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ORIGINAL ARTICLE

In vivo efficacy of β-lactam/tripropeptin C in a mouse septicemia model and the mechanism of reverse β-lactam resistance in methicillin-resistant *Staphylococcus aureus* mediated by tripropeptin C

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Natural lipopeptide antibiotic tripropeptin C (TPPC) revitalizes and synergistically potentiates the activities of the class of β-lactam antibiotics against methicillin-resistant *Staphylococcus aureus* (MRSA) but not against methicillin-sensitive *S. aureus in vitro*; however, the mode of action remains unclear. In the course of the study to reveal its mode of action, we found that TPPC inhibited the β-lactamase production induced by cefotiam. This prompted us to focus on the β-lactam-inducible β-lactam-resistant genes *blaZ* (β-lactamase) and *mecA* (foreign penicillin-binding protein), as they are mutually regulated by the *blaZ/I/R1* and *mecA/I/R1* systems. Quantitative reverse-transcription polymerase chain reaction analysis revealed that TPPC reversed β-lactam resistance by reducing the expression of the genes *blaZ* and *mecA*, when treated alone or in combination with β-lactam antibiotics. In a mouse/MRSA septicemia model, subcutaneous injection of a combination of TPPC and ceftizoxime demonstrated synergistic therapeutic efficacy compared with each drug alone. These observations strongly suggested that reverse β-lactam resistance by TPPC may be a potentially effective new therapeutic strategy to overcome refractory MRSA infections.
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INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (MRSA) strains have acquired multidrug resistance and are one of the major etiological agents of both hospital- and community-acquired infections.[1–4] The high-level resistance of MRSA to β-lactams is caused by acquisition of the foreign penicillin-binding protein (PBP2A; encoded by *mecA*), to which β-lactams have remarkably low binding affinity.[5–7] In addition to the *mecA* gene, ~90% of MRSA strains have also acquired a β-lactamase (penicillinase, BlaZ, encoded by *blaZ*) for penicillin resistance.[8,9] Recently, some MRSA strains have become resistant even to the 'last resort' antibiotic vancomycin (VAN).[10–12] Therefore, new effective drugs and strategies to overcome MRSA are urgently needed.

Tripropeptin C (TPPC) (Figure 1) is a natural, calcium-ion-dependent lipopeptide, which showed potent ant-imicrobial activity against MRSA both *in vitro* and in a mouse/MRSA ATCC33591-septicemia model when administered intravenously.[13–15] TPPC inhibits peptidoglycan biosynthesis through a different mechanism than that of other drugs targeting peptidoglycan biosynthesis, such as VAN, β-lactams and bacitracin, and shows no cross-resistance with these drugs.[15] Recently, we discovered an additional and unique effect of TPPC on revitalizing and synergistically potentiating the activity of the class of β-lactams against MRSA strains.[16,17] Interestingly, the synergy was observed against MRSA but not against methicillin-sensitive *S. aureus* (MSSA). We previously reported the synergistic anti-MRSA activity of the TPPC/β-lactam combination based on the fractional inhibitory concentration index and time-kill kinetics against MRSA strains, and the mechanism responsible for this effect was suggested to be distinct from its own anti-MRSA activity based on a study using an inactive TPPC semisynthetic derivative.[17] However, the mode of action mediating this interesting effect remains unclear.

The purpose of this study was to evaluate whether the synergy of TPPC/β-lactam treatment could also be demonstrated in an *in vivo* model of mouse MRSA septicemia and to determine the mechanism by which TPPC revitalizes the activity of β-lactams against MRSA. In this study, the *in vivo* efficacy of the TPPC/β-lactam combination, the inhibition of TPPC on the enzymatic activity and induction of the β-lactamase, BlaZ, and the transcription of β-lactam-inducible drug-resistant genes, both with TPPC alone and its combination with β-lactams, were evaluated. Clinically important anti-MRSA drugs, such as VAN and arbekacin (ABK), were also evaluated for comparison to provide further insight into the mode of revitalization of β-lactams by TPPC. The effects of TPPC/β-lactams against constructed MSSA strains harboring the plasmid containing the *mecA* gene were examined to reveal the role of this gene or its product.

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This article is dedicated to Prof. Hamao Umezawa on the occasion of the 60th anniversary of worldwide marketing of kanamycin.
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MATERIALS AND METHODS

Plasmids, bacterial strains and growth conditions

The plasmids and bacterial strains used in this study are listed in Table 1. The shuttle vector pHY300PLK was purchased from Takara (Shiga, Japan) and the β-lactamase gene was removed for this study. All parental strains are commercially available. *S. aureus* strains were grown at 37 °C with shaking at 140 r.p.m. in Mueller–Hinton broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 50 μg ml^{-1} calcium ion (MHCa broth). Recombinant *S. aureus* strains were selected and maintained with 10 μg ml^{-1} tetracycline.

Test compounds

TPPC was prepared as reported previously with slight modifications.[15] ABK was kindly provided by Meiji Seika Pharma (Tokyo, Japan). VAN was purchased from Shionogi (Osaka, Japan). Cefotiam (CTM), sulbactam and clavulanic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Ceftizoxime (ZOX) was purchased from Fujisawa Pharmaceutical (Osaka, Japan).

