



OPEN Restoration of Oxacillin susceptibility in MRSA strains by NPPB

Se Jin Jo¹, Rui Jiang¹, Sook In Jung², Joon Haeng Rhee³ & Young Ran Kim¹✉

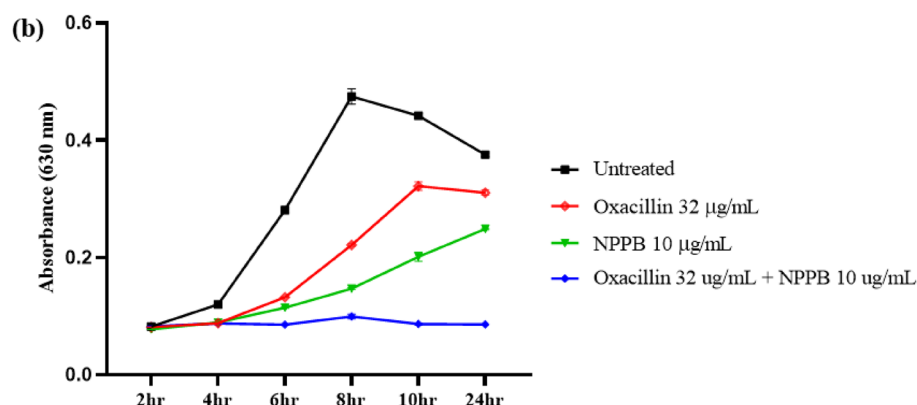
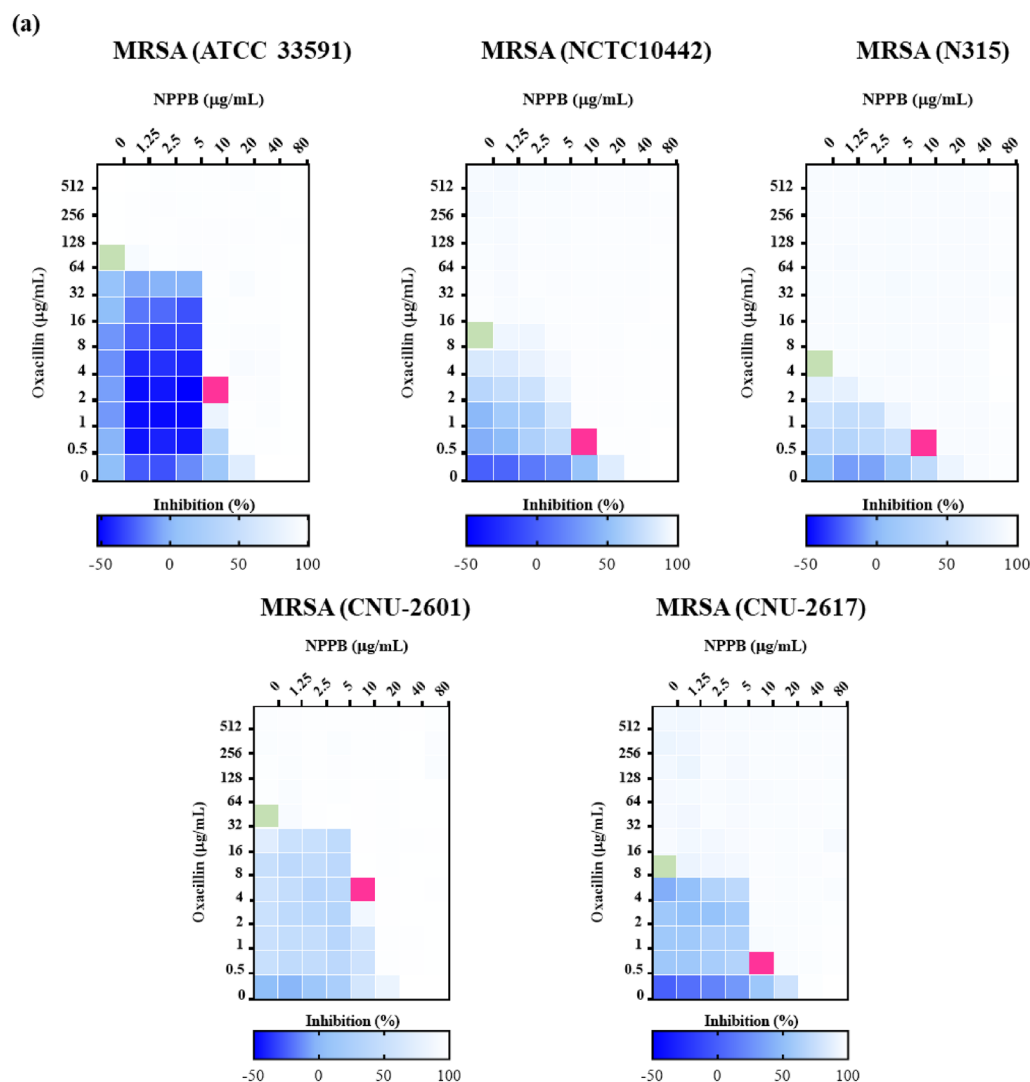
Methicillin-resistant *Staphylococcus aureus* (MRSA) is a critical public health issue because of its resistance to multiple antibiotics, including β -lactams such as methicillin and oxacillin. To search for antibacterial sensitizers, we have investigated the synergistic antibacterial effects of the combination of the β -lactam antibiotic oxacillin and 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) against the MRSA strains. Checkerboard assays showed the synergistic antibacterial efficacy of NPPB combined with oxacillin against five MRSA strains. In addition, the combined effect of NPPB and oxacillin was evaluated by disc diffusion assay and time-dependent growth assay in MRSA strain ATCC 33591, and real-time PCR results showed that the combined treatment decreased the expression levels of *abcA* and *norA* genes encoding efflux transporters. NPPB also decreased the oxacillin-induced biofilm formation of ATCC 33591. Field emission transmission electron microscopy showed that the combination treatment induced extensive damage to the bacterial cell wall and membrane, leading to cell lysis. NPPB and oxacillin treated to mice for one week did not cause toxicity on the liver and kidney functions. These findings highlighted the potential of combination therapy of oxacillin and NPPB to treat MRSA infection by enhancing antibiotic efficacy and overcoming resistance.

