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APPLIED MICROBIAL AND CELL PHYSIOLOGY

# Cell-penetrating peptide and antibiotic combination therapy: a potential alternative to combat drug resistance in methicillin-resistant *Staphylococcus aureus*

**Harmandeep Kaur Randhawa[1] · Ankur Gautam[2] · Minakshi Sharma[2] · Rakesh Bhatia[3] · Grish C. Varshney[3] · Gajendra Pal Singh Raghava[2] · Hemraj Nandanwar[1]**

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**Abstract** The diverse pattern of resistance by methicillin-resistant *Staphylococcus aureus* (MRSA) is the major obstacle in the treatment of its infections. The key reason of resistance is the poor membrane permeability of drug molecules. Over the last decade, cell-penetrating peptides (CPPs) have emerged as efficient drug delivery vehicles and have been exploited to improve the intracellular delivery of numerous therapeutic molecules in preclinical studies. Therefore, to overcome the drug resistance, we have investigated for the first time the effects of two CPPs (P3 and P8) in combination with four antibiotics (viz. oxacillin, erythromycin, norfloxacin, and vancomycin) against MRSA strains. We found that both CPPs internalized into the MRSA efficiently at very low concentration (<10 μM) which was non-toxic to bacteria as well as mammalian cells and showed no significant hemolytic activity. However, the combinations of CPPs (≤10 μM) and antibiotics showed high toxicity against MRSA as compared to antibiotics alone. The significant finding is that P3 and P8 could lower the MICs against oxacillin, norfloxacin, and vancomycin to susceptible levels (generally <1 μg/mL) for almost all five clinical isolates. Further, the bacterial cell death was confirmed by scanning electron microscopy as well as propidium iodide uptake assay. Simultaneously, time-kill kinetics revealed the increased uptake of antibiotics. In summary, CPPs assist to restore the effectiveness of antibiotics at much lower concentration, eliminate the antibiotic toxicity, and represent the CPP-antibiotic combination therapy as a potential novel weapon to combat MRSA infections.

**Keywords** Methicillin-resistant *Staphylococcus aureus* · Drug resistance · Drug delivery · Cell-penetrating peptides

Harmandeep Kaur Randhawa and Ankur Gautam equally contributed to this paper.

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✉ Gajendra Pal Singh Raghava
raghava@imtech.res.in

✉ Hemraj Nandanwar
hemraj@imtech.res.in

[1] Bioactive Screening Laboratory, CSIR-Institute of Microbial Technology, Chandigarh 160036, India

[2] Bioinformatic Centre, CSIR-Institute of Microbial Technology, Chandigarh 160036, India

[3] Cell Biology and Immunology Laboratory, CSIR-Institute of Microbial Technology, Chandigarh 160036, India

## Introduction

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a serious health problem that causes mild to life threatening infections (Lowy 1998). MRSA is resistant to multiple antimicrobial agents and has caused major outbreaks in nosocomial environments, limiting the therapeutic options (Kaatz and Seo 1993). It has acquired resistance to a broad array of β-lactam antibiotics, making it difficult to treat (Franciolli et al. 1991). The rate of antibiotic resistance by MRSA is way faster than the discovery of new compounds into clinical practice. Therefore, there is an urgent need to develop new approaches to tackle this drug resistance problem (Ling et al. 2015).

The poor penetration of drug molecules into the bacteria is the major cause of acquiring the drug resistance (Payne et al. 2007). Recently, cell penetrating peptides (CPPs) have emerged as potential mode of drug delivery (Gautam et al.

2012; Vasconcelos et al. 2013) and had been used to deliver small molecules (Pujals et al. 2006), nucleic acids (Lehto et al. 2012), nano-particles (Farkhani et al. 2014), peptides (Alves et al. 2014), and proteins (Ma et al. 2014), successfully into the cells both in vitro and in vivo (Snyder and Dowdy 2004; Vives et al. 2008). CPP-based drug delivery system offers a great potential for improving the intracellular delivery to overcome the cell permeability issue (Huang et al. 2013). CPPs share physical properties such as amphiphilicity and cationic nature with some antimicrobial peptides (AMPs). Recently, CPPs have also been reported to exhibit antimicrobial activity (Oh et al. 2014).

CPP, octaarginine, was used to improve the efficacy of fosmidomycin against *Plasmodium* and *Mycobacterium* species (Sparr et al. 2013). However, the conjugation of drug with the CPP makes it cost-ineffective. In the present study, we explored the potential occurrence of synergism between CPPs, P3 and P8 (Gautam et al. 2015), and antibiotics to combat the drug resistance in MRSA strains. To study the effect of CPPs over drug delivery, we selected four antibiotics: oxacillin, norfloxacin, erythromycin, and vancomycin. Oxacillin exerts its effect by inhibiting enzymes involved in cell wall synthesis, and hence the bactericidal effect is slower as compared to instantaneous membrane disruption by the peptide (Dartois et al. 2005). Norfloxacin, which enters cytoplasm through porins, inhibits intracellular enzymes, DNA-gyrase, and topoisomerase (Nagappa et al. 2004). The binding of vancomycin to the D-Ala-D-Ala prevents cell wall synthesis of the long polymers of *N*-acetylmuramic acid (NAM) and *N*-acetylglucosamine (NAG) that form the backbone strands of the bacterial cell wall, and it prevents the backbone polymers that do manage to form cross-linking with each other. Resistance to vancomycin is due to the presence of operons that encode enzymes for synthesis of low-affinity precursors, in which the C-terminal D-Ala residue is replaced by D-lactate (D-Lac) or D-serine (D-Ser), thus modifying the vancomycin-binding target (Courvalin 2006). Reduced expression of porins arrests the delivery of norfloxacin across the membrane which makes the drug impotent towards these enzymes. The cause of erythromycin resistance is meager uptake of antibiotic by the cells because of permeability barrier at the cell wall level (Piątkowska et al. 2012). In the present study, we have demonstrated that the combinations of CPP and antibiotic at their sub-inhibitory concentrations are highly effective, compared to antibiotics alone, against different strains of *Staphylococci* exhibiting wide degree of drug resistance. However, this combination therapy showed no significant effect against Gram negative strains.

We also demonstrated the sufficient cell membrane damage and cytoplasmic accumulation of oxacillin using scanning electron microscopy (SEM) and propidium iodide (PI) uptake assay. The present study has revealed that CPPs are able to enhance the sensitivity of traditional antibiotics significantly, thus providing a novel and effective way to withstand the bacterial drug resistance. The study has been carried out with special emphasis on combined regimen of CPPs and oxacillin against MRSA to enhance its sensitivity up to thousands times.

