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ORIGINAL ARTICLE

Natural lipopeptide antibiotic tripropeptin C revitalizes and synergistically potentiates the activity of beta-lactams against methicillin-resistant *Staphylococcus aureus*

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Tripropeptin C (TPPC) is a natural calcium-ion-dependent lipopeptide antibiotic that inhibits peptidoglycan biosynthesis by binding to prenyl pyrophosphate. It displays very potent antimicrobial activity both *in vitro* and in a mouse model of methicillin-resistant *Staphylococcus aureus* (MRSA) septicemia. The combination of TPPC with all classes of beta-lactams tested (including penam, carbapenem, cephem and oxacephem) showed highly synergistic (SYN) effects against MRSA strains, but not against methicillin-sensitive *S. aureus* strains. These SYN effects were observed with both a checkerboard methodology and a time-kill analysis. The TPPC analog, bis-methyl ester-TPPC, which has neither antimicrobial activity nor the ability to bind prenyl pyrophosphate, also potentiated the activity of beta-lactams. This result indicates that the mechanism of the SYN activity of TPPC is independent of its binding to prenyl pyrophosphate. Therefore, synergistically enhancing the anti-MRSA activities of TPPC and beta-lactams by combining them is a novel and potentially powerful therapeutic strategy for MRSA infections.
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INTRODUCTION

Staphylococcus aureus is one of the major etiological agents of hospital- and community-acquired infections. Strains of methicillin-resistant *S. aureus* (MRSA), first detected in the 1960s, have spread worldwide and acquired multidrug resistance. They now pose a significant health threat, especially for immunocompromised hosts.[1,2] Recently, MRSA strains have been detected that display heteroresistance, intermediate resistance or complete resistance to the antibiotic of last resort, vancomycin.[3–5] Therefore, new and effective strategies are urgently required to deal with and treat MRSA.

Tripropeptin C (TPPC; Figure 1) is a natural, calcium-ion-dependent lipopeptide antibiotic that shows potent antimicrobial activity *in vitro* against Gram-positive pathogens, including antibiotic-resistant strains such as MRSA, penicillin-resistant *Streptococcus pneumoniae* and vancomycin-resistant enterococci.[6,7] TPPC also displayed therapeutic efficacy *in vivo* in a mouse model of MRSA (ATCC33591) septicemia when administered intravenously, and its ED_{50} value was similar to that of vancomycin.[8] TPPC has a favorable toxicological profile, for example, TPPC exhibited no acute toxicity ($300\,mg\,kg^{-1}$) and no sub-acute ($100\,mg\,kg^{-1}$ per day for 14-days) toxicity in mice when administered intravenously.[9] TPPC inhibits peptidoglycan biosynthesis differently from other drugs targeting peptidoglycan biosynthesis, including vancomycin and bacitracin, and shares no cross-resistance with these drugs.[8]

It has been established that TPPC exerts potent anti-MRSA activity. Interestingly, when combined with beta-lactams, the antimicrobial activity of TPPC against MRSA is synergistically enhanced. In this study, the combined effects of TPPC and 14 beta-lactams were evaluated against three strains of methicillin-sensitive *S. aureus* (MSSA) and five strains of MRSA using a microbroth dilution checkerboard methodology.[10] The SYN effects of TPPC/beta-lactams were also observed with a time-kill kinetic analysis. Antibiotics in classes other than beta-lactams were also evaluated as references. The non-antibiotic TPPC derivative, bis-methyl ester-TPPC[11] (BM-TPPC; Figure 1), retains this ability to potentiate beta-lactams, providing some insight into the mechanism underlying the SYN effects of TPPC against MRSA.

We also found that TPPC reduced the bactericidal activity of daptomycin (DAP). The possible mode of action of this antagonism is discussed.

MATERIALS AND METHODS

Bacterial strains

The following strains were used to evaluate the combined effects of TPPC with beta-lactams or other antibiotics: MSSA strains (Smith,[12] RN4220 and FDA209P), clinically isolated MRSA strains (IMC B-1109, IMC B-1114, IMC B-1117 and ATCC 33591) and the community-acquired MRSA reference strain USA300 (ATCC BAA-1556).