Keywords Methicillin-resistant *Staphylococcus aureus*, 5-nitro-2-(3-phenylpropylamino) benzoic acid, Oxacillin, Anti-bacterial sensitizer, Antibiotic resistance, Synergistic antibacterial effects

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterium that commonly appears asymptotically in various body parts, such as the skin, skin glands, and mucous membranes of humans and animals^{1,2}. It is an opportunistic pathogen that can cause various infections when it breaches the host's immune defenses. Because of the thick peptidoglycan layer in the cell wall of gram-positive bacteria, such as *S. aureus*, they are susceptible to β -lactam antibiotics, which inhibit cell wall synthesis³. However, with the emergence of methicillin-resistant *S. aureus* (MRSA), many β -lactam antibiotics, including methicillin and oxacillin, have become ineffective. MRSA poses a serious threat to public health worldwide because it is resistant to multiple antibiotics and responsible for a range of infections such as skin and soft tissue infections, pneumonia, and bloodstream infections^{4–6}. The primary mechanism of resistance in MRSA is the acquisition of *mecA* in the staphylococcal cassette chromosome *mec* (SCC *mec*)^{7,8}. This gene encodes penicillin-binding protein 2a (PBP2a) that has a low affinity for β -lactam antibiotics; as such, antibiotics against MRSA are ineffective⁹. The efflux pump NorA, which is involved in fluoroquinolone antibiotic resistance, produces a protein that pumps antibiotics out of the cell through the cell membrane, leading to antibiotic resistance^{10,11}. AbcA, part of the ATP-binding cassette (ABC) transporter family, contributes to multidrug resistance in MRSA strains by exporting toxic substances, including antibiotics, out of the cell, thus leading to antibiotic resistance^{12,13}.

With the increasing prevalence of MRSA, novel therapeutic strategies should be explored to combat this pathogen. Among these strategies, combination therapy has emerged as a promising approach to enhance the efficacy of existing antibiotics through synergistic effects. In this approach, antibiotics are used in conjunction with other agents that can disrupt bacterial defense mechanisms or improve antibiotic penetration; thus, the effectiveness of antibiotic penetration can be restored. Recent studies have shown that combining β -lactam antibiotics with non-antibiotic adjuvants that can inhibit resistance mechanisms or compromise bacterial cell wall integrity can substantially improve antibacterial activities^{14,15}.

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Oxacillin, a semisynthetic penicillinase-resistant β -lactam antibiotic, has been largely ineffective against MRSA because of the presence of PBP2a¹⁶. However, this resistance can be overcome when oxacillin is used in combination with agents that target bacterial membranes or metabolic pathways. Its interactions with these adjuvant agents and the mechanisms of synergy should be elucidated during the development of effective treatment regimens against MRSA^{17,18}. Another challenge in treating *S. aureus* infections is the ability of this bacterium to form biofilms. These biofilms create a physical barrier that prevents antibiotics from reaching bacterial cells and protects the bacteria from various environmental threats¹⁹. Consequently, finding candidates that inhibit the biofilm formation of *S. aureus* can be an effective strategy to prevent the occurrence of antibiotic resistance. Previous studies revealed that pyocyanin from *Pseudomonas aeruginosa* isolates is a candidate activator for the growth inhibition and biofilm eradication of MRSA isolates²⁰. Another study reported that the combination of antimicrobial peptides, which are innate host immune agents, with silver nanoparticles exhibit

◀**Fig. 1.** Synergistic antimicrobial effects of oxacillin and NPPB against MRSA strains. **(a)** The effects of oxacillin and NPPB on the growth of five MRSA strains. The combined effect of oxacillin and NPPB on the growth MRSA strains was evaluated using the checkerboard assay. MICs of oxacillin, NPPB, and oxacillin plus NPPB were determined. The fractional inhibitory concentration index (FICI) ≤ 0.5 indicated synergism. The MICs of oxacillin against five MRSA strains were significantly reduced when combined with NPPB (10 $\mu\text{g}/\text{mL}$) compared to oxacillin alone. Green color indicates the MIC of oxacillin when administered alone, whereas pink represents the MIC of oxacillin in the presence of NPPB. **(b)** Time-dependent growth inhibition of the combination of oxacillin and NPPB on MRSA ATCC 33591. The bacteria were cultured in a 37°C incubator at 200 rpm shaking overnight. The bacterial solution 1000-fold diluted in fresh TSB media was inoculated in 96-well plates with oxacillin (32 $\mu\text{g}/\text{mL}$), NPPB (10 $\mu\text{g}/\text{mL}$), or a combination of oxacillin (32 $\mu\text{g}/\text{mL}$) and NPPB (10 $\mu\text{g}/\text{mL}$). Absorbance at 630 nm was measured at different time points (2, 4, 6, 8, 10, and 24 h). The combination treatment significantly reduced bacterial growth over time compared with the individual treatment.

Strains	Characteristics	MIC (Oxacillin)	Outcome (FICI)
ATCC 33591	<i>Staphylococcus aureus</i>	64 $\mu\text{g}/\text{mL}$	Synergistic (0.1562)
NCTC10442	<i>Staphylococcus aureus</i> (Scc mec type I)	8 $\mu\text{g}/\text{mL}$	Synergistic (0.3125)
N315	<i>Staphylococcus aureus</i> (Scc mec type II)	4 $\mu\text{g}/\text{mL}$	Synergistic (0.1875)
CNU-2601	<i>Staphylococcus aureus</i> (Clinical strains)	32 $\mu\text{g}/\text{mL}$	Synergistic (0.2656)
CNU-2617	<i>Staphylococcus aureus</i> (Clinical strains)	8 $\mu\text{g}/\text{mL}$	Synergistic (0.3125)

Table 1. Minimum inhibitory concentration (MIC) of oxacillin and NPPB against MRSA strains MIC (Oxacillin): minimum inhibitory concentration of oxacillin against MRSA strains when used alone. FICI ≤ 0.5 indicates a synergistic effect.

synergistic effects; therefore, they can be developed as potential agents to combat the antibiotic resistance of MRSA²¹. To overcome the antibiotic resistance of MRSA strains, researchers have been investigating candidates that exhibit synergistic effects with antibiotics^{22–24}.

This study aims to develop drug restoring oxacillin susceptibility towards MRSA strain. Through extensive screening to search for antibacterial sensitizers, we found that oxacillin and 5-nitro-2-(3-phenyl-propylamino) benzoic acid (NPPB) exhibited a synergistic effect against the five MRSA strains (ATCC 33591, NCTC10442, N315, CNU-2601, and CNU-2617). NPPB, which is primarily known as a chloride channel blocker in eukaryotic cells, has been investigated for its therapeutic benefits to conditions such as vasospasm²⁵ anxiety, and affective disorders²⁶; however, its potential as an antibacterial agent remains underestimated. Considering its mechanism of action, NPPB may enhance the permeability of bacterial membranes to antibiotics, potentially restoring the efficacy of antibiotics such as oxacillin against MRSA. In this study, we aimed to investigate how this combination could disrupt bacterial resistance mechanisms and restore antibiotic efficacy. By examining these interactions, we aimed to provide new insights into combating MRSA infections and solving the problem of antibiotic resistance.