## Materials and methods

### Bacterial strains

MRSA strains used in this study are shown in Table 1. The type strains of *Escherichia coli* viz. MTCC 739 and MTCC 618 were obtained from Microbial Type Culture Collection (MTCC), CSIR-IMTECH, Chandigarh. The wild-type clinical isolates of *E. coli* and *Klebsiella pneumoniae* were obtained from Government Medical College & Hospital (GMCH), Sector 32, Chandigarh. NorA-overproducing *Staphylococcus aureus* strain (SA-1199B) and its wild-type strain (SA-1199) was obtained as a kind gift by Dr. G. W. Kaatz (Wayne State University, USA). All strains were maintained, grown, and tested on Muller Hinton Broth and Agar (MHB, MHA from HiMedia, India).

### Antimicrobials and chemicals

CPPs (P3 and P8) with and without FITC-labeled were purchased from GL Biochem (China). Norfloxacin, oxacillin, linezolid, teicoplanin, vancomycin, and propidium iodide (PI) were purchased from Sigma-Aldrich, USA. Erythromycin and Mueller-Hinton broth were obtained from HiMedia, India.

### Cell culture

CHO-K1 cells were grown in Ham's F12 medium supplemented with 10 % heat-inactivated FBS and 1 % penicillin/streptomycin antibiotics. Cells were maintained at 37 °C in 5 % $CO_2$.

### Confocal microscopy

FITC-labeled P3 and P8 (5 μM each) were incubated with ATCC 33591 ($5 \times 10^5$ CFU/ml) at 37 °C for 1 h in PBS. Thereafter, the cells were washed twice with phosphate buffer saline (PBS) and resuspended in 500 μl in the same buffer. Trypsin digestion of the cell membrane-adsorbed peptide (Richard et al. 2003) was carried out by incubating the cells with trypsin (1 mg/ml) for 10 min at 37 °C. After trypsin treatment, cells were washed again with PBS and suspended in 250 μl PBS. Finally, cells were mounted on poly-L-lysine-coated slides with antifade reagent (Invitrogen, Molecular Probes). Localization of fluorescently labeled peptides in the

**Table 1** Susceptibility of various Staphylococcal strains to antibiotics and CPPs used in this study

| Strains of *Staphylococcus aureus* | Oxa (μg/ml) | Nor (μg/ml) | Ery (μg/ml) | Van (μg/ml) | Tei (μg/ml) | Lin (μg/ml) | P3 (μM) | P8 (μM) |
|---|---|---|---|---|---|---|---|---|
| MRSA 01 (Clinical isolate, GMCH) | 7.8 | 125 | 250 | 1.56 | 0.78 | 4 | 50 | 12.5 |
| MRSA 02 (Clinical isolate, GMCH) | 7.8 | 62.5 | 12.5 | 1.56 | 1.56 | 4 | 50 | 12.5 |
| MRSA 03 (Clinical isolate, GMCH) | 15.62 | 125 | 0.78 | 1.56 | 0.78 | 4 | 50 | 12.5 |
| MRSA 831 (Clinical isolate, GMCH) | 2000 | 100 | 25 | 3.12 | 12.5 | 4 | 25 | 12.5 |
| MRSA 839 (Clinical isolate, GMCH) | 7.81 | 500 | 500 | 1.56 | 6.25 | 4 | 6.25 | 12.5 |
| ATCC 33591 (+ve control, MRSA) | 500 | 250 | 250 | 1.56 | 0.78 | 2 | 50 | 12.5 |
| ATCC 43300 (+ve control, MRSA) | 64 | 1.56 | 128 | 1.56 | 0.78 | 2 | 50 | 12.5 |
| ATCC 25923 (−ve control, MSSA) | 1.56 | 3.9 | 0.78 | 1.56 | 0.78 | 1 | 50 | 12.5 |
| *S. aureus* 1199 (Clinical, wild type) | 0.48 | 0.78 | 0.39 | 0.78 | 0.19 | 1.95 | 50 | 12.5 |
| *S. aureus* 1199B (NorA over-expressed) | 1000 | 50 | 6.12 | 6.25 | 3.12 | 7.8 | 50 | 25 |
| *S. aureus* MTCC 96 (Non-clinical) | 3.12 | 0.39 | 0.78 | 1.56 | 1.56 | 1.56 | 50 | 6.25 |

*Oxa* oxacillin, *Nor* norfloxacin, *Ery* erythromycin, *Van* vancomycin, *Tei* teicoplanin, *Lin* linezolid, *GMCH* Govt. Medical College & Hospital, Sector 32, Chandigarh

bacterial cells was analyzed immediately using Nikon A1R (Japan) confocal microscope.

### Determination of MIC

MIC of peptides and antibiotics were determined against all bacterial strains in MHB with an initial inoculum of $5 \times 10^5$ CFU/ml as per CLSI broth micro-dilution method (CLSI 2006). After 24 h of incubation at 37 °C, MIC was interpreted as the lowest concentration of peptide or antibiotic that completely inhibited the visible growth of bacteria. Three independent sets of experiments in triplicates were performed for each agent.

### In vitro combination study and synergy

Two-fold serial dilutions of antibiotics (for *S. aureus*—norfloxacin, erythromycin, oxacillin, and vancomycin; for *E. coli and K. pneumoniae*—ampicillin and imipenem) in combination with CPP's were evaluated in a similar way as described above except that fixed concentration of CPP (≤1/4 MIC) at which peptide alone did not show any antibacterial activity against MRSA. Correlation between antibiotics with CPPs was evaluated by fractional inhibitory concentration index (FICI). The FICI is the sum of the FICs of each of the drugs, which were defined as the MIC of each drug when used in combination divided by the MIC of each drug when used alone. The FIC index was calculated as follows: FIC = (MIC of drug A in combination/MIC of drug A alone) + (MIC of drug B in combination)/MIC of drug B alone). FIC indices were interpreted as follows FICI was as follows: ≤0.5, synergy; >0.5 to ≤1.0, additive; >1.0 to ≤2.0, indifference; and >2.0, antagonism.

### Hemolytic activity

Hemolytic activity of the peptides was analyzed by following the standard protocol with slight modification (Ghosh et al. 1997). Human blood was washed three times with PBS (50 mM sodium phosphate, 150 mM NaCl, pH 7.2) by centrifugation at 900×*g* for 5 min at 4 °C. The cell suspension of $4 \times 10^8$ cell/ml was prepared, and 50 μl of this suspension was added to 96-well microtiter plate (Nunc, USA). Fifty micrometers of each peptide was subsequently added to the respective wells and incubated at 37 °C for 2 h. The supernatant was collected by centrifugation at 900×*g* for 5 min, and hemolytic activity was assessed by measuring the absorbance at 541 nm. For 100 % hemolysis or baseline values, control wells were treated with distilled water or PBS, respectively. Percentage hemolysis was calculated with respect to the absorbance of distilled water-treated wells.