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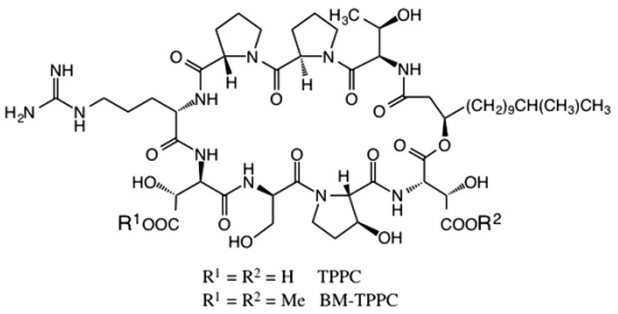

Figure 1 Structures of tripropeptin C (TPPC) and bis-methyl ester-TPPC (BM-TPPC).

Test compounds

TPPC was prepared as reported previously, with slight modifications.[6] BM-TPPC was synthesized as reported previously.[11] We purchased Penicillin G, ampicillin, amoxicillin, carbenicillin, oxacillin (OXA), meropenem, imipenem, cefotaxime, cefaclor, kanamycin, ciprofloxacin and tetracycline from Wako Pure Chemical Industries (Osaka, Japan). Chloramphenicol and cefoxitin were purchased from Sigma-Aldrich (St Louis, MO, USA). Erythromycin was purchased from Nacalai Tesque (Kyoto, Japan). Piperacillin was purchased from Toyama Chemical (Tokyo, Japan). Cefotiam (CTM) was purchased from Takeda Pharmaceutical Company Limited (Osaka, Japan). Ceftizoxime and teicoplanin were purchased from Asteras Pharma (Tokyo, Japan). Flomoxef and vancomycin (VAN) were purchased from Shionogi (Osaka, Japan). DAP and linezolid were purchased from Funakoshi Corporation (Tokyo, Japan). Arbekacin was kindly provided by Meiji Seika Pharma (Tokyo, Japan).

Evaluation of the MICs and fractional inhibitory concentration (FIC) indices

MICs were determined as follows. The 100 × assay concentrations of the test drugs were prepared as twofold dilutions. The final bacterial cultures were prepared by diluting overnight preculture broths to 5×10^5 CFU ml^{-1}. The assay was performed in 96-well microtiter plates. The drugs were added successively to bacterial cultures in Mueller–Hinton broth (Becton Dickinson and Company, Franklin Lakes, NJ, USA) supplemented with 50 μg ml^{-1} calcium ions. The plates were then incubated at 37 °C for 18 h. After culture, the MICs were recorded as the lowest concentrations that completely inhibited bacterial growth, assessed with visual inspection. The FICs were determined with the standard checkerboard methodology.[10] The FIC indices were calcd with 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); FIC index = (FIC of agent A)+(FIC of agent B)

The combined effects were defined as follows: SYN: FIC index ⩽ 0.5; additive (ADD): 0.5 < FIC index ⩽ 1; indifferent: 1 < FIC index ⩽ 4; antagonistic (ANT): FIC index > 4.

Each test was repeated at least three times and the average results are reported. The minimum FIC index was used when the combination effects were described as ADD or SYN, whereas the maximum FIC index was used when the combination effects were described as indifferent or ANT, according to a previous study.[10]

Time-kill analysis

A time-kill analysis was conducted using MRSA strain IMC B-1109 grown in Mueller–Hinton broth (Difco, Franklin Lakes, NJ, USA) supplemented with 50 μg ml^{-1} Ca^{2+} ions. The initial inocula contained 1–3 × 10^6 CFU ml^{-1}. The samples were incubated at 37 °C without shaking. The concentrations of the antibiotics are described in the figure legends. Viability counts in the antibiotic-treated cultures were made at the indicated times after the addition of the antibiotics. The CFU were measured on plates containing 30–300 bacterial colonies. Data are means ± s.d. ($n = 4$).

