Short Communication

In vitro activity of meropenem/piperacillin/tazobactam triple combination therapy against clinical isolates of Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus pseudintermedius and vancomycin-resistant Enterococcus spp

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A B S T R A C T

Objectives: To evaluate the activity of the reported synergistic and collaterally sensitive antibiotic combination, meropenem/piperacillin/tazobactam (ME/PI/TZ), against a panel of methicillin-resistant Staphylococcus aureus (MRSA) and other methicillin-resistant Staphylococcus species; and to investigate the relationship between ME/PI/TZ susceptibility and the genomic background of clinical isolates of MRSA.

Methods: ME/PI/TZ combination and single drug minimum inhibitory concentrations (MICs) were determined for 207 strains (including 121 MRSA, 4 methicillin-sensitive S. aureus [MSSA], 37 vancomycin-intermediate S. aureus [VISA], 6 ceftaroline non-susceptible MRSA, 29 coagulase-negative staphylococci [CoNS], 5 S. pseudointermedius and 5 vancomycin-resistant Enterococci [VRE]) by broth microdilution. Whole genomes of 168 S. aureus strains were sequenced, assembled, and comparatively analysed.

Results: USA300-SCCmec type IV isolates, clonal complex 8 (CC8)-MRSA isolates, including some VISA and ceftaroline (CPT)-intermediate strains, and all tested methicillin-resistant S. epidermidis isolates were highly susceptible to ME/PI/TZ. Isolates with elevated MICs (MICs of >16/16/16 mg/L) clustered with the USA100-SCCmec type II strain. Susceptibility of MRSA to ME/PI/TZ was correlated with susceptibility to ME. No obvious cross-resistance to CPT was observed among high-ME/PI/TZ MIC isolates.

Conclusions: The ME/PI/TZ combination is effective against a variety of clinical MRSA isolates, particularly of the USA300 lineage, which is expanding worldwide. ME/PI/TZ is also effective against drug-resistant CoNS and S. pseudointermedius clinical isolates.

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1. Introduction

Staphylococcus aureus is a Gram-positive bacterium frequently associated with community-acquired pneumonia, bacteremia, and nosocomial infections, resulting in a significant burden on healthcare systems [1]. The initial effectiveness of antibiotics in overcoming mortality associated with S. aureus infections has been lost because of the rise in antibiotic resistance [2].

Drug-resistant S. aureus strains, particularly methicillin-resistant S. aureus (MRSA), account for almost half the deaths caused by infections with drug-resistant bacteria in the United States [1]. A critical bridge between ineffective antibiotics and limiting antibiotic use to preserve efficacy involves combining existing drugs in novel ways. We have reported about an antibiotic combination (meropenem/piperacillin/tazobactam; ME/PI/TZ) that synergistically kills MRSA and suppresses development of drug resistance [3]. The most effective ratio against MRSA N315 using checkerboard assays was 1:1:1 [3]. This combination simultaneously targets β-lactamases and multiple penicillin-binding proteins (PBPs), including drug-resistant PBP2a in MRSA, in a collaterally sensitive manner. In addition to inhibiting the cell-division protein
PB2P1, ME binds PB2P2a at a site distal to the transpeptidase active site binding pocket, causing allosteric opening of the binding pocket to enable attack by PI or ME. TZ inhibits β-lactamase, thus protecting PI, and disrupts genetic cross-regulation in cell wall generation in MRSA [3]. The in vivo relevance of ME/PI/TZ activity was confirmed by complete clearance of an otherwise lethal MRSA systemic infection in neutropenic mice [3].

The current study investigated the spectrum of ME/PI/TZ activity in a diverse panel of clinical isolates from patients at Barnes Jewish Hospital (BJH), a tertiary-care academic medical center. To understand the genomic and phylogenetic basis of resistance and susceptibility, whole-genome sequencing (WGS) of 138 clinical S. aureus isolates from BJH was performed and these strains were compared to 30 isolates from outside sources, including reference "type" strains representing major strain types. Resistance genes were also identified that could explain variable patterns of resistance to the combination therapy.