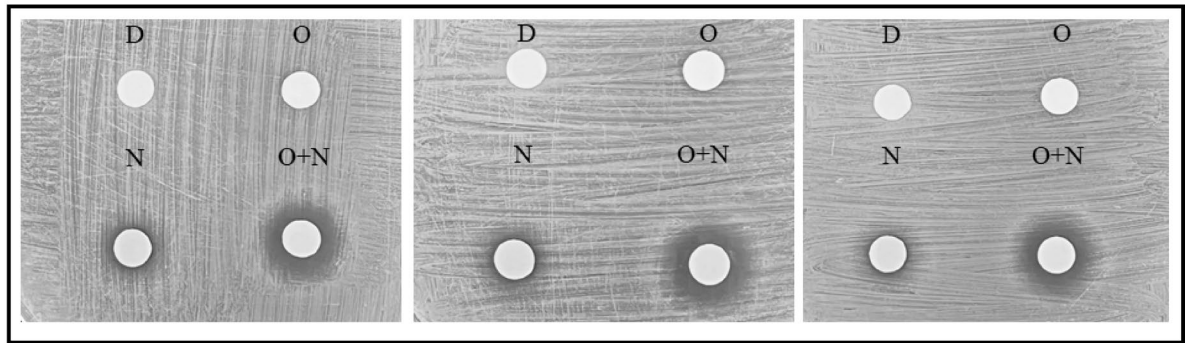
Results
Synergistic antimicrobial activities of Oxacillin and NPPB

Checkerboard assay analysis was performed using a combination of various concentrations of oxacillin with NPPB. The MICs of oxacillin, NPPB alone, and their combination were determined against the five MRSA strains. The fractional inhibitory concentration index (FICI) obtained from the checkerboard assay. MIC and FICI are showed in Fig. 1a; Table 1. The MICs of oxacillin in various MRSA strains were significantly decreased when administered in co-treated with NPPB (10 $\mu\text{g}/\text{mL}$). NPPB significantly enhances the efficacy of oxacillin by reducing its required concentration to inhibit MRSA growth.

The time-dependent growth of MRSA ATCC 33591 treated with oxacillin and NPPB was examined. Growth curve analysis revealed that the combination of oxacillin and NPPB elicited a substantial antibiotic activity on MRSA over time (Fig. 1b). Growth was monitored every 2 h in the presence or absence of oxacillin (32 $\mu\text{g}/\text{mL}$), NPPB (10 $\mu\text{g}/\text{mL}$), or a combination of oxacillin (32 $\mu\text{g}/\text{mL}$) and NPPB (10 $\mu\text{g}/\text{mL}$). While the treatments with oxacillin or NPPB alone slightly inhibited bacterial growth, the combination treatment completely inhibited bacterial growth. Differences in growth rates became apparent after 6 h of incubation. Therefore, the combination therapy exhibited a time-dependent efficacy.

Antibacterial effect of Oxacillin and NPPB against MRSA via the disc diffusion assay
To further confirme the synergistic antibacterial activity of the combination of oxacillin and NPPB, disc diffusion assay was conducted. The zone of inhibition of the combination treatment was larger than that of each drug alone (Fig. 2a). Specifically, the zones of inhibition of NPPB alone ranged from 7.79 mm to 8.03 mm, with an average diameter of 7.91 mm (Fig. 2b). Oxacillin alone did not produce a zone of inhibition. However, the zone of inhibition of the combination treatment ranged from 11.34 mm to 11.66 mm, with an average diameter of 11.5 mm (Fig. 2B), demonstrating the enhanced antibacterial activity when both agents were used together. Therefore, NPPB likely improved the antibiotic susceptibility of MRSA ATCC 33591 to oxacillin.

(a)



(b)

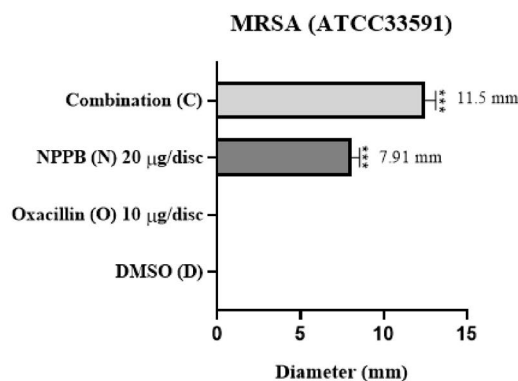


Fig. 2. Antibacterial effect of the combination of oxacillin and NPPB via the disc diffusion assay. **(a)** MRSA ATCC 33591 bacterial suspension ($100\ \mu\text{L}$ of 1.5×10^8 CFU/mL) was evenly spread over the surface of the TSA plates by using a sterile cotton swab. Sterile paper discs (6 mm in diameter) were placed on the agar plate and impregnated with oxacillin (10 µg/disc), NPPB (20 µg/disc), or the combination. The agar plates were incubated at $37\ ^\circ\text{C}$ for 24 h. oxacillin (O), NPPB (N), oxacillin + NPPB (O + N), the solvent control (0.1% DMSO, D) **(b)** The combination effect of oxacillin and NPPB was confirmed by measuring the diameter of the inhibition zone around the paper discs. The inhibition zone of the combination treatment was significantly larger than that of the individual treatments. *** $P < 0.001$ compared with the control group.

Effect of Oxacillin and NPPB on the mRNA expression of *abcA*, *norA*, and *MecA*

To investigate the action mechanisms of synergistic effects of oxacillin and NPPB, the gene expression related to the resistance mechanism of MRSA ATCC 33591 was evaluated. Real-time PCR analysis showed that the treatment with oxacillin and NPPB significantly downregulated the expression of *abcA*, *NorA*, and *mecA* genes in MRSA (Fig. 3). The *abcA* and *norA* genes encode efflux pump and transporters that contribute to antibiotic resistance by expelling antibiotics out of the cells; *mecA* encodes PBP2a, a protein with low affinity for β -lactam antibiotics. The combination treatment with NPPB inhibited the expression of antibiotic resistance genes in MRSA, suggesting that this result may be related to the mechanism underlying NPPB's synergistic effect with oxacillin.

Effect of NPPB on biofilm production by Oxacillin

Biofilm formation substantially influences the virulence, antibiotic resistance, persistence, and transmission of bacterial strains²⁷. Biofilm formation was quantified using crystal violet staining, and the optical density (OD) of the solubilized stained biofilms was spectrophotometrically measured. In Fig. 4, the biofilm formation of the MRSA ATCC 33591 was increased by oxacillin (8, 16, or 32 µg/mL), and the increased biofilm formation was significantly decreased by combination treatment with oxacillin and NPPB (5–10 µg/mL).