### Cellular toxicity of CPPs

The toxicity of P3 and P8 was assessed against eukaryotic cells (CHO-K1 and HeLa cells) using the CellTiter 96®AQueous Non-Radioactive Cell Proliferation Assay kit from Promega. In brief, $5 \times 10^3$ cells/well were seeded in 96-well microtiter plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum for at least 12 h. Cells were further incubated with different concentrations (5, 10, 20, 40, and 80 μM, respectively) of peptides for 24 h. Untreated cells were taken as control. At the end of incubation period, the peptide solution was discarded and replaced with the MTS assay reagent. Cells were placed back into 37 °C humidified atmosphere with 5 % $CO_2$ for 4 h. Samples were prepared in triplicate, and the absorbance was measured at 490 nm with an Infinite F200 plate reader (Tecan

Systems Inc.). The survival of cells relative to the control was calculated by taking the ratio of the $A_{490}$ values.

### Time kill assay

An overnight culture of two strains of MRSA (MRSA 831 and ATCC 33591) was diluted with fresh CaMHB ~$1.5 \times 10^5$ CFU/ml according to the method described previously (McKay et al. 2009), with slight modifications. P8 at synergistic MIC was added in combination with oxacillin, norfloxacin, erythromycin, and vancomycin at 1×, 2×, and 1/2× MIC, respectively. Aliquots taken at specific time points were serially diluted in PBS and plated in triplicates on MHA plate. Peptide free inoculation was taken as control. CFU was counted after 24 h incubation at 37 °C.

### Bacteriolysis by $OD_{600}$ measurement

The effectiveness of combination approach was evaluated on ATCC 33591 by previously described method (Isnansetyo and Kamei 2003), with slight modification. Briefly, the bacterial suspension was adjusted to $OD_{600} \approx 0.20$ by diluting logarithmic growth in MHB. P8 and antibiotics at their synergistic MIC were added in the plastic cuvette containing 1 ml of diluted bacteria. Cuvette without bacteria but with same corresponding dilutions of antibiotics and peptide served as a control. Samples were monitored at $OD_{600}$ at specific time points. The assay was performed twice in triplicates.

### Scanning electron microscopy

Standard inocula of MRSA 33591 ($1 \times 10^7$ bacteria/ml) were harvested by centrifugation, suspended in PBS, and exposed to the combination of oxacillin and P8 at their synergistic concentration. P8 and oxacillin alone were taken as control. After incubation at 37 °C for 4–5 h, the bacterial cells were centrifuged at 8000 rpm for 10 min, pellets were washed and re-suspended in PBS followed by overnight treatment with Karnovsky's fixative at 4 °C. Thereafter, samples were dehydrated through a graded series of ethanol (30, 50, 70, 90, and 100 %). The samples were treated with pure tertiary butyl alcohol for 30 min followed by lyophilization and gold coating. Specimens were examined using a scanning electron microscope.

### PI uptake assay

Alterations in membrane permeability was studied by measuring the change in fluorescence of PI in MRSA 33591 as described earlier (Nobles et al. 2013), with slight modifications. Briefly, the cell suspension was prepared in a similar manner as in $OD_{600}$ experiment with the only difference in the incubation time (24 h). Thereafter, cells were centrifuged; pellets were washed and re-suspended in PBS. Cells were treated with PI at 10 μM and aliquots of 200 μl were transferred in triplicate to a 96-well plate, incubated in dark at room temperature for 15 min. With excitation wavelength of 535 nm and emission wavelength of 625 nm, PI fluorescence was monitored for 30 min using Synergy HT Multi-Mode Microplate reader (BIOTEK, Winooski, VT, USA).

## Results

### Susceptibility of MRSA strains to antibiotics and CPPs

All strains, used in the bioassays for methicillin resistance, were as per the CLSI guidelines. About 20 clinical strains were procured from local hospital and their MRSA status was determined as per the CLSI guidelines. Based on the oxacillin resistance, strains viz. MRSA 01, MRSA 02, MRSA 03, MRSA 831, and MRSA 839 were selected for further studies. The resistance pattern of these strains against the battery of antibiotics, including oxacillin, norfloxacin, erythromycin, vancomycin, linezolid, and teicoplanin was determined and summarized in Table 1. In this study, we have used two recently characterized CPPs, P3 and P8 (Gautam et al. 2015), and their MIC values were determined (Table 1). The sequences and properties of these two CPPs are summarized in Table 2.

To evaluate whether CPPs traverse the *Staphylococcus* cell membrane, we examined the internalization of both CPPs in ATCC 33591 at sub-inhibitory concentrations using confocal microscopy. As seen in Fig. 1, both CPPs were internalized into the bacteria at concentration examined. Susceptibility profile of CPPs against ATCC 33591 as well as other *Staphylococcus* strains suggested that both CPPs did not show any potent antibacterial activity as the MIC values were ≥12.5 μg/ml.

### In vitro combination study and synergism in MRSA

Overall MIC pattern of various antibiotics against MRSA strains showed the predominant resistance to oxacillin, erythromycin, and norfloxacin (Table 1). Since vancomycin and daptomycin are the only antibiotics approved by US Food and Drug Administration for the treatment of *S. aureus* infections, we have also considered vancomycin. So, these antibiotics were selected for combinations studies with CPPs. The fold-reduction in MICs of all three four antibiotics in the combination of various sub-inhibitory concentrations of peptides is illustrated in Table 3. P3 and P8 could reduce the MIC of oxacillin, norfloxacin, and vancomycin in most of the MRSA strains, including NorA overexpressed strain (Table 4). We found the synergistic effect of P8 with norfloxacin against wild-type as well as NorA over-expressed strain up to 3 μM.

**Table 2** Cell-penetrating peptides used in this study

| Peptide designation | Amino acid sequence | Length | M. Wt. | Charge | Hydrophobic amino acid |
|---|---|---|---|---|---|
| P3 | RRRQKRIVVRRRLIR | 15 | 2061.5 | +9 | 33.3 % |
| P8 | RRWRRWNRFNRRRCR | 15 | 2274.64 | +9 | 20 % |

However, this synergistic effect was not observed when CPPs was used in combination with amoxycillin and carbapenem against Gram–ve strains (clinical isolates of *E. coli and Klebsiella* sp. Table S1).