In vivo efficacy of TPPC/ZOX in a model of mouse MRSA (ATCC33591) septicemia

Crl:CD1 (ICR) 4-week-old male mice (Charles River Laboratories Japan, Yokohama, Japan) were inoculated intraperitoneally with a lethal dose of MRSA ATCC33591 (4.8×10^7 colony-forming units per mouse) in 0.5 ml of brain heart infusion broth containing 5% mucin. TPPC, ZOX or a combination of both was dissolved in 10% dimethyl sulfoxide saline, and administered subcutaneously at 50, 25 and/or 12.5 mg kg^{-1}. TPPC was administered at 2 h and ZOX was administered two times at 2 and 6 h after the MRSA challenge. Mortality was recorded once daily for 9 days.

Evaluations of the MICs and FICs

MICs and fractional inhibitory concentration indices (FICIs) were evaluated as reported previously.[17] FICs were determined using the standard checkerboard methodology[18] in MHCa broth and were calculated using the following formulae: FIC of agent A = (MIC of agent A in combination)/(MIC of agent A alone); FIC of agent B = (MIC of agent B in combination)/(MIC of agent B alone); and FIC index (FICI) = (FIC of agent A)+(FIC of agent B).

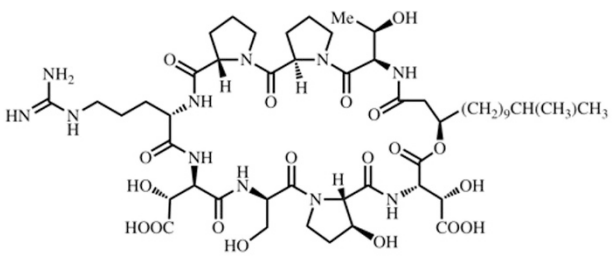

Figure 1 Structure of tripropeptin C (TPPC).

The combined effects were defined as follows: synergistic: FICI ⩽ 0.5; additive: 0.5 < FICI ⩽ 1; and indifferent: 1 < FICI ⩽ 4. Each test was repeated at least three times, and the average results are reported.

Evaluation of the inhibitory activity of TPPC on β-lactamase enzymatic activity and induction

Nitrocefin was used for evaluating β-lactamase enzymatic activity by measuring the absorbance at 490 nm (iMark Microplate Reader; Bio-Rad, Hercules, CA, USA). Nitrocefin is a chromogenic substrate for β-lactamase, which rapidly turns from yellow to red following β-lactamase degradation.[19]

The inhibition of TPPC on β-lactamase enzymatic activity was measured as follows. MRSA strain ATCC33591 was grown in MHCa broth at 37 °C with shaking until mid-log phase (OD_{600} 0.5). To this cultured broth, 1 μg ml^{-1} of CTM (MIC: 512 μg ml^{-1}) was added to induce β-lactamase, if needed. All of the concentrations of CTM tested (0.25–8 μg ml^{-1}) strongly induced the β-lactamase production to a similar extent (data not shown). The broth was cultured for a further 2 h at 37 °C with shaking, and then plated at 200 μl per well in a 96-well microtiter plate. TPPC was added to the wells at final concentrations of 100, 10, 1 and 0.1 μg ml^{-1}, followed by the addition of 100 μg ml^{-1} of nitrocefin. After 30 min incubation at 37 °C, the absorbance at 490 nm was measured. Sulbactam and clavulanic acid were used as positive controls.

The inhibition of TPPC on the induction of β-lactamase by adding the β-lactam antibiotic CTM was examined as follows. MRSA ATCC33591 strain was cultivated in MHCa broth at 37 °C with shaking until mid-log phase (OD_{600} 0.5). To cultured broths, 1 μg ml^{-1} of CTM was added to induce β-lactamase, and TPPC, VAN or ABK was also added, if needed, followed by further culture at 37 °C for 1 h with shaking. Then, the β-lactamase activity of each broth was measured as written above.

DNA manipulation

Polymerase chain reaction (PCR) was performed with PrimeSTAR HS DNA polymerase (Takara). Primers used in this study are listed in Supplementary Table S1. Restriction enzymes were used as recommended by the manufacturer (FastDigest; Thermo Fisher Scientific, Waltham, MA, USA). DNA bands were purified with a Wizard SV Gel and a PCR Clean-Up System (Promega, Madison, WI, USA). Total DNA from the MRSA strain N315 was isolated from a bacterial culture using a Genomic DNA Extraction Mini Kit (RBC Bioscience, Taipei, Taiwan) according to the manufacturer's instructions using lysostaphin (0.5 mg ml^{-1}) in the lysis step. Plasmid DNA was isolated from *Escherichia coli* cultures using the QuickLyse Miniprep Kit (Qiagen, Hilden, Germany). For plasmid DNA isolation from *S. aureus* cultures, cell pellets were treated with a bead beader before using the QuickLyse Miniprep Kit (Qiagen). Vector arms and insert ligation were performed with a DNA Ligation Kit Mighty Mix (Takara).