RESULTS

Combined effects of TPPC with beta-lactams and other key antibiotics

The SYN anti-MRSA activities of TPPC and beta-lactams were observed in an agar diffusion assay (see Supplementary Figure S1B). In contrast, when the MSSA strain Smith was tested, the inhibition zones caused by TPPC and CTM were independent, with no ADD antimicrobial effect (see Supplementary Figure S1A). These results prompted us to examine the combined effects of TPPC and beta-lactams against a range of MSSA and MRSA strains, and we evaluated the effects using the microbroth dilution checkerboard methodology. The combined effects of TPPC and other antibiotics were also evaluated. SYN effect was not observed against the MSSA strains tested, with a few exceptions (the combinations of TPPC and ceftizoxime or cefotaxime in strains Smith and RN4220) in the checkerboard analysis, as shown in Table 1A. ADD effects were observed in most assays on MSSA. In contrast, in all the MRSA strains, including hospital-acquired MRSA and community-acquired MRSA, the FIC indices of the combinations of TPPC and all the beta-lactams tested did not exceed 0.5, indicating that they all displayed SYN effects, as shown in Tables 1A, 2A and B. The SYN antimicrobial activities of TPPC and the beta-lactams against MRSA were also observed in the time-kill analysis, as shown in Figures 2a and b. When 0.125 μg ml^{-1} (1/4 × MIC) TPPC and 8 μg ml^{-1} (1/32 × MIC) OXA were combined, 4.07 $\log_{10}$ CFU ml^{-1} reduction in cell number of MRSA strain IMC B-1109 compared with the number in the control (no drug) culture was observed after 24 h (Figure 2a). In another example, combination of 0.125 μg ml^{-1} (1/4 × MIC) TPPC and 16 μg ml^{-1} (1/16 × MIC) CTM demonstrated 4.61 $\log_{10}$ CFU ml^{-1} reduction in cell number in the same conditions as above (Figure 2b). As shown here, the SYN effects of the combinations of TPPC and beta-lactams observed in the checkerboard assay were reflected in the results of the time-kill analysis. Significantly, the combined effects of TPPC and beta-lactams against MRSA were SYN in all cases.

The combined effects of TPPC and other classes of clinically important drugs were then examined (Table 1B). In contrast to the results for TPPC and beta-lactams, ADD or indifferent effects were observed in most cases. One interesting combination was TPPC and DAP, which displayed an ANT effect as shown in Tables 1B, 2A and B and Figure 2c. These ANT effects were often observed when sub-MIC TPPC was combined with supra-MIC DAP. And we also investigated the time-kill kinetics of the combination of TPPC and DAP against MRSA strain IMC B-1109. As shown in Figure 2c, although 1 × MIC DAP alone displayed potent bactericidal activity against the tested MRSA strain, the combination of 1/2 × MIC TPPC (0.25 μg ml^{-1}) and 1 × MIC DAP (1 μg ml^{-1}) exerted only a small growth inhibitory effect relative to the growth of the control culture. This growth inhibitory effect was somewhat more potent than that exerted by 1/2 × MIC TPPC alone. Therefore, sub-MIC TPPC reduced the bactericidal activity of DAP.

Antimicrobial potentiation with the combination of BM-TPPC and beta-lactams

To investigate the mechanism of this SYN effect, the non-antibiotic TPPC-derivative BM-TPPC[11] (Figure 1) was used. TPPC is reported to inhibit peptidoglycan biosynthesis by binding to the important lipid carrier prenyl pyrophosphate, and the free carboxylic acids of TPPC are essential for its complexation with prenyl pyrophosphate.[8,11] In contrast, BM-TPPC does not bind to the target molecule, resulting in the loss of antimicrobial activity.[11] Surprisingly, BM-TPPC still potentiated the anti-MRSA activity of the beta-lactams, as shown in

Table 1 FIC indices for the combinations of TPPC and clinically important drugs against MSSA (3) and MRSA (5) strains