The significant fold-reduction in MICs of antibiotics was observed even at 1/4th MIC (3.0 μM) of P8 whereas P3 at higher concentration (10 μM) could show synergy with oxacillin and vancomycin against a broad range of MRSA. In addition, P3 at 10 μM showed synergistic effect with erythromycin and norfloxacin against very selective MRSA strains but the lower concentration of P3 did not work effectively. Interestingly, P3 did not work synergistically with any antibiotic except vancomycin against control strain ATCC 33591 even at 10 μM concentration whereas it showed 65,536-fold reduction in MIC with oxacillin, 8192-fold reduction with norfloxacin, 32-fold reduction with erythromycin, and 2-fold reduction with vancomycin against MRSA 831. On the other hand, P8 at 3.12 μM had proved as better CPP against ATCC 33591, which exhibit 32-fold reduction with oxacillin, 128-fold with erythromycin, 1024-fold with norfloxacin, and 32-fold reduction with vancomycin. Therefore, the combination of P8 at ≥3.12 μM with sub-inhibitory concentration of antibiotics is the best approach and studied further on ATCC 33591. As per FICI index, synergism (FICI index, synergy ≤0.5 and partial synergy >0.5 to ≤1) was observed against most of the strains when treated with sub-inhibitory concentrations of both P8 as well as antibiotics. P8-antibiotic combinations showed the synergistic effect against all other MRSA strains at 3.0 μM irrespective of antibiotics or its nature or mode of action. In one of the clinical strain MRSA 831, P3 at 10 μM showed synergistic effect with all antibiotics summarized in Table 3.

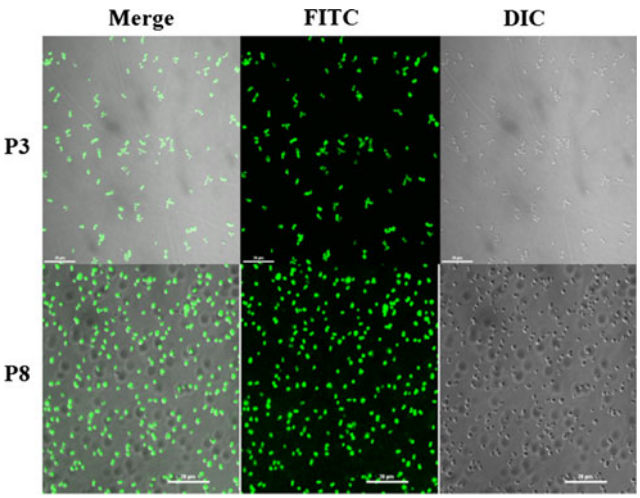


**Fig. 1** Internalization of CPPs in MRSA. Bacterial cells were incubated at 37 °C for 1 h in PBS containing the FITC-labeled P3 and P8 (5 μM each). At the end of the incubation period, bacterial cells were washed twice with PBS, treated with trysin, and re-suspended in PBS. Finally, cells were mounted on slides and analyzed immediately by confocal microscope

## Hemolytic activity and cellular toxicity of CPPs

We determined hemolytic activity of these CPPs in normal red blood cells and results are shown in Fig. 2a. As shown, none of the peptides showed significant hemolytic activity at 50 μM suggesting that these peptides could be used as drug delivery vehicle. We also determined the toxicity of both CPPs on normal cells. For this, we examined the cytotoxicity of P3 and P8 on CHO-K1 and HeLa cells. Both peptides did not show significant cytotoxicity up to 40 μM when tested on CHO-K1 cells. However, at much higher concentration (80 μM), 20–30 % cytotoxicity was observed (Fig. 2b). Similar results were observed in HeLa cells where both peptides did not show any significant cytotoxicity (Gautam et al. 2015).

## Bacterial killing kinetics

Time kill results of P8-oxacillin, P8-norfloxacin, P8-erythromycin, and P8-vancomycin against ATCC 33591 are illustrated in Fig. 3a–d. Antibiotics showing apparent synergy with all four antibiotics in the screening tests were confirmed by time kill kinetics. The rate of bacterial killing by oxacillin (15.62 μg/ml) when exposed to P8 at 3.0 μM exhibited a bactericidal effect with a 3-$Log_{10}$ bacterial population reduction (99.9 % clearance) at 5 h time, and similar effect was observed at 4 h when synergistic MIC increased to 2× (Fig. 3a). Erythromycin achieved >3-$Log_{10}$ bacterial population reduction by 1 h early at 1× MIC (1.92 μg/ml) and 2× MIC compared to oxacillin but at 0.5× synergistic MIC, regrowth occurred after 3-$Log_{10}$ reduction at 4 h (Fig. 3b). However, norfloxacin killed the bacteria, at 12 and 6 h at synergistic MIC (0.24 μg/ml) and 2× synergistic MIC, respectively. At 0.5× synergistic MIC, 2-$Log_{10}$ reduction occurred but re-growth was observed after 24 h (Fig. 3c). Similarly, with vancomycin and P8, bacterial killing was observed at 6 and 5 h at synergistic MIC and 2× synergistic MIC, respectively (Fig. 3d). It was observed that at 2× synergistic MIC for all antibiotics, there is a decrease in killing time to some extent. Taken all results together, it can be stated that the