Total RNA purification and quantitative PCR

Total cellular RNA was extracted with zirconia beads (1 μM, TZ-B125m; Tosoh, Tokyo, Japan) and Trizol (Thermo Fisher Scientific) from mid-exponential phase cells of *S. aureus* (OD_{600} 0.5) grown in 7 ml of MHCa broth, after 10 min exposure with or without drugs. Crude total RNA samples were treated with

Table 1 Plasmids, and parental and transformant *S. aureus* strains used in this study

	descriptions
pSTBlue-1	Multi-purpose bacterial cloning and expression vector with a versatile MCS, T7 and SP6 promoters, Kan^r, Amp^r
vector	pHY300PLK(ΔBla), *E. coli-S. aureus* shuttle vector, low copy number in *S. aureus*, Tet^r, β-lactamase region deleted
p*mecA*	pHY300PLK(ΔBla)::*mecA*, pHY300PLK(ΔBla) with *mecA* gene including promotor region from MRSA N315 strain
MSSA ATCC29213	β-lactamase positive (*blaR1/blaI/blaZ* positive)
MSSA ATCC29213-vector	MSSA ATCC29213 transformant with pHY300PLK(ΔBla)
MSSA ATCC29213-p*mecA*	MSSA ATCC29213 transformant with pHY300PLK(ΔBla)::*mecA*
MRSA N315	clinically isolated prototype MRSA strain, wild type *mecR1-mecI*
MRSA ATCC33591	clinically isolated MRSA, β-lactamase (*blaZ*) positive

Abbreviation: MRSA, methicillin-resistant *Staphylococcus aureus*.

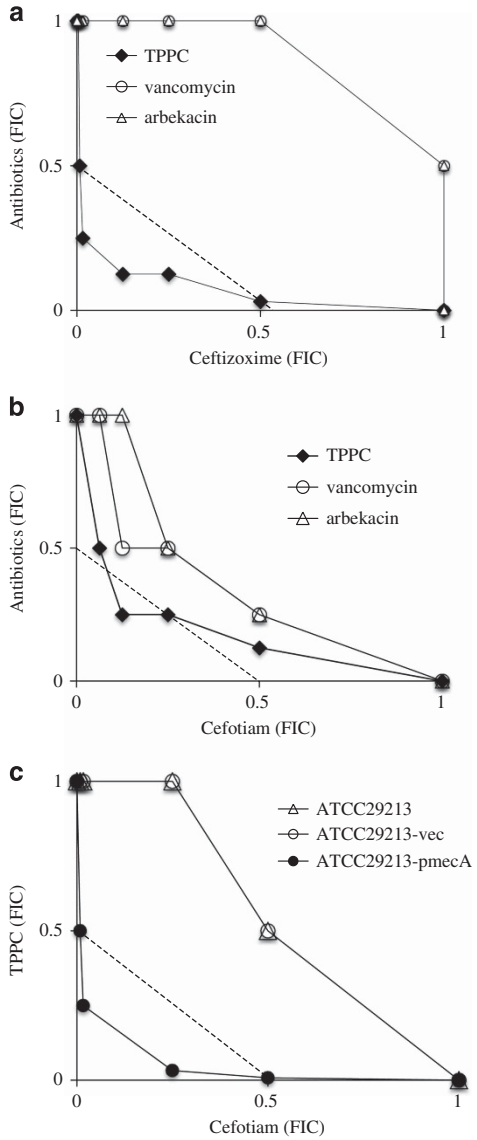

Figure 2 *In vitro* combination effects against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC33591, and against methicillin-sensitive *S. aureus* (MSSA) ATCC29213 and its mutant derivatives. Anti-MRSA activity of ceftizoxime and cefotiam was revitalized by tripropeptin C (TPPC) but not by vancomycin or arbekacin. The combination effects of TPPC (black diamonds), vancomycin (open circles) and arbekacin (open triangles) with ceftizoxime (**a**) or cefotiam (**b**) against the MRSA strain ATCC33591 in checkerboard analysis are shown. (**c**) The combination effects of TPPC and cefotiam against the MSSA strain ATCC29213 (open triangles), ATCC29213-vector control (open circles) and ATCC29213-*mecA* (black triangles) are shown. The fractional inhibitory concentration index (FIC) of each antibiotic combination pair is shown, and a total FIC < 0.5 (indicated by a diagonal dotted line) indicates a synergistic combination.

DNaseI (Takara) to remove any genomic DNA contamination in the presence of recombinant RNase inhibitor (Takara). Transcriptional modulation induced by CTM, ZOX, TPPC or their combinations were investigated by quantitative reverse-transcription PCR using the One Step SYBR PrimeScript Reverse-Transcription-PCR Kit II (Prefect Real Time; Takara) according to the manufacturer's instructions. Experiments were performed on a Thermal Cycler Dice Real Time System (model TP800; Takara). The newly designed, or previously published,[20–22] primers used in this study are listed in Supplementary Table S1. The thermal conditions were as follows: 5 min at 42 °C and 10 s at 95 °C, followed by 50 cycles at 98 °C for 5 s and 58 °C for 30 s. To verify the specificity of the PCR amplification products, melting-curve analyses were performed. The housekeeping gene *gyrA* was amplified for normalization. Gene markers *blaZ* and *mecA* were indicative of the β-lactam-resistant response, *vraS* and *pbpB* were indicative of cell wall stress and *vraD* was indicative of membrane stress.[22,23]

Construction of *mecA*-overexpressing MSSA

Recombinant plasmids were constructed in *E. coli* DH5α (Takara), and then electroporated into the MSSA strain ATCC29213 using the Gene Pulser II Electroporation System (Bio-Rad). The plasmid inserts were confirmed by restriction analysis, PCR and DNA sequencing (FASMAC, Kanagawa, Japan).