	MSSA			*MRSA*				
				HA-MRSA				*CA-MRSA*
TPPC + beta-lactams Combined with	*Smith*	*209P*	*RN4220*	*IMC B-1109*	*IMC B-1114*	*IMC B-1117*	*ATCC33591*	*USA300*
A								
Penam								
Penicillin G	1.00 (ADD)	0.75 (ADD)	0.75 (ADD)	**0.25 (SYN)**	**0.38 (SYN)**	**0.38 (SYN)**	**0.38 (SYN)**	**0.28 (SYN)**
Ampicillin	1.00 (ADD)	2.06 (IND)	2.25 (IND)	**0.38 (SYN)**	**0.25 (SYN)**	**0.38 (SYN)**	**0.38 (SYN)**	**0.50 (SYN)**
Amoxicillin	2.25 (IND)	0.75 (ADD)	0.75 (ADD)	**0.25 (SYN)**	**0.50 (SYN)**	**0.50 (SYN)**	**0.31 (SYN)**	**0.38 (SYN)**
Piperacillin	0.75 (ADD)	0.56 (ADD)	0.63 (ADD)	**0.28 (SYN)**	**0.31 (SYN)**	**<0.31 (SYN)**	**<0.38 (SYN)**	**0.50 (SYN)**
Carbenicillin	1.00 (ADD)	0.51 (ADD)	1.00 (ADD)	**0.25 (SYN)**	**0.31 (SYN)**	**<0.50 (SYN)**	**0.31 (SYN)**	**0.38 (SYN)**
Oxacillin	2.25 (IND)	0.63 (ADD)	1.00 (ADD)	**0.19 (SYN)**	**0.25 (SYN)**	**<0.50 (SYN)**	**0.38 (SYN)**	**0.50 (SYN)**
Carbapenem								
Meropenem	4.25 (ANT)	1.00 (ADD)	1.00 (ADD)	**0.19 (SYN)**	**0.25 (SYN)**	**<0.31 (SYN)**	**0.38 (SYN)**	**0.50 (SYN)**
Imipenem	0.75 (ADD)	1.00 (ADD)	0.75 (ADD)	**0.16 (SYN)**	**0.38 (SYN)**	**<0.50 (SYN)**	**<0.31 (SYN)**	**0.25 (SYN)**
Cephem								
Cefotiam	2.25 (IND)	0.75 (ADD)	0.75 (ADD)	**0.16 (SYN)**	**0.25 (SYN)**	**<0.50 (SYN)**	**0.50 (SYN)**	**0.18 (SYN)**
Ceftizoxime	**0.50 (SYN)**	<0.56 (ADD)	**0.37 (SYN)**	**0.13 (SYN)**	**0.25 (SYN)**	**0.50 (SYN)**	**0.26 (SYN)**	**0.38 (SYN)**
Cefaclor	2.25 (IND)	0.75 (ADD)	0.63 (ADD)	**<0.31 (SYN)**	**0.28 (SYN)**	**<0.38 (SYN)**	**0.38 (SYN)**	**0.18 (SYN)**
Cefoxitin	0.56 (ADD)	1.00 (ADD)	1.00 (ADD)	**0.25 (SYN)**	**0.38 (SYN)**	**0.50 (SYN)**	**0.28 (SYN)**	**0.38 (SYN)**
Cefotaxime	**0.50 (SYN)**	1.00 (ADD)	**0.50 (SYN)**	**0.14 (SYN)**	**<0.50 (SYN)**	**0.38 (SYN)**	**<0.50 (SYN)**	**0.50 (SYN)**
Oxacephem								
Flomoxef	1.00 (ADD)	0.75 (ADD)	0.75 (ADD)	**0.19 (SYN)**	**0.25 (SYN)**	**0.50 (SYN)**	**0.31 (SYN)**	**0.31 (SYN)**
B								
Chloramphenicol								
Chloramphenicol	1.00 (ADD)	1.50 (IND)	0.75 (ADD)	1.00 (ADD)	1.00 (ADD)	NT	1.00 (ADD)	0.75 (ADD)
Aminoglycoside								
Kanamycin	**0.50 (SYN)**	1.00 (ADD)	0.75 (ADD)	1.00 (ADD)	2.25 (IND)	NT	ND	**0.50 (SYN)**
Arbekacin	1.00 (ADD)	2.25 (IND)	2.25 (IND)	2.06 (IND)	1.00 (ADD)	4.25 (ANT)	1.50 (IND)	**0.50 (SYN)**
Oxazolidinone								
Linezolid	0.63 (ADD)	1.50 (IND)	0.75 (ADD)	1.00 (ADD)	1.00 (ADD)	0.75 (ADD)	0.75 (ADD)	1.00 (ADD)
Tetracycline								
Tetracycline	0.75 (ADD)	1.50 (IND)	0.75 (ADD)	1.50 (IND)	1.00 (ADD)	NT	0.63 (ADD)	0.75 (ADD)
Macrolide								
Erythromycin	1.00 (ADD)	1.50 (IND)	0.63 (ADD)	ND	ND	NT	ND	0.53 (ADD)
Quinolone								
Ciprofloxacin	1.50 (IND)	1.00 (ADD)	1.50 (IND)	1.00 (ADD)	1.50 (IND)	NT	1.00 (ADD)	**0.38 (SYN)**
Lipopeptide								
Daptomycin	4.50 (ANT)	4.50 (ANT)	2.50 (IND)	4.50 (ANT)	8.50 (ANT)	4.50 (ANT)	5.00 (ANT)	2.25 (IND)
Glycopeptide								
Teicoplanin	1.00 (ADD)	0.53 (ADD)	**0.50 (SYN)**	0.75 (ADD)	0.75 (ADD)	0.75 (ADD)	**0.50 (SYN)**	2.25 (IND)
Vancomycin	1.00 (ADD)	1.00 (ADD)	1.00 (ADD)	0.75 (ADD)	0.62 (ADD)	0.53 (ADD)	0.75 (ADD)	0.75 (ADD)