2. Materials and methods

2.1. Bacterial strains and antibiotic susceptibility testing

De-identified clinical strains of MRSA, coagulase-negative *Staphylococcus* (CoNS), VISA (both MRSA and MSSA), and vancomycin-resistant Enterococcus (VRE) were obtained from the BJH Clinical Microbiology Laboratory (St. Louis, MO), and strains ATCC 29213 (MSSA), 43300 (MRSA), 29212 (vancomycin-susceptible *E. faecalis*), and 51299 (vancomycin-resistant *E. faecalis*) were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Fourteen VISA strains were obtained from the CDC & FDA AR Bank ([https://www.cdc.gov/arisolatelab/index.html](https://www.cdc.gov/arisolatelab/index.html) Atlanta, GA). USA type strains (USA100 through USA1100) were a gift from Dr. Andrew Tomaras (St. Louis, MO). Strains were streaked out on tryptic soy agar before use. ME, PI, and TZ were purchased from Sigma-Aldrich (St. Louis, MO). Drugs/combinations were solubilized in DMSO at 512 g/L and diluted in cation-adjusted Mueller-Hinton broth (CAMHB; Sigma-Aldrich, St. Louis, MO) to a final concentration of 128, 256, 1024, and 2048 mg/L. Reference broth microdilution testing was performed according to the CLSI guidelines using appropriate quality control strains [4]. Strain N315 was used as an internal standard.

2.2. 3-D checkerboard analysis

ME, PI and TZ were dissolved in DMSO at 256 mg/L (ME) or 1024 mg/L (PI, TZ) to make master plates for PI (diluted 2-fold column-wise in CAMHB) and TZ (diluted 2-fold row-wise in CAMHB). 50 μL of serially diluted PI were aliquoted to 24 U-bottom 96-well plates. To the 24 plates, 25 μL of serially diluted TZ was added. ME (starting at 256 mg/L) was 2-fold serially diluted in 10 mL CAMHB in 8 levels, and 25 μL of each concentration was dispensed into triplicate plates that already contained PI-TZ 2D gradient. A bacterial suspension was made by resuspending freshly streaked colonies in CAMHB and adjusted to an optical density (OD) corresponding to McFarland Standard 5.0. The bacterial suspension was then diluted 1/1000 in 250 mL CAMHB, and 100 μL of the cell suspension was dispensed into each well using a multi-channel pipette. Quality control plates for ATCC 29213 were made using drug stock solutions and inoculated. After inoculation, plates were sealed with Breathe-easy membranes and incubated at 37 °C for 16-20 h. The presence/absence of bacteria in each well was determined by visual inspection.

2.3. Whole-genome sequencing, bioinformatics, and statistical analysis

Genomic DNA extraction, Illumina sequencing library preparation, raw read processing and assembly were performed as previously described [5]. Staphylococcal Chromosome Cassette (SCC) mec types were determined using SCCmecFinder [6]. Clinical S. aureus isolates were contextualized within the broader taxonomy by constructing a core-genome containing genes shared by 99% of all isolates, and maximum-likelihood phylogenetic trees were constructed and visualized using previously described programs and methods [5]. To increase phylogenetic resolution for the two dominant genomic clusters (mostly consisting of S. aureus CC5 and CC8 isolates), additional core-genomes and maximum-likelihood trees were constructed.

Antimicrobial resistance genes (ARGs) were annotated in silico, retaining ARGs that covered at least 90% of the reference sequence, with >90% sequence identity using previously described methods [5]. These thresholds were selected taking into account the frequently polymorphic nature of central elements of *S. aureus* antimicrobial resistance [7].