To clone the *mecA* gene, a fragment containing *mecA* and its native promoter was amplified by PCR with *mecA* primers (Supplementary Table S1) and extracted total DNA from the MRSA strain N315 was used as the template. The PCR product was then cloned into pSTBlue-1 (pSTBlue-1 Perfect Blunt Cloning Kit; Novagen, Darmstadt, Germany). This recombinant plasmid and pHY300PLK(ΔBla)[24] were digested with *Bam*HI and *Hin*dIII, and the obtained *mecA*-containing DNA fragment and vector arm were ligated. The resulting recombinant plasmid (pHY300-p*mecA*) was confirmed by PCR and sequencing, and then electroporated into the MSSA strain ATCC29213.

Ethics statement

All animal experiments were approved by the Institutional Committee for Animal Experiments at the Institute of Microbial Chemistry (BIKAKEN, Tokyo, Japan) and were performed in accordance with the relevant guidelines and regulations to minimize animal suffering.

RESULTS

MICs and FICIs against MRSA ATCC33591

The MICs against the MRSA strain ATCC33591 of CTM, ZOX, TPPC, VAN and ABK were 512, 1024, 1, 1 and 2 μg ml^{-1}, respectively, as determined by the broth dilution assay. The combination effects of CTM or ZOX with TPPC, ABK or VAN against MRSA strain ATCC33591 are shown in Figures 2a and b. When ZOX or CTM was combined with TPPC, synergistic anti-MRSA activities were observed (minimum FICI = 0.26 and 0.38, respectively). Meanwhile, VAN and ABK demonstrated only additives, and their minimum FICIs were 0.63 and 0.75 when combined with CTM and 1.0 and 1.0 when combined with ZOX, respectively.

Combination effect of TPPC/ZOX in a mouse MRSA (ATCC33591) septicemia model

The 50% effective dose for subcutaneous injection of TPPC or ZOX alone were 50 and >50 mg kg^{-1} in this animal model (data not shown). As, among β-lactams, ZOX exhibited the highest synergism *in vitro* against MRSA ATCC33591 when combined with TPPC,[17] ZOX was used in this animal model. Interestingly, despite the finding that TPPC alone (12.5 mg kg^{-1}) exhibited no *in vivo* anti-MRSA activity, the combined administration of TPPC with sub-50% effective dose doses of ZOX remarkably enhanced the efficacies in a dose-dependent manner, as shown in Figure 3. All MRSA-infected mice survived after the combination administration of 12.5 mg kg^{-1} of TPPC with 25 mg kg^{-1} of ZOX. The combination 50% effective dose values of TPPC and ZOX were 12.5 and 13.5 mg kg^{-1}, respectively. This established that the TPPC/β-lactam combination treatment exhibits synergistic anti-MRSA activity both *in vitro* and *in vivo*, which prompted us to investigate its mode of action.

Inhibition of TPPC on β-lactamase activity

Positive controls clavulanic acid and sulbactam inhibited β-lactamase activity at half-maximal inhibitory concentration of 0.37 and 5.7 μg ml^{-1}, respectively, as shown in Figure 4a. However, TPPC

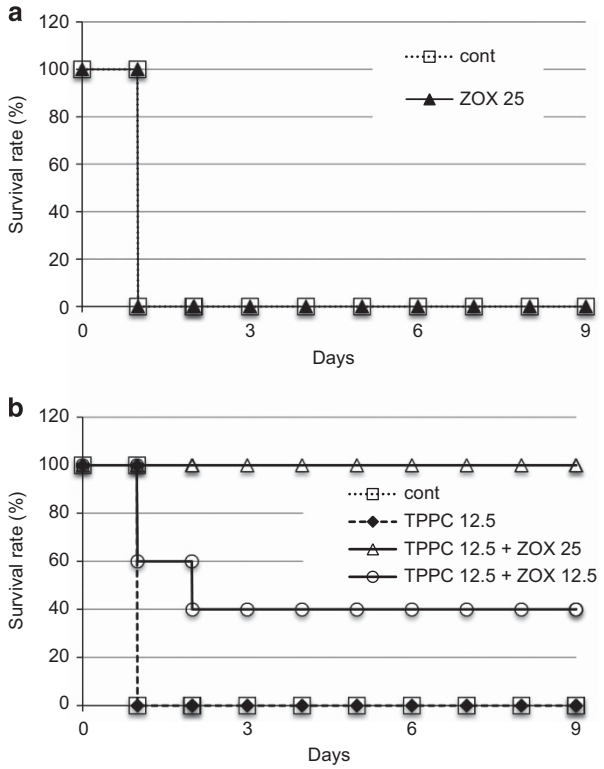

Figure 3 Therapeutic efficacy of ceftizoxime (ZOX) and tripropeptin C (TPPC) alone or in combination in a mouse methicillin-resistant *Staphylococcus aureus* (MRSA) septicemia model. (**a**) Non-treated control (open squares); ZOX, 12.5 mg kg^{-1} (solid diamonds); ZOX, 25 mg kg^{-1} (solid triangles). (**b**) Non-treated control (open squares); TPPC, 12.5 mg kg^{-1} (solid diamonds); TPPC, 12.5 mg kg^{-1}+ZOX 25 mg kg^{-1} (open triangles); TPPC, 12.5 mg kg^{-1}+ZOX, 12.5 mg kg^{-1} (open circles).