Abbreviations: ADD, additive; ANT, antagonistic; CA, community acquired; FIC, fractional inhibitory concentration; HA, hospital acquired; IND, indifferent; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; ND, not determined; NT, not tested; SYN, synergistic; TPPC, tripropeptin C.
Combined effects of TPPC and beta-lactams, and TPPC and other clinically important antibiotics are described as FIC indices.
Bold letters indicate synergistic combinations.

Table 2A Representative results for the checkerboard analysis of the effects on MSSA and MRSA strains of TPPC and beta-lactams, and TPPC and DAP

Test organism	*Combined antibiotic*	*FIC index*	*MICs at minimum FIC index*[a] *(TPPC and combined antibiotic)*	MIC_{TPPC} ($\mu g\,ml^{-1}$)	FIC_{TPPC}	$MIC_{combined\ antibiotic}$ ($\mu g\,ml^{-1}$)	$FIC_{combined\ antibiotic}$
MSSA Smith	Oxacillin	2.25 (IND)	0.13 and 0.25	0.5	0.25	0.125	2
	Imipenem	1.00 (ADD)	0.25 and 0.004	0.5	0.5	0.008	0.5
	Cefotaxime	**0.50 (SYN)**	**0.13 and 0.25**	0.5	0.25	1	0.25
	Cefotiam	2.25 (IND)	0.13 and 1	0.5	0.25	0.5	2
	Daptomycin	4.50 (ANT)	0.25 and 0.25	0.5	0.5	0.063	4
MSSA RN4220	Oxacillin	1.00 (ADD)	0.5 and 0.063	1	0.5	0.125	0.5
	Imipenem	0.75 (ADD)	0.5 and 0.002	1	0.5	0.008	0.25
	Cefotaxime	**0.50 (SYN)**	**0.25 and 0.25**	1	0.25	1	0.25
	Cefotiam	0.75 (ADD)	0.5 and 0.25	1	0.5	1	0.25
	Daptomycin	2.50 (IND)	0.5 and 2	1	0.5	1	2
MRSA IMC B-1109	**Oxacillin**	**0.19 (SYN)**	**0.063 and 16**	0.5	0.13	256	0.06
	Imipenem	**0.16 (SYN)**	**0.063 and 1**	0.5	0.13	32	0.03
	Cefotaxime	**0.14 (SYN)**	**0.063 and 4**	0.5	0.13	512	0.01
	Cefotiam	**0.16 (SYN)**	**0.063 and 4**	0.5	0.13	128	0.03
	Daptomycin	4.50 (ANT)	0.25 and 4	0.5	0.5	1	4
MRSA ATCC33591	**Oxacillin**	**0.38 (SYN)**	**0.13 and 32**	0.5	0.25	256	0.13
	Imipenem	**⩽0.38 (SYN)**	**0.25 and 8**	1	0.25	>64	⩽0.13
	Cefotaxime	**⩽0.50 (SYN)**	**0.13 and 128**	0.5	0.25	>512	⩽0.25
	Cefotiam	**0.50 (SYN)**	**0.13 and 64**	0.5	0.25	256	0.25
	Daptomycin	5.00 (ANT)	0.5 and 4	0.5	1	1	4

Abbreviations: ADD, additive; ANT, antagonistic; DAP, daptomycin; FIC, fractional inhibitory concentration; IND, indifferent; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; SYN, synergistic; TPPC, tripropeptin C.
[a]MICs at the maximum FIC indices are shown when the combined effect was antagonistic.
Bold letters indicate synergistic combinations.