Effect of Oxacillin and NPPB on the morphological characteristics of MRSA

FE-TEM revealed significant morphological alterations in the MRSA ATCC 33591 cells treated with the combination of oxacillin and NPPB. The untreated MRSA cells and those treated with either oxacillin or

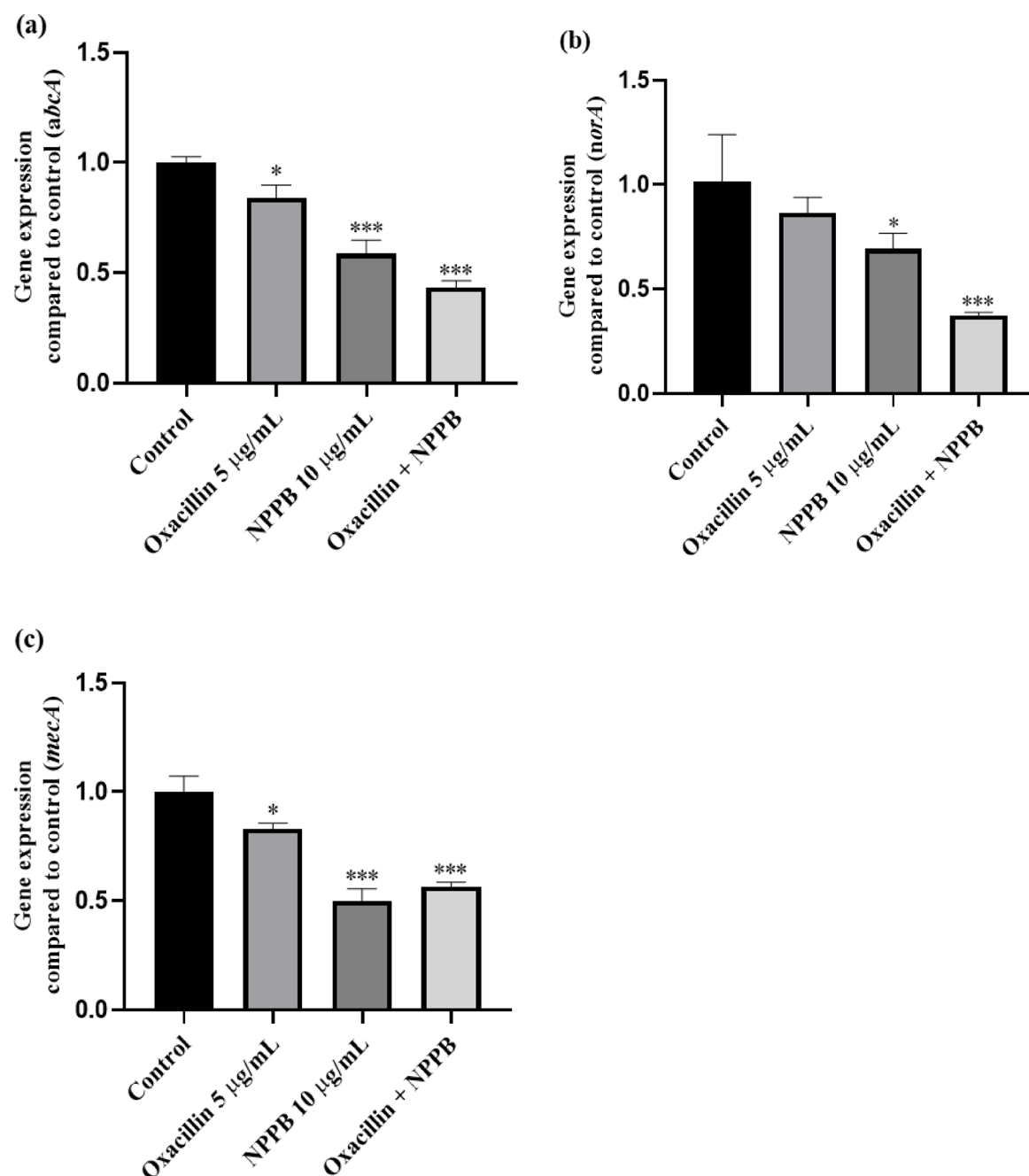


Fig. 3. Effect of oxacillin and NPPB on the mRNA expression of *abcA*, *norA*, and *mecA* genes. MRSA ATCC 33591 bacteria were treated with oxacillin and NPPB for 7 h. Real-time PCR analysis was conducted to investigate the mRNA expression levels of *abcA*, *norA*, and *mecA* genes. The sequences of the primers used are listed in Table 2. The transcription of *abcA* and *norA* genes was significantly downregulated in the combination treatment compared with that in the individual treatments. * $P < 0.05$ and *** $P < 0.001$ compared with the control group.

NPPB alone showed intact cell walls and membranes. Conversely, the MRSA cells exposed to the combination of oxacillin and NPPB exhibited extensive cell wall and membrane damage, cytoplasmic content leakage, and abnormal cell division (Fig. 5). These observations suggested that the combination treatment of oxacillin and NPPB to MRSA ATCC 33591 disrupted the bacterial cell integrity, leading to cell lysis.

Safety test of Oxacillin and NPPB

The safety of oxacillin and NPPB was evaluated on splenocytes purified from mouse via MTS assay. As shown in Fig. 6a, co-treatment with the highest concentration of oxacillin (128 µg/mL) and NPPB (40 µg/mL) did not show cytotoxicity to splenocytes. In addition, whole-blood elements were isolated from the mice treated