3. Results

3.1. Susceptibility of clinical isolates to meropenem/piperacillin/tazobactam

The ME/PI/TZ combination at 1:1:1 mass ratio was empirically determined as the optimal ratio for MRSA N315 in our previous study [3]. The present study confirmed that this ratio offers broad coverage for isolates with various resistance profiles (Table S1) and was effective against a spectrum of MRSA and methicillin-resistant (MR) *Staphylococcus* species: 82% (99/121) of vancomycin and CPT-susceptible MRSA isolates (Fig. 1A) and all (29/29) MR-CoNS (28 *S. epidermidis* and 1 *S. simulans*) were inhibited at concentrations of 16/16/16 mg/L (Table 1). The majority of MR-s. *pseudintermedius* were resistant to PI but, like MSSA, highly susceptible to ME and ME/PI/TZ (Tables 1, S2E). MR-s. *pseudintermedius* causes similar infections to MRSA and is an increasingly recognized pathogen that was commonly misidentified as MRSA in the pre-MALDI-TOF era [8]. Some multidrug-resistant MRSA, such as VISA (17/27, Table 1) and CPT-intermediate and -resistant isolates (4/6, Tables 1, 2, S2B, S2C), were susceptible to ME/PI/TZ, with no cross-resistance to CPT observed (Table S2H, Figure S5).

Three of five VRE strains had ME/PI/TZ MICs of ≤16/16/16 mg/L, although the efficacy of ME/PI/TZ against VREs appeared to be linked to PI susceptibility for *E. faecalis* (Tables 1, S2G). The triple combination inhibited (fractional inhibitory concentration index = 0.30) at 16/16/16 mg/L one *E. faecium* strain ("PT20") that showed high levels of ME and PI resistance synergistically (MICs of 64 mg/L and >512 mg/L, respectively, Table S2G).

For MRSA isolates, susceptibility to ME (correlation coefficient 0.85, Fig. 1B) and not PI susceptibility (correlation coefficient 0.18, Fig. 1C) was strongly positively correlated with susceptibility to the ME/PI/TZ combination. The β-lactamase inhibitor TZ did not have antimicrobial activity alone.

3.2. Comparative genomics reveals a strong link between ME/PI/TZ susceptibility and strain background

To explore the relationship between ME/PI/TZ susceptibility and the genomic background of the clinical isolates, whole genomes of strains tested for ME/PI/TZ susceptibility were sequenced to reconstruct the population structure at BJH (strain IDs "BJH###" denote isolates collected prior to 2013; "BJH18_###" denotes strains isolated after 2018) and identify associations between phylogenetic
Fig. 1. Minimum inhibitory concentrations of MRSA strains. (A) Heatmap of MICs of all 121 MRSA isolates against the triple combination of ME/PI/TZ (labeled as “MPT”) as well as single treatment with each drug. (B) Scatterplot of MICs of MRSA isolates for meropenem against MICs for ME/PI/TZ. The linear regression line is plotted. (C) Scatterplot of MICs of MRSA isolates for piperacillin against MICs for ME/PI/TZ. The linear regression line is plotted.
background and ME/PI/TZ resistance. Strains from external sources, including USA-types, N315, 4 MSSAs and 13 VISAs, were included in our analysis to place our clinical isolates in the larger epidemiological context of multidrug-resistant S. aureus lineages. Overall, genomes of 138 clinical isolates were sequenced and assembled.