Table 2B Representative results for the checkerboard analysis of the effects on MSSA and MRSA strains of BM-TPPC and beta-lactams, and BM-TPPC and DAP

Test organism	*Combined antibiotic*	*FIC index*	*MICs at minimum FIC index*[a] *(BM-TPPC and combined antibiotic)*	$MIC_{BM\text{-}TPPC}$ ($\mu g\,ml^{-1}$)	$FIC_{BM\text{-}TPPC}$	$MIC_{combined\ antibiotic}$ ($\mu g\,ml^{-1}$)	$FIC_{combined\ antibiotic}$
MSSA Smith	Oxacillin	⩽2.00 (IND)	>16 and 0.13	>16	⩽1	0.13	1
	Imipenem	⩽1.50 (IND)	>16 and 0.002	>16	⩽1	0.004	0.5
	Cefotaxime	**⩽0.50 (SYN)**	**4 and 0.5**	>16	⩽0.25	2	0.25
	Cefotiam	⩽1.50 (IND)	>16 and 0.13	>16	⩽1	0.25	0.5
	Daptomycin	⩽5.00 (ANT)	>16 and 0.064	>16	⩽1	0.016	4
MSSA RN4220	Oxacillin	⩽1.50 (IND)	>16 and 0.13	>16	⩽1	0.25	0.5
	Imipenem	⩽1.50 (IND)	8 and 0.004	>16	⩽0.5	0.004	1
	Cefotaxime	**⩽0.50 (SYN)**	**4 and 0.25**	>16	⩽0.25	1	0.25
	Cefotiam	⩽2.00 (IND)	>16 and 0.5	>16	⩽1	0.5	1
	Daptomycin	⩽3.00 (IND)	>16 and 0.13	>16	⩽1	0.063	2
MRSA IMC B-1109	Oxacillin	⩽0.56 (ADD)	8 and 16	>16	⩽0.5	512	0.06
	Imipenem	**⩽0.50 (SYN)**	**4 and 16**	>16	⩽0.25	>64	⩽0.25
	Cefotaxime	**⩽0.25 (SYN)**	**2 and 64**	>16	⩽0.13	>512	⩽0.13
	Cefotiam	**⩽0.38 (SYN)**	**4 and 64**	>16	⩽0.25	>512	⩽0.13
	Daptomycin	⩽5.00 (ANT)	>16 and 0.5	>16	⩽1	0.13	4
MRSA ATCC33591	Oxacillin	**⩽0.50 (SYN)**	**4 and 128**	>16	⩽0.25	512	0.25
	Imipenem	**⩽0.38 (SYN)**	**2 and 16**	>16	⩽0.13	>64	⩽0.25
	Cefotaxime	**⩽0.31 (SYN)**	**4 and 32**	>16	⩽0.25	512	0.06
	Cefotiam	**⩽0.38 (SYN)**	**4 and 64**	>16	⩽0.25	>512	⩽0.13
	Daptomycin	⩽3.00 (IND)	>16 and 0.5	>16	⩽1	0.25	2

Abbreviations: ADD, additive; ANT, antagonistic; BM, bis-methyl ester; DAP, daptomycin; FIC, fractional inhibitory concentration; IND, indifferent; MRSA, methicillin-resistant *Staphylococcus aureus*; MSSA, methicillin-sensitive *S. aureus*; SYN, synergistic; TPPC, tripropeptin C.
[a]MICs at the maximum FIC indices are shown when the combined effect was antagonistic.
Bold letters indicate synergistic combinations.