**Table 3** Effect of cell penetrating peptides (P3 and P8) on MIC modulation of various antibiotics

| Strains of *Staphylococcus aureus* | Fold reduction (FICI) | | | |
|---|---|---|---|---|
| | P3 (5 μM) | P3 (10 μM) | P8 (5 μM) | P8 (3.12 μM) |
| Oxacillin | | | | |
| ATCC 33591 | – (1.1) | – (1.2) | 512 (0.40) | 32 (0.27) |
| ATCC 43300 | – (1.1) | 128 (0.20) | 2048 (0.40) | 512 (0.24) |
| ATCC 25923 | – (1.1) | 4 (0.45) | 32 (0.43) | 8 (0.36) |
| MRSA 01 | – (1.1) | 8 (0.32) | 16 (0.46) | 4 (0.49) |
| MRSA 02 | – (1.1) | 4 (0.45) | 8 (0.52) | 4 (0.49) |
| MRSA 03 | – (1.1) | 16 (0.26) | 32 (0.43) | 8 (0.36) |
| MRSA 831 | 512 (0.2) | 65,536 (0.40) | 65,536 (0.40) | 4096 (0.24) |
| MRSA 839 | 65 (0.81) | – (0.81) | 128 (0.40) | 32 (0.27) |
| Erythromycin | | | | |
| ATCC 33591 | – (1.1) | – (1.2) | 1024 (0.40) | 128 (0.25) |
| ATCC 43300 | – (1.1) | – (1.2) | 512 (0.40) | 32 (0.27) |
| ATCC 25923 | – (1.1) | – (1.2) | 4 (0.65) | 2 (0.74) |
| MRSA 01 | – (1.1) | – (1.2) | 128 (0.40) | 32 (0.27) |
| MRSA 02 | – (1.1) | – (1.2) | 4 (0.65) | 2 (0.74) |
| MRSA 03 | – (1.1) | – (1.2) | 2 (0.90) | 2 (0.74) |
| MRSA 831 | 4 (0.45) | 32 (0.43) | 2048 (0.40) | 256 (0.24) |
| MRSA 839 | – (1.8) | – (1.8) | 8192 (0.40) | 512 (0.24) |
| Norfloxacin | | | | |
| ATCC 33591 | – (1.1) | – (1.20) | 8192 (0.40) | 1024 (0.24) |
| ATCC 43300 | – (1.1) | – (1.2) | 8 (0.52) | 2 (0.72) |
| ATCC 25923 | – (1.1) | 4 (0.44) | 8 (0.52) | 2 (0.74) |
| MRSA 01 | 4 (0.35) | 4 (0.45) | 4 (0.65) | 2 (0.74) |
| MRSA 02 | 2 (0.34) | 8 (0.32) | 8 (0.52) | 4 (0.49) |
| MRSA 03 | – (1.1) | – (1.2) | 4 (0.65) | 2 (0.74) |
| MRSA 831 | 16 (0.26) | 8192 (0.40) | 8192 (0.40) | 1024 (0.24) |
| MRSA 839 | – (1.8) | – (1.8) | 128 (0.40) | 16 (0.30) |
| Vancomycin | | | | |
| ATCC 33591 | – (1.1) | 4 (0.45) | 128 (0.40) | 32 (0.27) |
| ATCC 43300 | – (1.1) | 16 (0.26) | 256 (0.40) | 32 (0.27) |
| ATCC 25923 | – (1.1) | 2 (0.70) | 32 (0.43) | 8 (0.36) |
| MRSA 01 | – (1.1) | – (1.2) | 8 (0.52) | 2 (0.74) |
| MRSA 02 | – (1.1) | 4 (0.45) | 64 (0.41) | 16 (0.30) |
| MRSA 03 | – (1.1) | 2 (0.70) | 16 (0.46) | 8 (0.37) |
| MRSA 831 | – (1.2) | 2 (0.90) | 512 (0.40) | 64 (0.26) |
| MRSA 839 | – (1.1) | – (1.2) | 32 (0.43) | 8 (0.36) |

–No fold reduction in the presence of CPPs. FICI was as follows ≤0.5 synergy; >0.5 to ≤1 partial synergy; >1 to ≤4 additive or indifference; and >4 antagonism

**Table 4** Effect of CPP on norfloxacin delivery in engineered strain

| | MIC of antibiotics and CPP | | | Fold reduction (FICI) | | |
|---|---|---|---|---|---|---|
| | Norfloxacin (μg/ml) | CPP-3 (μM) | CPP-8 (μM) | CPP-3 (10 μM) | CPP-8 (5 μM) | CPP-8 (3.0 μM) |
| *S. aureus* 1199 (wild-type clinical isolate) | 0.78 | 50 | 12.5 | 2 (0.7) | 16 (0.46) | 4 (0.49) |
| *S. aureus* 1199B (NorA overexpressed strain) | 50 | 50 | 25 | 2 (0.7) | 8(0.32) | 4 (0.37) |

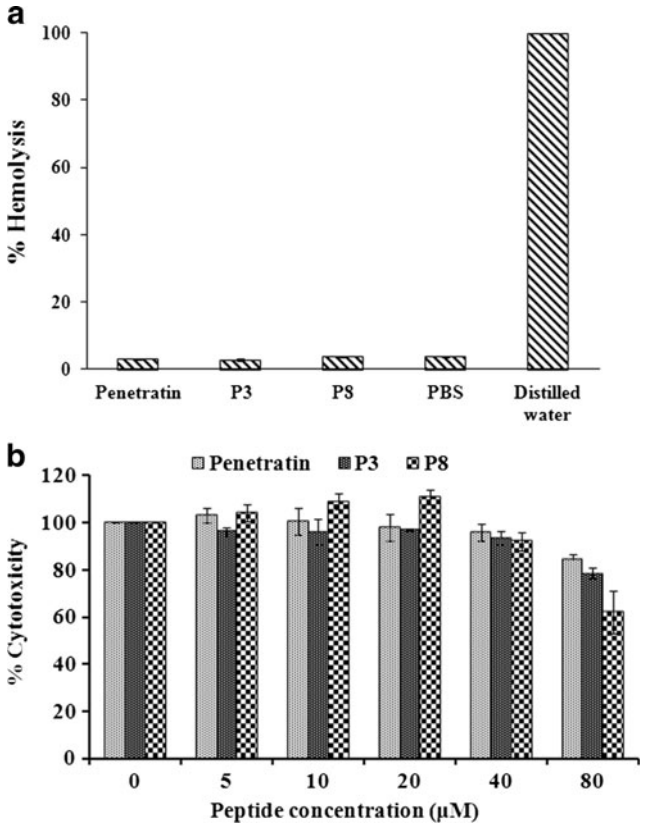


**Fig. 2** Toxicity study of CPPs. **a** The hemolytic activity of peptides. Human erythrocytes were treated with P3 and P8 (50 μM) for 2 h at 37 °C. Distilled water was taken as positive control, and PBS was taken as negative control for which hemolysis was defined as 100 and 0 %, respectively. **b** *In vitro* cytotoxicity of peptides. Overnight grown CHO-K1 cells were incubated with increasing concentrations (0, 5, 10, 20, 40, and 80 μM) of P3 and P8 for 24 h at 37 °C. Cell viability was measured by MTS assay. Viability of control (cells without peptide treatment) was taken as 100 %, and viabilities of cells treated with increasing concentrations of peptides were plotted as percentage of control. Error bar in all figures represent the standard errors of the mean ($n = 3$)

combination approach reduced the viable count >3log$_{10}$ when compared with the starting live bacterial population and the bactericidal effect was demonstrated.