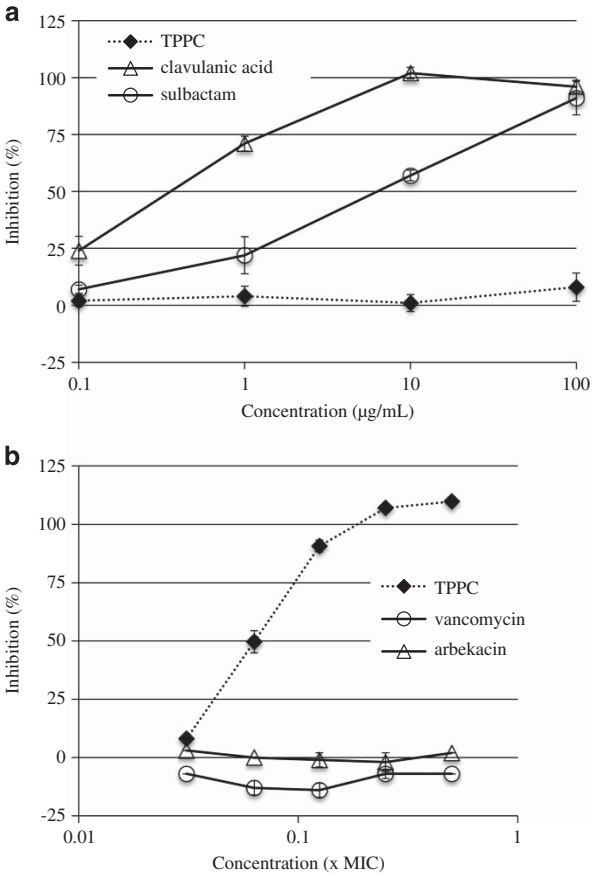

Figure 4 Inhibition of tripropeptin C (TPPC) and other antibacterials on β-lactamase activity and production in the methicillin-resistant *Staphylococcus aureus* (MRSA) strain ATCC33591. (**a**) Effect of TPPC and other inhibitors on β-lactamase activity. TPPC (solid diamonds), clavulanic acid (open triangles) and sulbactam (open circles). Values are expressed as the mean ± s.d. for three replicates. (**b**) Inhibition of TPPC, vancomycin (VAN) and arbekacin (ABK) on cefotiam (CTM)-inducible β-lactamase production. X axis indicates the concentration (μg ml^{-1}) of treated drugs. TPPC (solid diamonds), VAN (open circles) and ABK (open triangles). Values are expressed as the mean ± s.d. for three replicates of one of two independent experiments.

did not inhibit BlaZ β-lactamase activity even at 100 μg ml^{-1}, which was 100× its MIC.

Effect of TPPC on β-lactam-inducible β-lactamase production

CTM-induced β-lactamase production was not reduced by VAN or ABK even at concentrations around their MICs, as shown in Figure 4b. By contrast, TPPC reduced CTM-induced β-lactamase production with an half-maximal inhibitory concentration of 0.06× the MIC, as shown in Figure 4b. Unlike VAN and ABK, TPPC inhibited β-lactam-inducible β-lactamase expression. As the expression of the *mecA* and *blaZ* genes is mutually regulated by the *mecA/I/R1* and *blaZ/I/R1* systems,[21] our findings indicated the possibility that the expression of not only *blaZ* but also *mecA* may be inhibited by TPPC.

Effect of TPPC on the transcription of drug-response genes

To clarify the inhibition of TPPC on the expression of β-lactam-inducible resistance genes, together with the major two-component regulatory systems involved in membrane and cell wall stress, the transcriptions of *gyrA* (control), *blaZ*, *blaI*, *blaR1*, *mecA*, *mecI*, *mecR1*, *vraS*, *vraD* and *pbpB* were examined. MRSA strain ATCC33591 was treated with 1/1024× MIC of CTM, 1/128× MIC of ZOX and/or 1/2× MIC of TPPC and the results are shown in Figure 5a and Supplementary Figures S1 and S2. As expected, CTM treatment greatly enhanced the expressions of *mecA* (3.8-fold) and *blaZ* (35.4-fold) compared with the non-treated group, whereas changes in expressions of *vraS* (1.0-fold), *pbpB* (1.1-fold) and *vraD* (1.1-fold) were not observed. TPPC alone reduced the expressions of β-lactam-inducible resistance genes *mecA* (0.2-fold) and *blaZ* (0.5-fold) and positively induced the expressions of *vraS* (15.3-fold), *vraD* (2.0-fold) and *pbpB* (2.8-fold), which might reflect cell wall biosynthesis inhibition and membrane interactions with TPPC. Even under CTM-induced conditions, the addition of TPPC reduced the expressions of β-lactam-inducible resistance genes *blaZ* (1.9-fold) and *mecA* (0.5-fold) to lower than those of CTM alone and near to the levels observed in the absence of CTM. Under these conditions, TPPC enhanced the expressions of *vraS* (14.4-fold), *pbpB* (2.6-fold) and *vraD* (2.0-fold), which were comparable to those of TPPC alone, indicating that TPPC still affects both cell wall biosynthesis and membrane interactions. The combination effects of TPPC with ZOX on the transcriptions of the genes of interest are shown in Supplementary Figure S2, and their results were similar to those of TPPC with CTM.