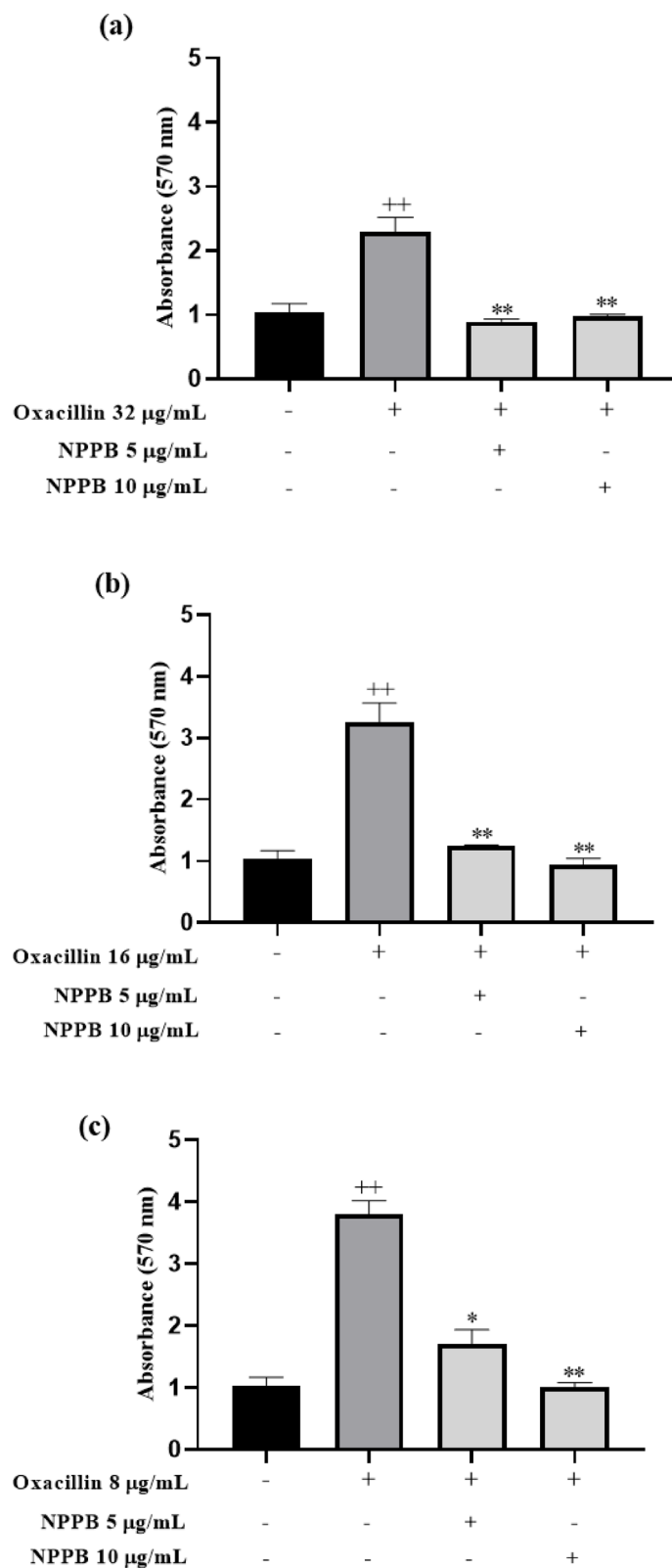


Fig. 4. Effect of NPPB on the biofilm formation of oxacillin-treated MRSA bacteria. MRSA ATCC 33591 bacteria were cultured in TSB containing 0.5% glucose overnight and diluted to $OD_{600} = 1$ in fresh media. The bacterial suspension (100 µL) was added to each wells of 96-well plates with oxacillin (8, 16, or 32 µg/mL) and NPPB (5–10 µg/mL) and incubated at 37 °C for 24 h. The bacterial pellets were washed, dried, and fixed with methanol. The biofilm was stained with 1% crystal violet for 15 min and solubilized with ethanol. Absorbance was detected at 570 nm by using an ELISA microplate reader. The combination treatment significantly inhibited biofilm formation compared with individual treatments. ⁺⁺ $P < 0.01$ compared with the untreated group. ^{*} $P < 0.05$ and ^{**} $P < 0.01$ compared with the oxacillin-treated group.

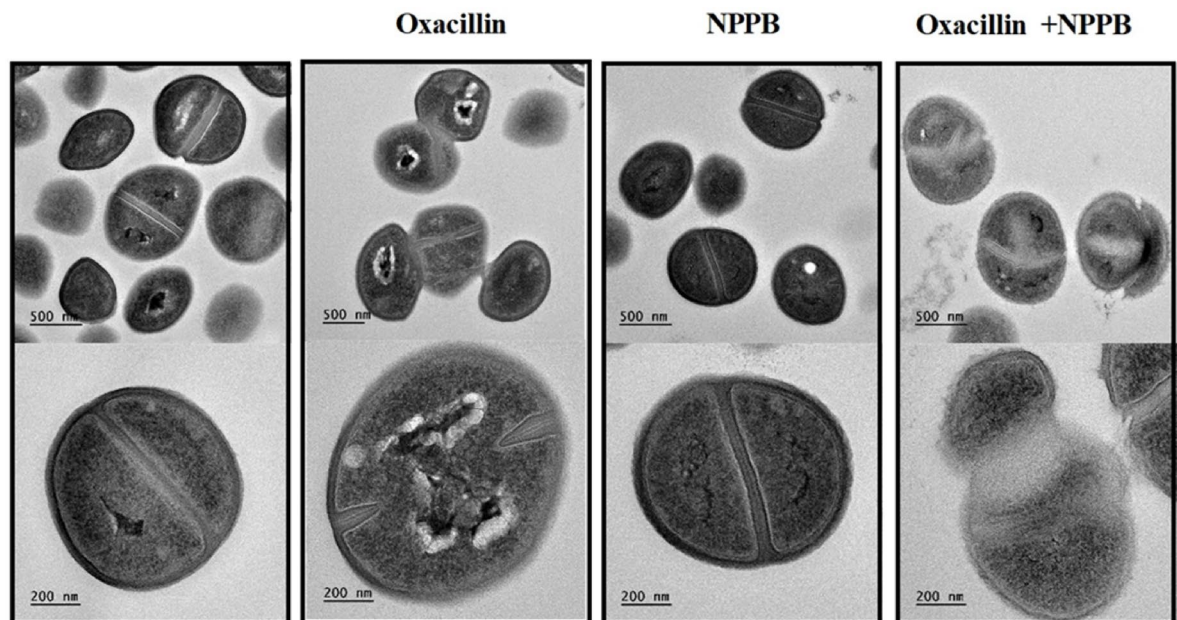


Fig. 5. Morphological changes of MRSA ATCC 33591 bacterial cells treated with oxacillin and NPPB. MRSA bacterial cells cultured overnight were diluted 200-fold and treated with oxacillin (5 $\mu\text{g}/\text{mL}$), NPPB (10 $\mu\text{g}/\text{mL}$), and oxacillin plus NPPB in a shaking incubator at 37 $^{\circ}\text{C}$ for 7 h. The bacterial cell pellets were collected through centrifugation, washed with PBS, and prepared on nanoparticle grids. Field emission transmission electron microscopy (FE-TEM) images illustrate that the cell walls and membranes of the bacteria were significantly destroyed after the combination treatment of oxacillin and NPPB.

with oxacillin, NPPB, or oxacillin with NPPB for 1 week. Serum AST and ALT levels were analyzed to assess hepatotoxicity, and serum UA, CRE, and BUN levels were examined to assess nephrotoxicity. Oxacillin, NPPB, and oxacillin with NPPB did not induce hepatotoxicity and nephrotoxicity to the ICR mice (Fig. 6b, c, d, e, and f).