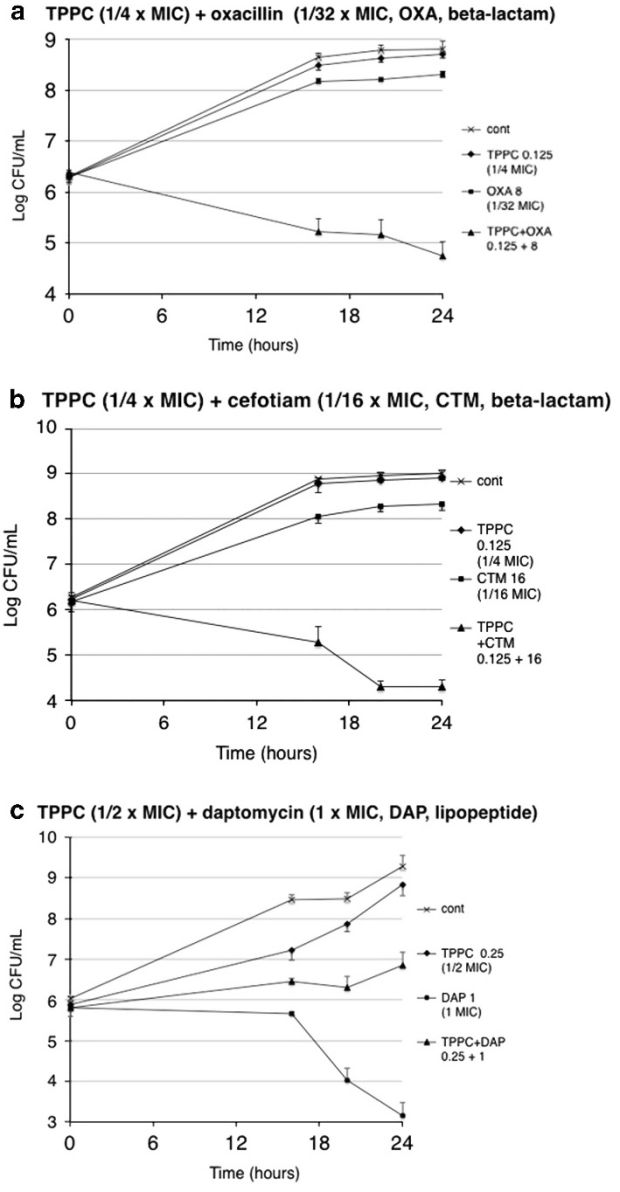

Figure 2 Time-kill kinetic analysis of combinations of (**a**) Tripropeptin C (TPPC) and oxacillin (OXA), (**b**) TPPC and cefotiam (CTM) and (**c**) TPPC and daptomycin (DAP) against methicillin-resistant *Staphylococcus aureus* strain IMC B-1109. Viable cell numbers were counted at the indicated times, where no antibiotic, TPPC alone, another antibiotic alone or a combination of these drugs was used to treat MRSA strain IMC B-1109. The concentrations of the antibiotics used are described in the figures. The *x*-axis and *y*-axis indicate the culture period and the viable cell number in log (CFU ml^{-1}), respectively. Control culture (crosses), TPPC (diamonds), oxacillin, cefotiam or daptomycin (squares, combined antibiotic) and combination treatment (triangles). Data are mean ± s.d. ($n=4$).

Table 2B, despite displaying no anti-MRSA activity when used alone. For example, CTM (>512 μg ml^{-1}) and BM-TPPC (>16 μg ml^{-1}) alone showed no antimicrobial activity against MRSA strain IMC B-1109. However, in combination, these two compounds showed lethal SYN activity against MRSA. In the presence of 16 μg ml^{-1} BM-TPPC, only 2 μg ml^{-1} CTM was sufficient to inhibit the growth of MRSA strain IMC B-1109. This was also true of 16 μg ml^{-1} CTM in the presence of 4 μg ml^{-1} BM-TPPC. Lethal SYN activity was also observed with the combination of BM-TPPC and other beta-lactams, as shown in Table 2B. Lethal SYN activities were also observed in tests with MRSA strain ATCC33591.

Antagonistic effects of TPPC plus DAP against both MSSA and MRSA

The effect of the combination of TPPC and DAP on both MSSA and MRSA was ANT, as shown in Tables 2A and B and Figure 2c. Because TPPC and DAP are both calcium-dependent antibiotics,[8] we evaluated if this antagonism between these two compounds resulted from their competition for limited calcium ions. To investigate this, the combined effects of TPPC and DAP against MSSA and MRSA were evaluated in Mueller–Hinton broth in the presence of excess calcium ions (50, 100, 150 or 200 μg ml^{-1}). TPPC and DAP displayed similar ANT effects at all Ca^{2+} concentrations tested (data not shown). The basal calcium ion concentration, 1.25 mM (50 μg ml^{-1}), was significantly higher than those of TPPC (3.47 μM, 4 μg ml^{-1}) and DAP (9.88 μM, 16 μg ml^{-1}).

DISCUSSION

More than 50 years have passed since the emergence of MRSA. During this period, MRSA has evolved and acquired multidrug resistance, not only against beta-lactams but also against other major antibiotic classes. Therefore, there is an urgent need for the discovery and development of new antibiotics that are effective against contemporary MRSA strains, which are structurally different from existing drugs and are capable of exerting their inhibitory activities *via* novel modes of action. TPPC is structurally different from the compounds already launched or in clinical trials,[13–15] and has a distinct mode of action.[8]

TPPC is effective against MRSA both *in vitro* and *in vivo*.[8] Here, we have demonstrated that in combination with beta-lactams, TPPC exerts SYN antimicrobial effects against MRSA. Because the therapeutic efficacy of the TPPC/beta-lactam combination is expected to be higher than that of each drug alone, the dose required can be reduced. Therefore, the combination therapy may also reduce both adverse effects and costs.