The effect of VAN on transcription of drug-response genes was also evaluated for comparison (Supplementary Figure S1). VAN enhanced *vraS* expression (3.6-fold) but did not reduce the expression of β-lactam resistance genes *blaZ* (1.4-fold) and *mecA* (1.8-fold). Following VAN/CTM cotreatment, the expressions of *blaZ* (20.7-fold) and *mecA* (3.5-fold) were much higher than VAN alone, which was

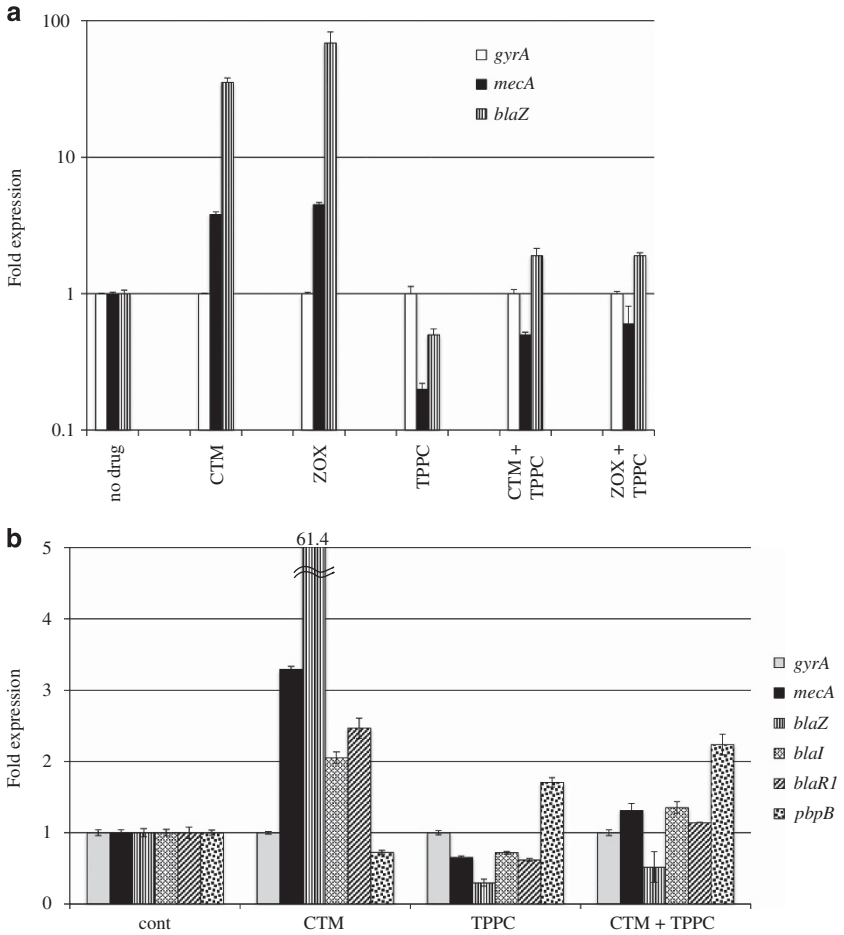

Figure 5 Effect of tripropeptin C (TPPC) and β-lactams on the transcription of drug-response genes. (**a**) Effects of TPPC, cefotiam (CTM) and ceftizoxime (ZOX) on the expression of the *blaZ* and *mecA* genes in methicillin-resistant *Staphylococcus aureus* (MRSA) strain ATCC33591. Values are expressed as the mean ± s.d. for three replicates of one of three independent experiments. (**b**) Effects of TPPC and CTM on transcription levels of drug-response genes in methicillin-sensitive *S. aureus* (MSSA) strain ATCC29213-p*mecA.* Values are expressed as the mean ± s.d. for three replicates of one of two independent experiments.

expected as VAN exhibited neither synergistic anti-MRSA activity nor inhibition of the induction of β-lactamase when treated with β-lactams.