Discussion

MRSA bacteria can cause infections in various body parts, such as skin and soft tissue infections, pneumonia, and bloodstream infections. They are challenging to treat because of their antibiotic resistance. The term MRSA initially referred specifically to resistance to methicillin. Since its emergence in the 1960s, MRSA has posed a serious public health problem due to its resistance to nearly all β -lactam antibiotics, including methicillin and oxacillin²⁸. While oxacillin and methicillin were developed to combat penicillinase-producing staphylococci, methicillin has largely been replaced by more stable derivatives such as oxacillin²⁹. Oxacillin is more stable than methicillin; as such, it can be orally and parenterally administered. Methicillin is largely historically but no longer clinically used because of stability issues and availability of more stable alternatives^{30,31}. Oxacillin is preferred for infections caused by penicillinase-producing staphylococci³². Because of antibiotic resistance, the treatment of MRSA infections become complicated, thereby increasing morbidity and mortality^{33,34}. Vancomycin and linezolid, which are used to treat MRSA, have considerable side effects and many limitations^{35,36}.

This study aims to develop drug restoring oxacillin susceptibility towards MRSA strain. There have been studies aiming to develop or identify compounds or strategies that restore oxacillin susceptibility in MRSA. These studies highlight diverse strategies including genetic modifications, molecular interventions targeting *mecA* or *vraTSR* regulatory pathways^{37,38} and natural compound synergists such as myricetin and sophoraflavanone G^{39,40} to combat MRSA by restoring oxacillin efficacy. We recently reported that inhibition of an efflux pump TolCV1 by NPPB renders *Vibrio vulnificus* less virulent and more susceptible to antibiotics⁴¹. In this study, we found that NPPB combined with oxacillin elicited a synergistic antibacterial activity against MRSA strains. Checkerboard and disc diffusion assays showed that NPPB significantly enhance the efficacy of oxacillin by lowering its MIC against MRSA strains (Figs. 1 and 2). In contrast, NPPB did not show any synergistic effects with erythromycin, tetracycline, and kanamycin antibiotics on MRSA ATCC 33591 (Supplement Fig. 1). These results suggest that NPPB specifically synergizes β -lactam antibiotics against MRSA.

To study restoration mechanism of oxacillin susceptibility in MRSA strains by NPPB, we have performed biofilm assay, real time PCR of *abcA* and *norA* genes encoding efflux transporters, and TEM analysis. First, inhibitory effect of NPPB on transcription of MRSA ATCC 33591 genes is stronger on *abcA* and *norA* than on *mecA* (Fig. 3). Gene *mecA* encodes penicillin-binding protein 2a (PBP2a) that has a low affinity for β -lactam antibiotics. In contrast, *norA* gene encodes an efflux pump and *abcA* gene does a transporter. Additionally, we assayed to biofilm formation of MRSA ATCC 33591. To improve the experimental sensitivity of the biofilm formation assay, TSB medium containing 0.5% glucose was used⁶. NPPB significantly decreased the biofilm