In this report, we also describe the lethal SYN activity of the inactive TPPC analog, BM-TPPC and beta-lactams against MRSA. This result indicates that neither the binding of TPPC to prenyl pyrophosphate nor the presence of free carboxylic acids on the TPPC molecule are essential for the potentiation of beta-lactam activity, but that the inhibition of peptidoglycan biosynthesis is a distinct biological activity of TPPC.

While evaluating the combined effects of TPPC and other antibiotics, we identified a strong antagonism between TPPC and DAP against both MSSA and MRSA. TPPC inhibits peptidoglycan biosynthesis,[8] whereas DAP causes ion leakage and rapid membrane depolarization by the formation of a DAP/phosphatidylglycerol complex on the bacterial membrane.[16,17] A comparison of the modes of action of TPPC and DAP has been reported.[8] Although TPPC and DAP exert their antimicrobial activities *via* different mechanisms, they share similar structural and biological properties.[8] They are both calcium-dependent antibiotics, have an acyl side chain and exert potent antimicrobial activity against Gram-positive pathogens.[8,16] Therefore, we examined if the antagonism was resulted from their competition for calcium ions, but similar ANT effects were observed at all Ca^{2+} concentrations tested. This implies that calcium ion deficiency resulting from their competitive capture by TPPC and DAP is an unlikely explanation of their ANT effects. This antagonism was observed regardless of the order of treatment with these two drugs (data not shown), suggesting that when TPPC is bound to the membrane DAP may be unable to bind and *vice versa*.

However, the anti-MRSA activity of DAP is known to be potentiated when DAP is combined with beta-lactams; clinically relevant studies and the characterization of this effect are in progress.[18,19] It has been reported that beta-lactams targeting penicillin-binding protein 1 (PBP-1) selectively enhance DAP activity against MRSA, whereas beta-lactams with minimal PBP1-binding activity, such as cefoxitin, ceftriaxone, cefaclor and cefotaxime, are less effective.[20] In contrast, the anti-MRSA activity of TPPC was enhanced by all the beta-lactams tested, including the non-selective PBP binders ampicillin, OXA and piperacillin, the PBP1-selective binder meropenem, the PBP2-selective binders cefotaxime and ceftizoxime, the PBP3-selective binder cefaclor and the PBP4-selective binder cefoxitin (Figure 2).[20,21] These results suggest that the mechanism of TPPC SYN anti-MRSA activity differs from that of DAP.

The strategy of beta-lactam resistance in MRSA involves the addition of newly acquired PBP2A to complement the functions of four native staphylococcal PBPs.[22] When combined with beta-lactams, TPPC demonstrated a SYN antimicrobial effect against MRSA, but not MSSA. Because the major difference between MSSA and MRSA is the presence of PBP2A, TPPC is inferred to interact with PBP2A or its substrates. Further analysis of the mechanism of this SYN effect is now in progress.

Beta-lactam antibiotics are ideal because they exert highly selective toxicity against bacteria. However, the emergence and global spread of beta-lactam-resistant pathogens has led to a great demand for effective alternatives. An attractive alternative approach would be to re-sensitize resistant pathogens to existing beta-lactams. It has been clearly demonstrated that TPPC revitalizes and synergistically potentiates the activity of beta-lactams against MRSA, as shown in Tables 1, 2A and B. Many other researchers also have been eagerly screening for beta-lactam potentiators, which has resulted in the recent discovery of the femA binder, cyslabdan;[23–25] the teichoic acid synthesis inhibitors tunicamycin[26] and ticlopidine;[27] and per-6-(4-methoxybenzyl)amino-6-deoxy-beta-cyclodextrin.[28] Although these compounds and TPPC share the capacity to potentiate beta-lactams, TPPC is distinctive in both its structure and biological activity.

This study has demonstrated that TPPC revitalizes and synergistically potentiates the activity of beta-lactams against MRSA strains. This unique effect of TPPC is very interesting as the therapeutic efficacy of TPPC/beta-lactam combination treatment would be expected to be much more effective than for each drug alone and the required dose would therefore be decreased. We hope that observations reported here will contribute the future drug development combating against the refractory multidrug-resistant pathogen, MRSA.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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