Combination effects of β-lactam and TPPC against a *mecA*-expressing MSSA strain

Since the β-lactam revitalizing activity of TPPC was observed selectively against MRSA but not against MSSA, we then focused on the role of the resistance determinant gene, *mecA*, to investigate further the difference between MSSA and MRSA. We constructed a *mecA*-expressing MSSA strain (ATCC29213-p*mecA*) and evaluated the susceptibility and combinatorial effect of CTM and TPPC against MSSA strain ATCC29213-p*mecA* transformants ($n=5$) and the vector-only control strain, ATCC29213-vector ($n=3$). The MICs of TPPC and CTM against ATCC29213 and its mutant derivatives are summarized in Supplementary Table S3. The *mecA*-overexpressing mutants acquired resistance against CTM and its MIC was 512 μg ml^{-1}. Meanwhile, ATCC29213-vector retained sensitivity to CTM (MIC: 1 μg ml^{-1}), similar to that of the parent strain. By contrast, the MIC of TPPC against ATCC29213-p*mecA* ($n=5$) was 1 μg ml^{-1}, which was the same as its vector control strain ATCC29213-vector. CTM/TPPC combination treatment against ATCC29213-p*mecA* mutants exhibited synergy and their minimum FICI was 0.28, as shown in Figure 2c. By contrast, no synergy was observed in ATCC29213-vector by CTM/TPPC.

These results prompted us to question whether inhibition of *mecA* gene expression is affected by the synergistic effects of TPPC/β-lactam cotreatment. And to investigate this, the inhibitions of TPPC/β-lactam cotreatment on the transcriptions of the *mecA* and *blaZ/I/R1* genes in *mecA*-overexpressing MSSA ATCC29213 were examined. In MSSA ATCC29213-p*mecA*, *blaZ* and *mecA* expression was expected to be β-lactam inducible because of this strain harbors its own *blaZ/I/R1* system, and the *blaZ* (61.4-fold) and *mecA* (3.3-fold) genes were actually found to be induced by CTM, as shown in Figure 5b. As expected, in response to TPPC treatment alone or in combination with CTM, the expression of the *mecA* (0.7- and 1.3-fold) and *blaZ* (0.3- and 0.5-fold) genes was much lower than that of the CTM-treated group, and was comparable to that of the none CTM treatment group, as shown in Figure 5b. These results indicated that TPPC inhibited the transcription of β-lactam-inducible β-lactam resistance genes, leading to reverse β-lactam resistance.

DISCUSSION

In this study, we confirmed the revitalization and synergistic potentiation of β-lactam activity against MRSA by TPPC in an *in vivo* model of mouse MRSA septicemia. We previously reported that the

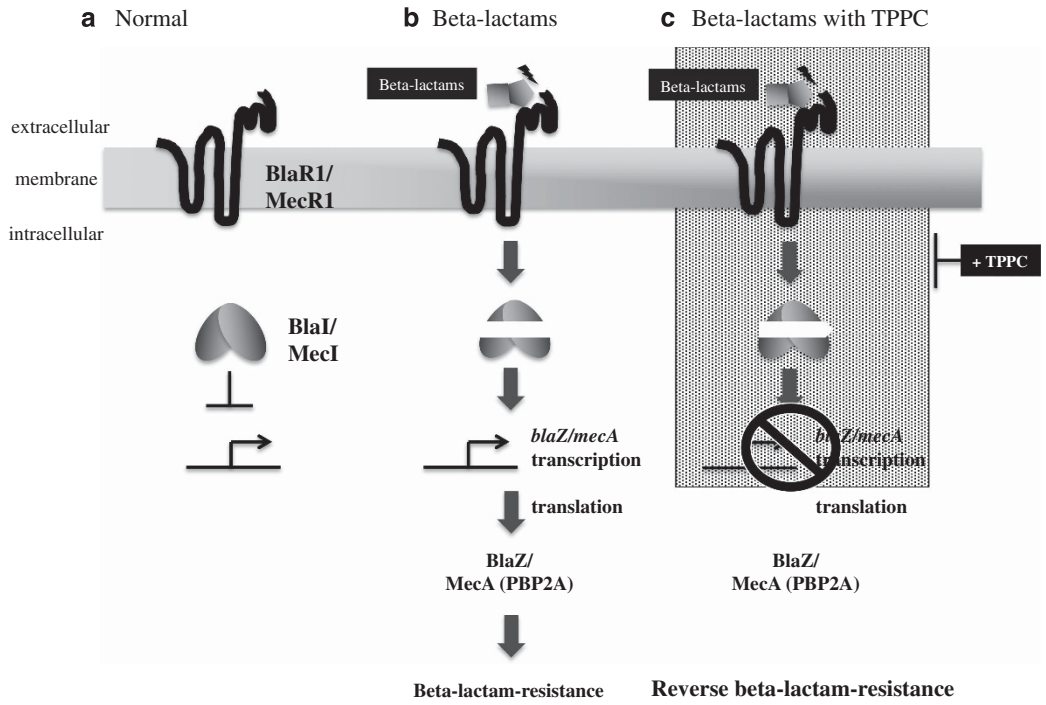

Figure 6 Possible mode of action of reverse β-lactam resistance mediated by tripropeptin C (TPPC). (**a**) In the absence of β-lactam, DNA-binding proteins of BlaI/MecI bind to the operator regions between the *blaZ* and *blaR1* or *mecA* and *mecR1* genes, which results in repression of the expression of these genes. (**b**) β-lactam sensing by the transmembrane transducer BlaR1 or MecR1 activates BlaR1 or MecR1 protease activity, which digests and inactivates the BlaI/MecI repressors via *blaZ/mecA* RNA transcription and leads to *blaZ/mecA* expression and the resulting β-lactam resistance. (**c**) Regardless of the presence of β-lactams, TPPC reduced the β-lactam-inducible β-lactam resistance genes. Possible TPPC inhibitory steps are shown in a gray rectangle.