### Bacteriolysis

Bacteriolytic effect was assessed by measuring $OD_{600}$ value of synergistically treated ATCC 33591 at various time points. When these cells were treated with combination of P8 (3.0 μM) and three different antibiotics at their synergistic MICs (erythromycin 1.92, norfloxacin 0.24, and oxacillin 15.52 μg/ml), there was a rapid decreased in $OD_{600}$ of synergistically treated cells compared to untreated cells and peptide control (Fig. 4). Erythromycin–P8 combination reduced the turbidity to 99 % after 6 h whereas norfloxacin–P8 combination took more than 12 h to reduce it at the same level. Oxacillin–P8 lysed the cells at a higher rate than norfloxacin–P8 with more than 70 % reduction in turbidity in 6 h and 99.9 % occurred in 8 h. In contrast, culture treated with peptide alone at 3 μM had no effect on culture turbidity within the same time period.

### Influence of CPPs on membrane permeability and antibiotic delivery

The findings obtained in time kill assay and bacteriolysis assay were further confirmed by SEM and PI assay at killing concentration of oxacillin and P8.

### Scanning electron microscopy

To gain insight into morphological changes in *Staphylococcus* cell membrane, SEM studies were carried out for untreated, P8 treated, oxacillin treated, and P8-oxacillin-treated cells of ATCC 33591 at synergistic concentration of oxacillin. Untreated bacteria showed bright smooth surface with no apparent cellular debris (Fig. 5a); however, most of oxacillin treated (15.52 μg/ml) cells were found intact without any cell-membrane damage, although cell aggregation was observed (Fig. 5b). P8 (3.0 μM) treated cells (Fig. 5c) showed only slight aggregation of bacterial cells. But, combination of P8 and oxacillin treatment resulted in huge amount of aggregated cells along with collapsed structure and disruption of the cell membrane and prominently visible debris from the cells (Fig. 5d). Hence, the SEM studies support the data obtained in various combination experiments conducted previously. Morphology of the oxacillin–P8 treated bacteria is reasonably altered as compared with controls.

### PI uptake assay

Since PI shows enhanced fluorescence only when it binds to DNA (Nobles et al. 2013), therefore, we have evaluated the effect of P8–oxacillin combination of membrane integrity, if any, using PI uptake assay. Cells treated with combination of P8 and oxacillin showed a significant increase in the PI fluorescence after 24 h whereas negligible PI fluorescence was observed in cells treated with either P8 alone or oxacillin alone and untreated cells (Fig. 6). These results suggested that the combination of P8 and oxacillin caused loss of membrane integrity of ATCC 33591 after 24 h incubation and membrane became more permeable. Hence, cellular material such as DNA exposed to PI when added after 24 h of incubation and showed enhanced fluorescence.

## Discussion

Life-threatening infections caused by MRSA have become one of the most serious public health concerns. Developing new antibiotics is a time-consuming, costly process, and

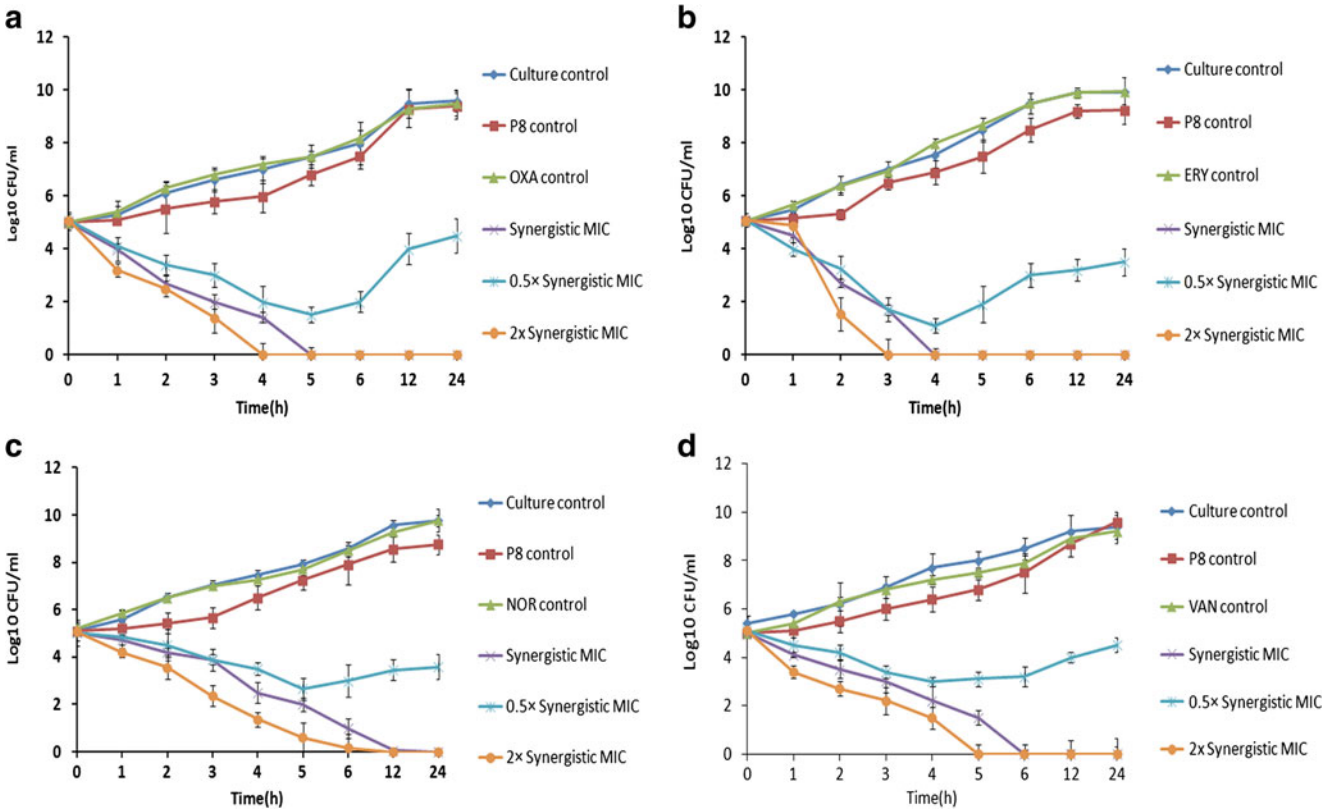


**Fig. 3** Synergistic killing of MRSA 33591 at 0.25× MIC of P8 along with four different antibiotics by time-kill assay. **a** Cultures were incubated at 7.76 μg/ml (0.5× Synergistic MIC), 15.52 μg/ml (Synergistic MIC), and 31.04 μg/ml (2× Synergistic MIC) of oxacillin; **b** at 0.96 μg/ml (0.5× Synergistic MIC), 1.92 μg/ml (Synergistic MIC), and 3.84 μg/ml (2× Synergistic MIC) of erythromycin; **c** at 0.12 μg/ml (0.5× Synergistic MIC), 0.24 μg/ml (Synergistic MIC), and 0.48 μg/ml (2× Synergistic MIC) of norfloxacin; and **d** at 0.024 μg/ml (0.5× Synergistic MIC), 0.048 μg/ml (Synergistic MIC), and 0.097 μg/ml (2× Synergistic MIC) of vancomycin in combination with P8 at 3 μM. Samples were obtained at different time points and then plated and counted. The control with peptide diluent (sterile water) was as untreated/blank samples. Samples treated with only peptide and with only antibiotic at synergistic MIC were used as a control. The killing curves were identical (overlapping in the figure) for untreated culture control and antibiotic control at MIC concentration. The results are given as mean SD ($n = 3$)

