ml flasks to mid-log phase in CAMHB ± piperacillin at 11.1 μg/ml or tazobactam at 33.3 μg/ml. To harvest cells at mid-log phase, each culture flask was split into two 50-ml screw-cap tubes and centrifuged at 4 °C for 10 min at 3,500 r.p.m. The supernatant was then removed, and the pellets were combined carefully with a 2-ml serological pipette. 1 ml RNAprotect Bacteria Reagent (Qiagen) was added to pellets to stabilize the RNA, vortexed briefly, and incubated for 5 min at RT. After incubation, tubes were spun again at 4 °C for 10 min at 3,500 r.p.m., the supernatant removed, and the pellets stored at −80 °C.

Total RNA was extracted by resuspending cell pellets in Buffer B (200 mM NaCl, 20 mM EDTA), addition of 20% SDS, acid-washed sterile glass beads (Sigma, Inc.), phenol-chloroform–isoamyl alcohol (phenol:chloroform:IAA), and bead-beating using the Mini-Beadbeater-24 (BioSpec Products, Inc.) on setting 'high' for 5 min. Extraction mix was spun at 8,000 r.p.m. at 4 °C for 3 min (to separate the phases), and top aqueous phase was transferred to a new tube. RNA was precipitated with isopropanol and 3M sodium acetate, mixed thoroughly by inversion then centrifuged at 4 °C at 13,200 r.p.m. for 10 min, and supernatant was aspirated. The pellet was washed with ice-cold 70% ethanol and centrifuged at max r.p.m. at 4 °C for 5 min, then the supernatant was aspirated and the pellet air-dried. The pellet was resuspended in nuclease-free water and incubated in a 50 °C heat block, vortexing periodically. To remove DNA contamination, TURBO-DNase buffer (Ambion, Inc.) and RNase-free TURBO-DNase was added to each sample, which was then incubated at 37 °C for 30 min and purified using MEGAClear columns and kit (Thermo Fisher Scientific, Inc.) per manufacturer's protocol. Samples were repurified using Baseline-ZERO DNase buffer and Baseline-ZERO DNase (Epicentre, Inc.) following the manufacturer's protocol. Final RNA samples were eluted with 30 μl TE buffer, pH 7.0.

First-strand cDNA was synthesized from total RNA with SuperScript First-Strand Synthesis System for RT-PCR (Life Technologies). qRT-PCR of *pbp2*, *mecA* and *blaZ* in MRSA N315 was performed against *gyrB* using SYBR Select Master Mix for CFX (Life Technologies) on a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc.). Primer sequences were as follows (0.3 μM each): pbp2_F, CGTGCCGAAATCAATGAAAGACGC; pbp2_R, GGCACCTTCAGAACCAAATCCACC; mecA_F, TGGAACGAT GCCTATCTCATATGC; mecA_R, CAGGAATGCAGAAAGACCAAAGC; blaZ_F, TTTATCAGCAACCTTATAGTCTTTTGGAAC; blaZ_R, CCTGCT GCTTTCGGCAAGAC; gyrB_F, CGATGTGGATGGAGCGCATATTAG; gyrB_R, ACAACGGTGGCTGTGCAATATAC. CFX96 thermal cycler protocol: 2 min at 50 °C, 2 min at 95 °C, (15 s at 95 °C, 1 min at 60 °C) × 40 cycles. Gene expression was determined using the ΔΔCt method of normalized quantification, where Ct indicates threshold cycle (the cycle number at which exponential growth phase increases above threshold fluorescence signal).

Sequencing library preparation. Genomic DNA (gDNA) was extracted from wild-type and adapted MRSA N315 using lysostaphin digestion and phenol:chloroform:IAA extraction. 1-ml aliquots from 5-ml cultures of *S. aureus* strains (grown overnight in shaker) were centrifuged at 13,000 r.p.m. for 3 min, the media poured off and the process repeated with an additional 1 ml of culture. Cell pellets were resuspended in Buffer A (NaCl 200 mM, Tris-HCl 200 mM, EDTA 20 mM (pH 8.0)) at 4 °C and vortexed briefly. 2.5 μl of 10 mg/ml (200×) lysostaphin (Sigma-Aldrich, Inc.) was added to tubes, which were then 'flick-mixed' and centrifuged, then incubated in a 37 °C dry bath for 1 h. 0.1-mm zirconium beads (BioSpec Products 1107910), 20% SDS, and phenol:chloroform:IAA (25:24:1, pH 7.9) were added to the tubes, and samples chilled on ice. Cells were lysed by bead-beating on the "homogenize" setting for 4 min (beat 2 min, ice 2 min, beat 2 min), spun at 6800 rcf (4 °C) for 3 min, and aqueous phase (~500 μl) transferred to pre-spun phase-lock gel tubes (5Prime, 2302820). An equal amount (500 μl) of phenol:chloroform:IAA (25:24:1, pH 7.9) was added to tubes and mixed by inversion. Tubes were centrifuged at 20,800 r.c.f. at room temperature (RT) for 5 min, aqueous phase transferred (~500 μl) to a new Eppendorf tube, precipitated with −20 °C isopropanol and 1/10 volume of 3M sodium acetate at pH 5.5 (Ambion, AM9740), and mixed thoroughly by inversion. Mixture was stored at −20 °C overnight, then centrifuged at 13,200 r.p.m. at 4 °C for 20 min. Pellet was washed with 100% ethanol (RT) and centrifuged at 4 °C for 3 min, then ethanol was carefully drawn off, and the pellet was air-dried for 15 min. Pellet was resuspended in 30 μl of TE (Ambion, AM 9861) and incubated at 50 °C for 5 min, then DNA was purified with Qiagen QIAQuick PCR purification columns per the manufacturer's recommendations with the following modifications: RNaseA treatment at beginning of column clean-up was done with 4 μl Qiagen RNase (100 mg/ml) combined with every 300 μl buffer PB used, and incubation in buffer PB and RNase was done for 15 min at RT. PE wash buffer was held in column at RT for 2 min and genomic DNA was eluted with 35 μl of EB buffer pre-heated to 55 °C and then left to sit for 1 min before final spin.