combination effect of TPPC was observed against only MRSA but not MSSA, and the carboxylic acids in the TPPC molecule are not essential for the synergy with β-lactams, although the existence of carboxylic acids in TPPC is essential to exhibit its potent anti-bacterial activities.[25] This indicates that the mode of action of TPPC-reversing β-lactam resistance in MRSA would be different from that of the TPPC-bactericidal effect caused by peptidoglycan biosynthesis inhibition.[17] To clarify the mode of revitalization of β-lactams by TPPC, we focused on the β-lactam-inducible resistance factors BlaZ and PBP2A, which are encoded by the *blaZ* and *mecA* genes, respectively. Most of MRSA strains have acquired the β-lactamase BlaZ[9] that hydrolyses the β-lactam ring and results in deactivation of anti-bacterial properties. We found that TPPC inhibited the BlaZ production induced by cefotiam treatment in the MRSA strain ATCC33591. This indicated that TPPC likely inhibits the expression of the β-lactam-inducible resistance genes *blaZ* and *mecA*, as they are mutually regulated by the *blaZ/I/R1* and *mecA/I/R1* systems.[21] A characteristic feature of MRSA is the acquisition of the foreign *mecA* gene, of which product PBP2A can polymerize peptidoglycan in the presence of β-lactams because of its low affinity to this class of antibiotics. This results in high-level β-lactam resistance. The possibility we proposed in the previous report[17] that TPPC inhibited MecA (PBP2A) to potentiate the anti-MRSA activity of β-lactam antibiotics is unlikely, as TPPC exhibited the same MIC against both *mecA*-overexpressing ATCC29213 and *mecA*-non-expressing vector control strain. Instead, our results revealed that TPPC reduced the transcription of β-lactam-inducible-β-lactam resistance genes *mecA* and *blaZ* to levels comparable to those not induced by β-lactam. However, regardless of the presence of β-lactams, TPPC affected the cell wall and membrane, as deduced by the enhanced expression of *vraS*, *vraD* and *pbpB* (Supplementary Figure S1). The interaction between TPPC and the bacterial membrane was consistent with our previously reported findings that TPPC led to slight membrane depolarization but did not disrupt membrane function.[15] These observations demonstrated that TPPC disrupts the drug response specifically caused by β-lactam antibiotics.

In the MSSA strain ATCC29213-p*mecA* harboring the *mecA*-expressing plasmid, *blaZ*, *mecA* expression was β-lactam-inducible as expected, probably via its own *blaZ/I/R1* system. When strain ATCC29213-p*mecA* was exposed to CTM, TPPC exhibited synergistic anti-MRSA activity and reverse β-lactam resistance, which differed from its effect on MSSA strains[17] and the vector control strain ATCC29213-vector (BlaZ positive). This demonstrated that the expression of the β-lactamase *blaZ* gene alone is insufficient for TPPC to exhibit synergism with β-lactams. By contrast, it was suggested that both *mecA* expression system and reduction of *mecA* expression are essential for TPPC-synergistic anti-MRSA activity with β-lactams. In fact, TPPC exhibited reduction of the expression of the β-lactam-inducible resistance gene *mecA* (and *blaZ*) in both MRSA ATCC33591 and MSSA ATCC29213-p*mecA*.

Other researchers have previously reported that blocking the signal pathway or transcription of *mecA* and *blaZ* restores β-lactam susceptibility in MRSA.[26–28] Thus, we concluded that TPPC exhibited reverse β-lactam resistance by reducing the expression of inducible-β-lactam resistance genes in *mecA*-expressing strains, including clinically isolated MRSA strains. On the other hand, how β-lactams potentiate the anti-MRSA activity of TPPC remains to be determined and is under investigation.

Next, we considered how TPPC reduces the expression of both *blaZ* and *mecA*. These genes are mutually regulated by the *blaZ/I/R1* and *mecA/I/R1* systems[21] as follows (Figure 6). Binding of the β-lactams to the transmembrane sensor-transducer BlaR1/MecR1 activates their

protease activity resulting in inactivation of BlaI/MecI, repressors of *blaZ/mecA* RNA transcription, and leading to β-lactam-inducible *blaZ/mecA* expression resulting in β-lactam resistance. Within this process, there are at least three possible TPPC inhibitory steps (Figure 6): (1) TPPC interferes with the sensing of β-lactams by BlaR1/MecR1 or their autocatalytic activations; (2) TPPC inhibits the BlaR1/MecR1 protease activity toward BlaI/MecI or the release of inactivated BlaI/MecI from DNA-binding sites; (3) TPPC selectively inhibits the transcription of *blaZ/mecA* genes. As demonstrated in Supplementary Figure S2, TPPC treatment reduced the expression of both the *mecA/I/R1* and *blaZ/I/R1* systems, suggesting that TPPC might block pathways upstream of these signal transductions.

In this study, we revealed that TPPC inhibited the transcription of the β-lactam-inducible resistance genes *mecA* (PBP2A) and β-lactamase (*blaZ*) in MRSA, resulting in the reverse β-lactam resistance. However, the target of TPPC remains to be determined, and identification of the target molecule is currently under investigation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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