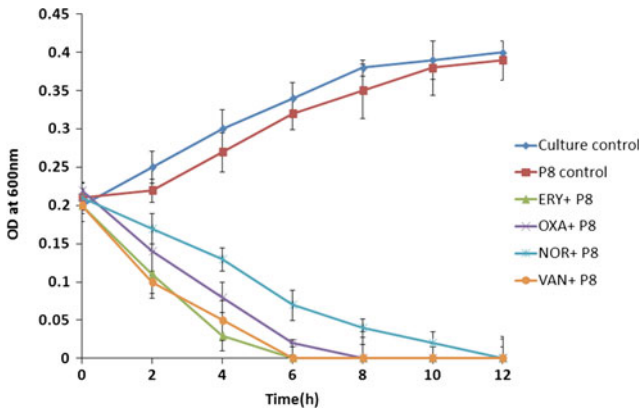


**Fig. 4** Time kill assay. MRSA ATCC 33591 was exposed to synergistic MIC of oxacillin, erythromycin, norfloxacin, and vancomycin with P8 at 3 μM were observed by measuring the $OD_{600}$ values at various time points. Culture with only sterile water as a control, culture with only peptide (3 μM) served as peptide control. Results are representative of two separate experiments, each done in triplicate. Error bars represent standard deviation values

success rate is very low. Therefore, there is a need to explore the novel therapeutic strategies to overcome the alarming drug-resistance in MRSA (Kaatz and Seo 1993; Ling et al. 2015). Till now, various antibiotics have been tested against different strains of MRSA. The poor transmembrane drug permeability, which is one of the major causes for resistance development (Nagappa et al. 2004; Piątkowska et al. 2012), can be improved by CPP-mediated intracellular drug delivery. Previous reports have depicted the potential of short polycationic CPP to penetrate most of the biological cell membranes without causing significant membrane damage (Pujals et al. 2006; Vasconcelos et al. 2013). Therefore, the focus of the present study was to develop the novel antibiotic–CPP combination to increase the susceptibility of MRSA to conventional antibiotics.

Although CPPs have been extensively used as a drug delivery vehicle in eukaryotic systems (Gautam et al. 2012), particularly in cancer cells, their use as transport vehicle in the bacterial system is comparatively less explored. Only a

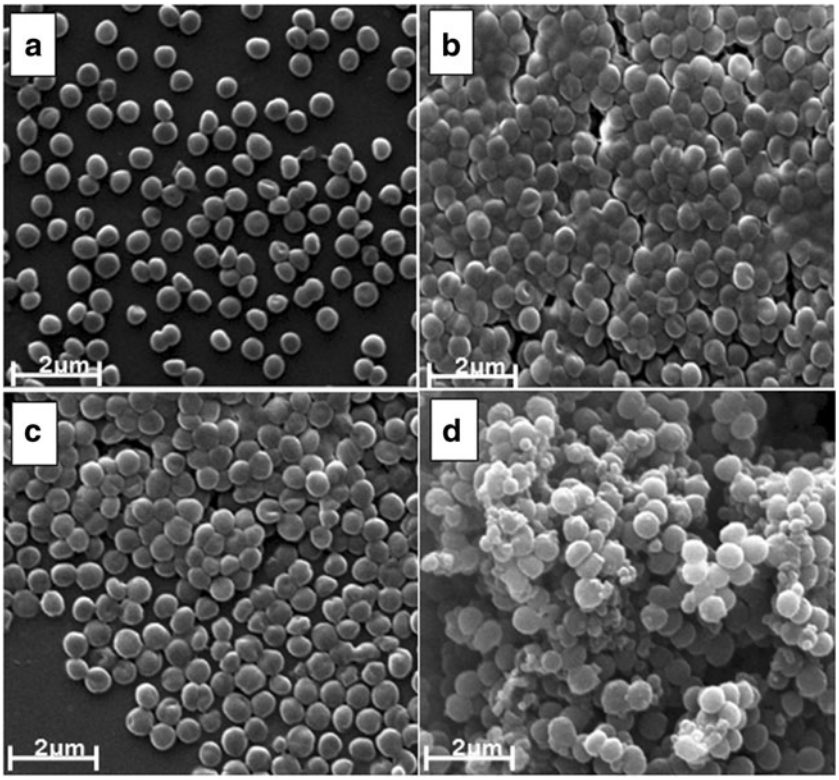


**Fig. 5** Scanning electron microscopic images of MRSA (ATCC 33591) treated with peptide P8. **a** Culture untreated, **b** culture treated with oxacillin at 15.52 μg/ml, **c** culture treated with P8 at 3 μM conc., **d** treated with both P8 and oxacillin at their synergistic MIC mentioned above

handful studies have been reported the successful delivery of therapeutics into the bacterial cells using CPPs (Sparr et al. 2013). In the present study, we sought to investigate whether CPPs, P8 and P3 if used in combination with the conventional antibiotics, can improve the efficacy and delivery of these antibiotics against MRSA. Therefore, we have determined the MIC of both CPPs and five antibiotics against MRSA. Both CPPs have shown poor (≥12.5 μM) antibacterial activity, while out of five, three antibiotics viz. oxacillin, norfloxacin, and erythromycin were found to be least effective and thus selected for further experiments. In addition to these less effective antibiotics, FDA approved antibiotic against MRSA viz. vancomycin was also used to demonstrate the combination effect of CPP.