The core genome, constructed from all clinical isolates and reference strains, contained 1381 genes shared at 95% nucleotide identity. The relatively large accessory genome (5485 genes) highlights genetic plasticity of clinical S. aureus isolates, enabling its success as a human pathogen [9]. Maximum Likelihood phylogenetic trees generated showed that the majority of clinical isolates (92/138 clones) clustered with the USA100 type-strain (Fig. 2, S3). In silico MLST and SCCmec typing indicated that these isolates, as characteristic for the USA100 lineage, predominately belonged to CC5 and carried the SCCmec type II cassette harboring a complete version of the mec-operon (mecl, mecR1, and mecA). A total of 77 of these isolates were characterized as MRSA, 10 were VISA, 4 were CPT non-susceptible, and one clone had an MSSA phenotype. Two isolates clustering with the USA100 type strain, BJH003 and BJH74, harbored the SCCmec type IV cassette and were highly susceptible to ME/PI/TZ, indicating an apparent link between SCCmec type and resistance to the combination (Figure S3). This assumption is supported by reports that link SCCmec types and antibiotic susceptibilities of MRSA [10]. The majority of MRSA clones were associated with the USA100 lineage, which is consistent with the epidemiological trends in the United States during the time of their isolation before 2013 [11]. Generally, isolates clustering with USA100 tended to have higher ME/PI/TZ MICs than isolates that clustered with the USA300 type strain (Fig. 2), the second epidemic strain present in our cohort. In silico MLST and SCCmec typing of isolates from the USA300 cluster identified SCCmec type IV and CC8 for most clones. The majority of isolates from this cluster were MRSA (24/26), and two clones were VISA. Interestingly, all MRSA isolates clustering with USA300 were highly susceptible to ME/PI/TZ (Fig. 2, S4), indicating that detectable genetic signatures associated with MRSA isolates might contribute to resistance against the triple combination.

3.3. The absence of mecA regulatory genes is associated with ME/PI/TZ susceptibility

This study showed that susceptible isolates clustering with the USA300 type strain harbored the SCCmec IV cassette (Fig. 2, S4). Interestingly, isolates with the SCCmec type IV lack a functional mec regulatory system, consisting of a mecA-repressor encoded by mecl and β-lactam-sensing transmembrane protein encoded by mecR1 that induces the PBP2a-encoding gene, mecA. Clones carrying the SCCmec type IV cassette have a truncated version of mecR1 and lack mecl, altering the regulatory patterns of mecA [12]. Altered expression of PBP2a, which provides resistance to ME, could explain increased susceptibility of SCCmec IV-positive clones and is consistent with reports of higher susceptibility of SCCmec IV strains to other drugs [11,13]. Notably, resistance against the triple combination was not correlated with the presence of other resistance genes, indicating that SCCmec status might be the defining factor for resistance against the triple combination (Figure S6).

4. Discussion

Novel antibiotic drug development is a slow process; therefore, combining established drugs that regain treatment efficacy through synergistic interaction is a promising avenue in the fight against rising antibiotic resistance [14]. In the present study, ME/PI/TZ was evaluated against a range of clinical isolates and was shown to be effective against a wide variety of S. aureus and MR-Staphylococcus species (Tables 1, S2). These findings, combined with our previous observation that the triple combination prevents low-MIC MRSA from becoming resistant to β-lactams, highlight the clinical potential of ME/PI/TZ [3]. The majority of clinical isolates included in the analysis were genetically similar to the USA100 type strain (Fig. 2). USA100 has historically been considered a “hospital-associated” lineage, whereas the USA300 lineage, representing the second cluster of isolates, has traditionally been considered “community-associated” [15]. In recent years, these demarcations have blurred, with strains previously classified as community-associated now frequently causing nosocomial infections. In the clinical setting, USA300 is increasingly important, partly due to its superior infectivity compared with the USA100 lineage, and MRSA infections in the US are now predominantly caused by the USA300 strains [13,16]. Most of the isolates in the present study were collected prior to 2013, which may explain the relatively higher abundance of clones grouping with USA100 compared with isolates of the USA300 lineage (Fig. 2). Isolates of CC5 were observed to be more resistant to the triple combination than isolates of CC8. Considering the trend for increasing USA300 strains in the US and elsewhere [10,13], ME/PI/TZ may be an effective therapeutic option for severe MRSA infections.