500 ng of total DNA from each genome was sheared to ~300-bp fragments in nine 10-min rounds of shearing on the BioRuptor XL. In each round the power setting was 'H' and samples were treated for 30 s and allowed to rest for 30 s. Each sample was concentrated using the Qiagen MinElute PCR purification kit per the manufacturer's protocol. End repair of the sheared DNA fragments was initiated with the addition of 2.5 μl of T4 DNA ligase buffer with 10 mM ATP (NEB, B0202S), 1 μl of 1 mM dNTPs (NEB), 0.5 μl T4 Polymerase (NEB, M0203S), 0.5 μl T4 PNK (NEB M0201S), and 0.5 μl Taq polymerase (NEB, M0267S). This mixture was incubated at 25 °C for 30 min, then at 75 °C for 20 min. Barcoded adaptors were then added to the solution along with 0.8 μl of T4 DNA ligase (NEB, M0202M), for the purpose of ligating the adaptors to the DNA fragments. This solution was then incubated at 16 °C for 40 min, then at 65 °C for 10 min. The adaptor-ligated DNA was then purified using the Qiagen MinElute PCR purification kit per the manufacturer's protocol.

The DNA fragments were then size selected on a 2% agarose gel in 1× TBE buffer stained with Biotium GelGreen dye (Biotium). DNA fragments were combined with 2.5 μl 6× Orange loading dye before loading on to the gel. Adaptor-ligated DNA was extracted from gel slices corresponding to DNA of 250–300 bp using a Qiagen MinElute Gel Extraction kit per the manufacturer's protocol. The purified DNA was enriched by PCR using 12.5 μl 2× Phusion HF Master Mix and 1 μl 10 μM Illumina PCR Primer Mix in a 25 μl reaction using 1 μl of purified DNA as template. DNA was amplified at 98 °C for 30 s followed by 18 cycles of 98 °C for 10 s, 65 °C for 30 s, 72 °C for 30 s with a final extension of 5 min at 72 °C. The DNA concentration was then measured using the Qubit fluorometer, and 10 nmol of each sample (up to 106 samples per lane of sequencing) were pooled. Subsequently, samples were submitted for Illumina HiSeq-2500 Paired-End (PE) 101 bp sequencing at GTAC (Genome Technology Access Center, Washington University in St. Louis) at 9 pmol per lane.

DNA sequence analysis. *Alignment and variant calling.* For wild-type and adapted MRSA N315, all sequencing reads for each genome were de-multiplexed by barcode into separate genome bins. Reads were quality trimmed to remove adaptor sequence and bases on either end with a quality score <19. Reads shorter than 31 bp after quality trimming were not used in further analysis. All reads were mapped to the *Staphylococcus aureus* N315 chromosome (GenBank BA000018.3) and pN315 plasmid (GenBank AP003139) using command

bowtie2 –x <reference_genome_index_name> -1 <forward_read_file> -2 <reverse_read_file> -q –phred33 –very-sensitive-local -I 200 -X 1000 -S <sam_output>. Variants from the reference were called using samtools[54] (commands: samtools view -buS <sam_file> | samtools sort -m 4000000000 - <sample_prefix> ### samtools index <bam_file> ### samtools mpileup -uD -f <reference_genome> <bam_file> | bcftools view -bcv - > <bcf_file> ### bcftools view <bcf_file>). The variant call format (VCF) file was then filtered to remove single nucleotide polymorphisms (SNPs) with a quality score lower than 70 or coverage greater than twice the average coverage expected per base. Absence of read coverage or overabundant read coverage indicated plasmid loss or large duplication respectively. Any variant position found from the wild-type alignment was determined to be a result of alignment error or to be derived from lab specific drift in N315 and was removed from all other VCF files. Each variant position was then compared to known ORF locations in N315 to search for causal variants.