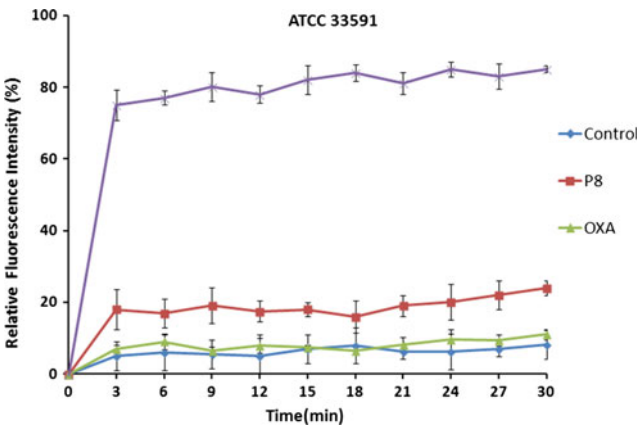


**Fig. 6** Propidium iodide uptake assay: MRSA ATCC 33591 treated with oxacillin (15.62 μg/ml) alone, P8 (3 μM) alone as control, and both combine at same concentration taken as test. Error bars indicate the standard deviation of the means of three independent experiments with three replicates each ($n = 9$)

In order to deliver a drug into the bacterial system, CPPs must be internalized into the MRSA without causing significant cell death. Since both P3 and P8 are found to be poor antibacterial, we examined whether they can internalize into bacteria at low concentration (below MICs). Confocal microscopic studies (Fig. 1) revealed that both CPPs were internalized into the MRSA at very low concentration. Also, at the same concentration, none of the peptides showed significant hemolysis (Fig. 2a) as well as cytotoxicity on CHO-K1 cells (Fig. 2b) and HeLa cells (Gautam et al. 2015), suggesting that these CPPs could be used as antibiotic delivery vehicle. Today, one of the major challenges in the therapeutic applications of peptides is associated with their cytotoxicity (Zasloff 2002). However, in the present study, peptides have demonstrated high therapeutic profiles, and there was no toxicity observed in mammalian cells at concentrations several times higher than their bactericidal concentrations.

Considering the poor membrane permeability of antibiotics is one of the probable reasons for drug resistance in MRSA (Ling et al. 2015; Franciolli et al. 1991), we examined the CPP-antibiotic combination therapy against MRSA. Interestingly, in combinations with CPPs, the MIC of oxacillin, norfloxacin, erythromycin, and vancomycin was reduced significantly (Table 3). The P8–antibiotics combination was

more effective than P3-antibiotics combination. P8 at 5 μM, when used in combinations with antibiotics, was highly effective against most of the strains. However, P3 at 10 μM, when used with antibiotics, has shown a reasonable reduction in MIC of oxacillin, norfloxacin, and vancomycin but no apparent reduction in the MIC of erythromycin. Interestingly, P8 was highly effective even at 3 μM when used in combination with antibiotics. Beside the same charge and hydrophobicity, the superior efficiency of P8 compared to P3 could be due to its tendency to adapt amphipathic helical conformation and presence of tryptophan residues (Gautam et al. 2015). These results suggest that besides having poor antibacterial activity, both P3 and P8 act as cell-permeable peptides at lower concentrations (below MICs) in MRSA and reduction of MICs of antibiotics could be due to the higher delivery of antibiotics in the presence of peptides. Similar observation has also been reported previously that cationic peptides have a dose-dependent antibacterial activity for many bacteria but in lower concentrations (compare to MIC) these peptide can be acted as cell permeable peptides (Heiat et al. 2014). We further demonstrated the delivery of norfloxacin in wild-type clinical strains of *S. aureus* 1199 (SA-1199) and its NorA over-expressed strain (SA-1199B). P3 did not show synergy even at 10 μM concentration and this may be due to its less penetration potential. But P8 even at 3 μM has shown synergistic effect against SA-1199 as well as against SA1199B. Although there was positive efflux of norfloxacin due to NorA pump, P8 empowered its effect by enhancing the permeability of cell membrane. In our recent study, the concept of efflux pump inhibitors as the adjuvant to reduce drug-resistance has been discussed (Roy et al. 2013).

It is interesting to note that the combinations of CPPs and broad-spectrum antibiotics such as ampicillin and imipenem did not show any effect on *E. coli* and *K. Pneumoniae* as no reduction in MICs was observed. These results are in accordance with the previous findings where no synergy was detected when the combination of peptides with ampicillin was tested. Antibiotics usually excluded by the LPS and, therefore, inactive against *E. coli* (Vaara and Nurminen 1999). Hence, P3 and P8 did not facilitate the delivery of antibiotics to act at sub-inhibitory concentration and MIC remained unchanged (Table S1).

The reduction of MIC of antibiotics could be due to the increased membrane permeability in the presence of CPPs, and this hypothesis is well-supported by various experiments. The uniform patterns in the kill-curves (Fig. 3a–d) irrespective of antibiotics used suggest that the role of CPP is non-specific to single antibiotic although degree of killing potential may slightly differ with respect to type of antibiotics and synergistic MIC concentration of CPP. This finding was also validated and supported by bacteriolysis (Fig. 4) where the role of P8 is uniform and killing rate differs with respect to antibiotics. For visualizing this effect, we examined the effect of oxacillin on the cell membrane by SEM with appropriate controls. We did not find membrane damage in the presence of either oxacillin or CPP alone at sub-inhibitory concentrations, though *Staphylococcus* cells were aggregated (Fig. 5b–c). But the combination of P8 and oxacillin significantly damaged the cell membrane (Fig. 5d). These results are in accordance with the previous finding, as oxacillin exerts their activity by inhibiting enzymes involved in cell wall synthesis (Dartois et al. 2005). These results are consistent with the PI uptake assay. The increased membrane permeability by CPP was also observed in PI uptake assay in which a significant effect of oxacillin on *Staphylococcus* cells was observed in the presence of P8. The fluorescence intensity of PI in combined therapy of oxacillin-P8 provides increased fluorescence as compared to either P8 or oxacillin alone. One interesting observation from time-kill kinetics, bacteriolysis, SEM study, and PI uptake assay is that although P8 is not bactericidal at sub-inhibitory concentrations but it has permeabilized the membrane which reflects in P8 control graphs compare to other control graphs.

In the recent past, few studies (Sparr et al. 2013; Nuding et al. 2014) have investigated the effect of combination therapy (consisting of AMPs and conventional antibiotics) against bacterial pathogens and reported an increased efficacy of the therapy compared to antibiotics alone. In this paper, we demonstrated that CPP, particularly P8 as adjuvant, significantly improved the sensitivity of antibiotics to overcome the drug resistance and has a strong potential to be used with variety of antibiotics for the treatment of MRSA infections. Further, in vivo studies for antibiotic delivery in combination with CPP may add a new dimension for the control of increasing antibiotic resistance in MRSA.

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**Compliance with ethical standards** This article does not contain any studies with animals performed by any of the authors. This study was approved by the Institutional Biosafety Commitee.

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