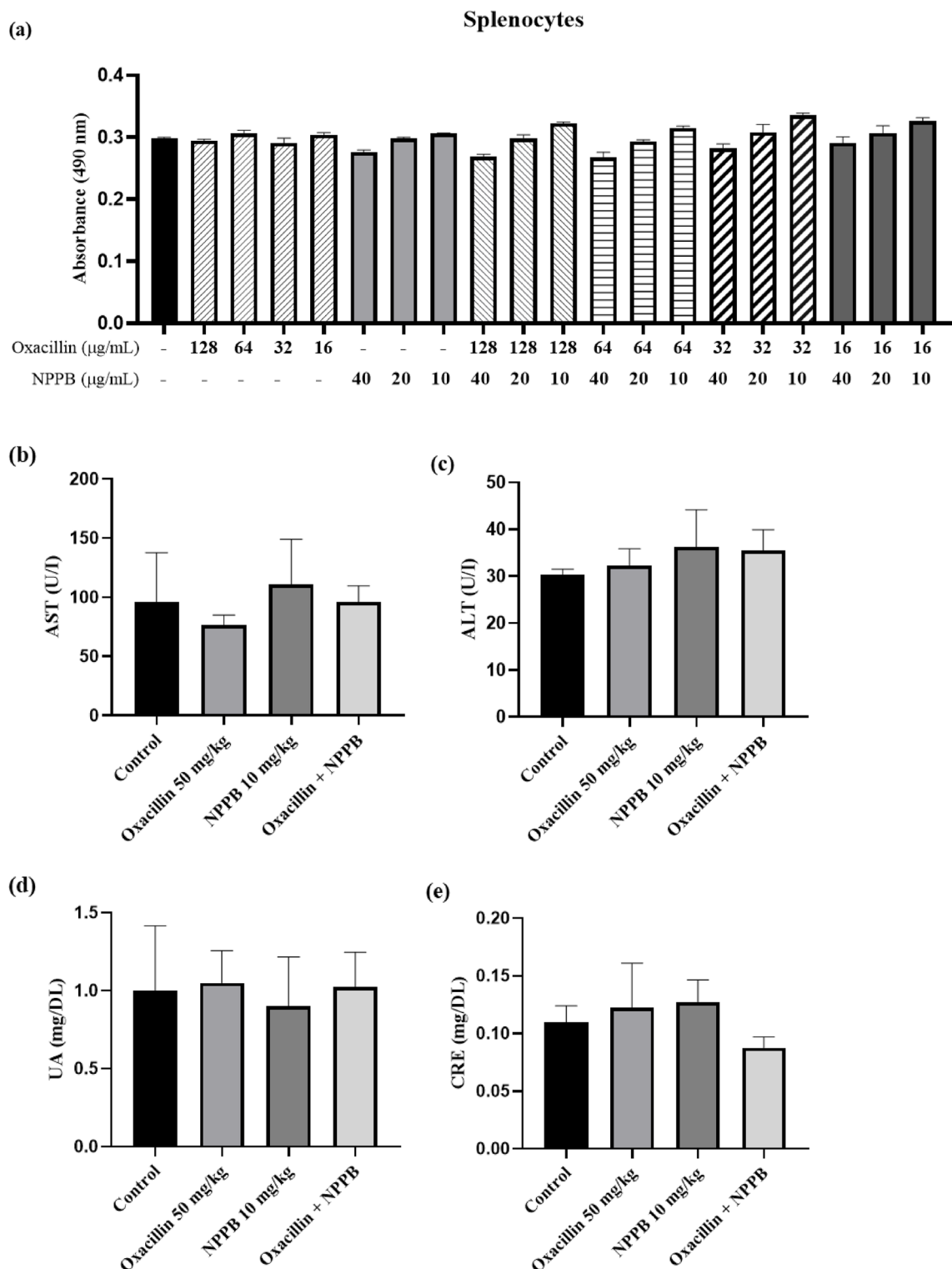


Fig. 6. Safety test of oxacillin and NPPB on mice. **(a)** Safety of oxacillin and NPPB on purified mouse splenocytes. BALB/c female mice were sacrificed by cervical dislocation and the single-cell suspension was prepared from the isolated spleen using 50 μ m mesh strainer at room temperature. Splenocytes were seeded in 96-well plates with oxacillin, NPPB, and oxacillin + NPPB and cultured in a CO₂ incubator at 37 °C for 1 day. The cytotoxicity was evaluated via MTS assay. Oxacillin, NPPB, and the combination did not show any cytotoxicity on purified mouse splenocytes. **(b–f)** Safety test of oxacillin and NPPB to mice. Mice were intraperitoneally injected with oxacillin (50 mg/kg), NPPB (10 mg/kg), or the combination once a day for one week. The mice were anesthetized in a sealed acrylic glass with 2% isoflurane and mouse blood was taken from the heart using a syringe with a needle. The mouse serum was analyzed using a serological analyzer. **(b)** aspartate transaminase (AST), **(c)** alanine transaminase (ALT), **(d)** uric acid (UA), **(e)** creatinine (CRE), and **(f)** blood urea nitrogen (BUN). Data were expressed as mean $\pm$ standard error of the mean ($n=4$).

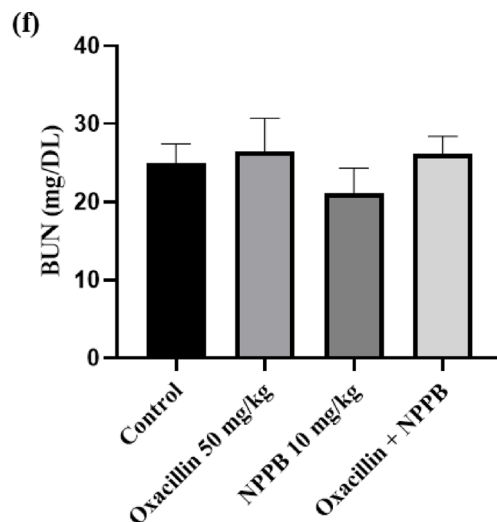


Fig. 6. (continued)

formation of MRSA bacteria (Fig. 4). The ability of the combination treatment to inhibit biofilm formation suggests that NPPB has a strong antibiotic activity over time highlighted its potential as a powerful therapeutic strategy against MRSA (Fig. 4). The reduction in biofilm formation is particularly vital because biofilms are associated with persistent infections and increased resistance to antibiotics. FE-TEM analysis (Fig. 5) also indicated that this combination disrupted bacterial cell integrity. In addition, preliminary toxicity studies to confirm the safety of NPPB showed that NPPB alone and its combination with oxacillin did not induce substantial cytotoxic effects on purified primary mouse cells, suggesting a favorable safety profile (Fig. 6A). NPPB treated to mice once a day for 1 week did not show toxicity to the liver and kidney (Fig. 6b, c, d, e, and f). There appears to be a need for more extensive evaluation of the safety profile of NPPB. These results suggest the potential of NPPB to restore or increase susceptibility to oxacillin in MRSA treatment.

Materials and methods

Strains and culture

The MRSA strain ATCC 33591 was purchased from the American Type Culture Collection (ATCC, Manassas, USA). NCTC10442 strain was purchased from TCS Biosciences (Buckingham, UK), and MRSA strains NRS70 (N315) was obtained through the Network on Antimicrobial Resistance in *Staphylococcus aureus* (NARSA). The clinical isolates CNU-2601 and 2617 were isolated from Chonnam National University Hospital (CNU, GJ, KR) and used in this study. The bacterial cells were stored at -70°C using 20% glycerol (Sigma-Aldrich, MO, USA) and were cultured in tryptic soy agar (TSA) or tryptic soy broth (TSB; BD, MD, USA)^{42,43}.

Chemical and reagents

Oxacillin, NPPB, crystal violet, glucose, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Phosphate buffered saline (PBS) was bought from HiMedia Laboratories Pvt. Ltd. (MB, IND). NPPB was dissolved in DMSO and the working concentration of DMSO is under 0.1%.

Checkerboard assay

NPPB (1.25 $\mu\text{g/mL}$ to 80 $\mu\text{g/mL}$) and oxacillin (0.5 $\mu\text{g/mL}$ to 512 $\mu\text{g/mL}$) at 2-fold serial dilution were added in 96-well plates. MRSA bacteria strains cultured overnight were diluted at a dilution of 1:10000 in a fresh TSB medium and then added to the each well of 96-well plates. After incubation at 37°C for 24 h, absorbances were read at 630 nm by using a microplate reader (KLAB MRX A2000, DJ, KR). The combination effect of oxacillin and NPPB was evaluated in terms of FICI according to a previously described formula⁶. $\text{FICI} \leq 0.5$ was interpreted as synergistic effects.

Bacterial growth curve measurements

MRSA bacteria were incubated in a shaking incubator at 200 rpm and 37°C overnight and diluted 1000-fold with a fresh TSB medium. The diluted bacteria were inoculated in 96-well plates with oxacillin (32 $\mu\text{g/mL}$), NPPB (10 $\mu\text{g/mL}$) alone, or a combination of oxacillin (32 $\mu\text{g/mL}$) and NPPB (10 $\mu\text{g/mL}$) and the plates were incubated at 37°C . Absorbances were measured at 630 nm by using a microplate reader (KLAB MRX A2000, DJ, KR) every 2 h for 24 h.