Geneious (Biomatters Ltd.) was used for alignment visualization (http://www.biomatters.com).

***In vivo* mouse model of MRSA infection.** *Animals.* Outbred ICR female mice (6–8 weeks old, 17–25 g body weight; Harlan Laboratories, Inc.) were used. Mice were given Teklad 2019 Extruded Rodent Diet (Harlan Laboratories, Inc.) and water *ad libitum*. Mice were maintained in polycarbonate shoebox cages containing corncob (The Andersons, Inc.) and Alpha-dri (Shepherd Specialty Papers, Inc.) bedding under 12-h light/12-h dark cycle at 22 ± 1 °C. All procedures involving animals were approved by the University of Notre Dame Institutional Animal Care and Use Committee.

Neutropenic mouse peritonitis model of MRSA infection. Doses of cyclophosphamide (100 µl of 50 mg/ml in 0.9% saline corresponding to 200 mg/kg; Alfa Aesar, Inc.) were given intraperitoneally (IP) at 4 and 1 d before infection. The *S. aureus* strain N315 was streaked onto Brain-Heart Infusion (BHI; Becton Dickson and Company) agar and grown overnight at 36 °C. The MRSA N315 bacterial inoculum was adjusted to approximately 1×10^8 c.f.u./ml (corresponding to $OD_{540} = 0.5$), then diluted to give 2×10^7 c.f.u./ml. A 10% porcine mucin (Sigma-Aldrich Co.) suspension was prepared and adjusted to pH 7. Immediately before infection, the bacterial inocula were diluted 1:1 with 10% mucin to a final concentration of 1×10^7 c.f.u./ml in 5% mucin. The mice were then infected IP with 0.5 ml of this inoculum. *In vivo* dosing of compounds in mice was compared with mean or range peak human plasma concentrations of studied β-lactams[39,40].

Antibiotic preparation. Meropenem was obtained from AK Scientific, Inc. Piperacillin and tazobactam were obtained from Sigma-Aldrich Co. Linezolid (CAS 165800-03-3) was obtained from AmplaChem. Antibiotics were dissolved at a concentration of 16.67 mg/ml in 30% DMSO, 30% propylene glycol, 40% water. Linezolid was used as positive control and was prepared at 7.5 mg/ml. Vehicle (30% DMSO, 30% propylene glycol, 40% water) was included as negative control. The dosing formulations were sterilized by passing through 0.2 µm filter before injection.

Bacterial isolation from blood. Blood samples were checked for bacterial growth by plating and liquid culture. Whole blood (100 µl, three samples per group) was spread onto BHI agar plates and incubated at 36 °C overnight. Colonies were counted, and three colonies were selected, grown overnight in liquid BHI culture at 36 °C, then mixed 1:1 with 30% LB-glycerol and stored at −80 °C. The remaining three blood samples of each group (50 µl) was added to 5 ml BHI medium and incubated overnight at 36 °C. When growth was noted, cultures were mixed 1:1 with 30% LB-glycerol and stored at −80 °C.

Statistical analysis. Data for minimal inhibitory concentrations (MICs) of drugs against bacteria were derived from triplicate measurements in plate and medium cultures. Adaptation data were taken from two replicate experiments for each drug combination condition. Data for qRT-PCR expression profiling were derived from three replicate experiments taken from three biological replicates each, with standard error of measurement calculated. Mice were treated with antibiotics or vehicle in groups of six ($n = 6$) as described[55]. Animal studies were not randomized or blinded, as the end point was either survival or death. Fisher's exact test with Bonferroni correction was used for 8 independent tests (comparing each treatment to vehicle). Fisher's exact test was used because planned experiments (with $n = 6$ mice) used small sample sizes, with independent tests.

51. Cai, Y., Wang, R., Pei, F. & Liang, B.-b. Antibacterial activity of allicin alone and in combination with β-lactams against *Staphylococcus* spp. and *Pseudomonas aeruginosa*. *J. Antibiot.* (*Tokyo*) **60**, 335–338 (2007).
52. Berenbaum, M.C. A method for testing for synergy with any number of agents. *J. Infect. Dis.* **137**, 122–130 (1978).
53. NCCLS. Methods for determining bactericidal activity of antimicrobial agents: approved guideline. (NCCLS document no. M26-A) (NCCLS, Wayne, Pennsylvania, 1999).
54. Li, H. *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* **25**, 2078–2079 (2009).
55. Ford, C.W. *et al. In vivo* activities of U-100592 and U-100766, novel oxazolidinone antimicrobial agents, against experimental bacterial infections. *Antimicrob. Agents Chemother.* **40**, 1508–1513 (1996).

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