ME is a broad-spectrum antibiotic of the carbapenem family. MRSA is usually resistant to ME because of its alternative penicillin-binding protein, PBP2a [3]. The data in the present study indicate that ME susceptibility is a major determinant of MRSA susceptibility to ME/PI/TZ. Combining ME with PI and TZ lowers drug concentrations via synergy, rendering ineffective antibiotics effective against MRSA. Surprisingly, ME/PI/TZ MICs were corre-

<table>
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<tr>
<th>Table 1</th>
<th>Distribution of ME/PI/TZ MICs by various clinical isolate groups</th>
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<tr>
<td>ME/PI/TZ concentration g/L (each)</td>
<td>MRSAa</td>
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<tr>
<td>&gt;32</td>
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<td>32</td>
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<td>&lt;2</td>
<td>5</td>
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<tr>
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CPT, ceftaroline; ME, meropenem; MIC, minimum inhibitory concentration; MRSA, methicillin-resistant Staphylococcus aureus; MSSA, methicillin-sensitive Staphylococcus aureus; VISA, vancomycin-intermediate Staphylococcus aureus; VRE, vancomycin-resistant Enterococci.

a MICs of non-VISA and CPT-susceptible MSSA not shown.

b VISA (MRSA) and CPT-non-susceptible MRSA not included.
lated to a similar extent with ME and PI resistance in *S. epidermidis* (Figure S2). This might be an effect of the generally lower resistance of *S. epidermidis* isolates to PI compared with MRSA isolates (Tables S2A, F).

The study data indicate that resistance to ME/PI/TZ may be tied to meca/PBP2a activity. Clones carrying the SCCmec type IV cassette, which lacks mecl and harbors a truncated version of mecR1, exhibited increased susceptibility to the triple combination. Previous studies have shown that crosstalk between the bla-locus and mec-locus additionally affects meca expression, with presence of both operons resulting in high levels of PBP2a activity in the presence of antibiotic stress [17,18]. However, there were no differences in susceptibility to the triple combination between isolates harboring and isolates lacking the bla-operand (Figure S6). This observation was independent of whether mecl/R1 were present in the isolate genome. As TZ inhibits the β-lactamase activity encoded by blaZ, and disrupts blaZ gene expression, the regulatory impact of the bla-operand on meca expression may be inhibited by the triple combination [3]. However, this disruption is seemingly counteracted by a functional mecl/mecR1 regulatory system, which may drive meca expression even under bla-inactivated conditions and lead to high levels of resistance to ME/PI/TZ. Taken together, these observations indicate that meca expression or overall PBP2a activity may be responsible for the differential resistance observed between clinical isolates.

As previously described, ME/PI/TZ targets multiple nodes in the cell wall machinery, limiting evolution of resistance [3]. High-dose ME has been used to treat technically “resistant” bacterial isolates with high ME MICs, and it is expected that constant unbound serum levels of ME, PI, and TZ of ~20 mg/L can be achieved through continuous infusion, maximizing the time above MIC for these time-dependent drugs [19]. Additional studies are necessary to determine a breakpoint for clinical therapy; however, the present data show that achievable serum concentrations would be effective against >80% of all investigated clinical MRSA isolates (Table 1). The data also indicate that ME/PI/TZ can be particularly
effective against virulent clones of the USA300 lineage for which MICs <12 mg/l (each) were detected. Furthermore, a recent study indicates that USA300 isolates are susceptible to β-lactam + β-lactamase combination if they possess mutations both within the mecA promoter and mecA itself [20]. As this lineage has become a relevant problem in the epidemiology of nosocomial S. aureus infections, ME/PI/TZ holds promise for controlling this worrisome trend [13,16].

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Declarations

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Competing Interests: G.D. is a co-founder of Viosera Therapeutics, which is evaluating the meropenem, piperacillin, tazobactam triple combination as a potential commercial therapeutic. A.Y., C-A.D.B., and G.D. have consulting and advisory relationships with Viosera Therapeutics. However, Viosera Therapeutics was not involved in any aspect of the work described herein, including funding or evaluation of any data or analyses.

Ethical Approval: Not required

Supplementary materials


References