Disc diffusion assay

MRSA ATCC 33591 bacteria were cultured in TSB media and the bacterial suspension (100 μL of 1.5×10^8 CFU/ mL) was evenly spread over the surface of the TSA plates by using a sterile cotton swab. Sterile paper discs (6 mm in diameter) were placed on the agar plate and impregnated with oxacillin (10 $\mu\text{g/disc}$), NPPB (20 $\mu\text{g/disc}$) alone,

Gene	Primers	Sequences
<i>abcA</i>	<i>abcA-F</i>	5'-CAA GAA CCT ATT GAA CCG ACA GAA-3'
	<i>abcA-R</i>	5'-GTG GGA TTT GGA ACG ACA CA-3'
<i>norA</i>	<i>norA-F</i>	5'-ATC GGT TTA GTA ATA CCA GTC TTG C-3'
	<i>norA-R</i>	5'-GCG ATA TAA TCA TTT GAG ATA ACG C-3'
<i>MecA</i>	<i>MecA-F</i>	5'-CCG TGG ATT TAG TCG TGA AG-3'
	<i>MecA-R</i>	5'-GGC ATG TAC TTG AAT CCA TAA TGG-3'
<i>16S rRNA</i>	<i>16S-F</i>	5'-CGT GCT ACA ATG GAC AAT AC-3'
	<i>16S-R</i>	5'-ATC TAC GAT TAC TAG CGA TT-3'

Table 2. Primers used in real-time PCR analysis List of primers used for the amplification of *abcA*, *norA*, *mecA*, or *16S rRNA* genes in real-time PCR experiments. Primer sequences are provided for each target gene.

or a combination of oxacillin (10 µg/disc) and NPPB (20 µg/disc). The agar plates were incubated at 37 °C for 24 h. The combination effect of oxacillin and NPPB was confirmed by measuring the diameter of the zone of inhibition around the paper discs.

Gene expression analysis

MRSA ATCC 33591 bacteria were treated with oxacillin (5 µg/mL), NPPB (10 µg/mL) or the combination of oxacillin and NPPB for 7 h. Total RNAs were purified from the bacterial cells by using TRIzol reagent (MRC, CVG, OH, USA) in accordance with the manufacturer's instructions and synthesized into cDNA with TOPscript™ RT DryMIX (Enzynomics, DJ, KR). The transcription of *abcA*, *norA*, and *mecA* genes was quantified by real-time PCR (qPCR) using Rotor-Gene Q (QIAGEN, GmbH, HD, DEU) and a qPCR 2x premix SYBR Green PCR Master Mix (Enzynomics, DJ, KR). Data were normalized to the expression of 16 S rRNA via the threshold cycle ($\Delta\Delta C_T$). The primer sequences used in this study were designed as shown in Table 2.

Biofilm formation assay by crystal Violet staining

MRSA bacteria were cultured in TSB containing 0.5% glucose to increase biofilm production and experimental sensitivity. The bacteria cultured overnight was diluted to optical density $OD_{600} = 1$ in fresh media; then, 150 µL of the solution was added to the 96-well plates. Afterward, 50 µL of oxacillin (8, 16, or 32 µg/mL) alone or a combination of oxacillin and NPPB (5–10 µg/mL) was added to the 96-well plates and the plates were subsequently incubated at 37 °C for 24 h. The supernatants were gently removed and the bacterial pellets were washed thrice with PBS and dried. After fixation with 200 µL of methanol, the biofilm was stained with 200 µL of 1% crystal violet solution for 15 min. The plates were washed with PBS thrice, 200 µL of ethanol was added to solubilize the crystal violet. Absorbance was detected at 570 nm by using an ELISA microplate reader (ELx808; BioTek Instruments, Inc., VT, USA).

Field emission transmission electron microscope (FE-TEM) analysis

The bacterial cells cultured overnight were diluted 200-fold with a fresh TSB medium, and treated with oxacillin (5 µg/mL), NPPB (10 µg/mL) alone, or the combination in a shaking incubator at 37 °C for 7 h. The bacterial cell pellets collected through centrifugation at 3000 rpm for 15 min were washed with PBS and prepared on nanoparticle grids. The morphological characteristics of MRSA cells were examined using FE-TEM 2100 F (200 kV; JEOL Ltd., TYO, JP).

Safety test of the combination treatment of Oxacillin and NPPB

All animal experiments were performed following the guidelines of the Animal Care and Use Committee of Chonnam National University (CNU IACUC-YB-2024-99) in accordance with ARRIVE guidelines. The cytotoxicity of oxacillin and NPPB was evaluated on splenocytes purified from mouse via MTS assay (Promega, WI, USA). First, 7-week-old BALB/c female mice (Damool Science, Daejeon, Korea) were sacrificed by cervical dislocation. The single-cell suspension was prepared from the isolated mouse spleen using 50 µm mesh strainer (BD Falcon, San Diego, CA, USA). Splenocytes seeded in 96-well plates were treated with oxacillin, NPPB, and oxacillin plus NPPB and cultured in CO₂ incubator at 37 °C for 1 day. The cytotoxicity was evaluated via MTS assay (Promega, WI, USA).

In addition, 8-week-old female ICR mice weighing approximately 22 g were maintained in a clean animal room with controlled temperature and a 12 h light/dark cycle. Twenty mice were randomly divided into four groups of 5 mice for control, oxacillin, NPPB, and oxacillin + NPPB groups. Oxacillin (50 mg/kg), NPPB (10 mg/kg) alone, and a combination of oxacillin (50 mg/kg) and NPPB (10 mg/kg) were intraperitoneally administered to mice once a day for 1 week. At the end of the experiment, the mice were anesthetized in a sealed acrylic glass with 2% isoflurane (Harvard apparatus vaporizer, Massachusetts, USA) and mouse blood was taken from the heart using a syringe with a needle. The mouse serum was analyzed using a serological analyzer (Fujifilm Corp., Tokyo, Japan). The mice used in the experiment were all used as analysis, with no exclusion. Serum aspartate transaminase (AST) and alanine transaminase (ALT) were determined to evaluate the liver function; serum creatinine (CRE) and blood urea nitrogen (BUN) were determined to assess the kidney function.

Statistical analysis

All results were expressed as means $\pm$ standard error of the mean (SEM). Data were statistically analyzed via Tukey's post hoc test and one-way ANOVA in GraphPad Prism version 8 (GraphPad Software, SD, CA, USA), and those with $p < 0.05$ were considered statistically significant. Data were obtained at least in triplicate, and all experiments were repeated at least thrice. The results shown were from representative experiments.

List of primers used for the amplification of *abcA*, *norA*, *mecA*, or *16 S* rRNA genes in real-time PCR experiments. Primer sequences are provided for each target gene.

Data availability

Data is provided within the manuscript or supplementary information files. The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

SJ Jo and R Jiang conducted all experiments and wrote manuscript text. SI Jung and JH Rhee wrote manuscript text and gave some test method. YR Kim did writing, review & editing, supervision, and conceptualization.

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Declarations

Compliance with ethical standards

All experimental protocols were approved by the committee of Chonnam National University in accordance with ARRIVE guidelines. Experiments involving MRSA were carried out under biosafety level 2 (BSL-2) conditions, following the institutional biosafety regulations approved by the Chonnam National University Medical School. All animal experiments were performed following the guidelines of the Animal Care and Use Committee of Chonnam National University (CNU IACUC-YB-2024-99) in accordance with ARRIVE guidelines.

Competing interests

The authors declare no competing interests.

Additional information

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