Diclofenac Resensitizes Methicillin-Resistant *Staphylococcus aureus* to β-Lactams and Prevents Implant Infections

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Implant infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) can cause major complications during the perioperative period. Diclofenac, one of the most widely used nonsteroidal anti-inflammatory drugs, is often used to relieve pain and inflammation. In this study, it is found that high-dose diclofenac can inhibit the growth of MRSA, and does not easily induce drug-resistant mutations after continuous passage. However, low-dose diclofenac can resensitize bacteria to β-lactams, which help to circumvent drug resistance and improve the antibacterial efficacy of conventional antibiotics. Further, low-dose diclofenac in combination with β-lactams inhibit MRSA associated biofilm formation in implants. Transcriptomic and proteomic analyses indicate that diclofenac can reduce the expression of genes and proteins associated with β-lactam resistance: mecA, mecR, and blaZ; peptidoglycan biosynthesis: murA, murC, femA, and femB; and biofilm formation: altE and fnbP. Murine implant infection models indicate that diclofenac combined with β-lactams, can substantially alleviate MRSA infections in vivo. In addition, it is investigated that low dose diclofenac can inhibit MRSA antibiotic resistance via the mecA blaZ pathway and related biofilms in implants. The synergistic effect of diclofenac and β-lactams might have promising applications for preventing perioperative infection, considering its multitarget effects against MRSA.

1. Introduction

With the ever-growing global elderly population, many medical implant devices have been developed to improve quality of life, including joint prostheses and catheters. However, the increasing application of medical implants also increases the incidence of postoperative infection.[1] In fact, an estimated 25.6% of all healthcare-related infections in the United States are attributed to device-associated infections.[2] Specifically, patients with weakened immune systems, including those with diabetes, rheumatoid arthritis, malnutrition, and others, are generally at higher risk for developing implant-related infections. Moreover, clinical infections often require long-term antibiotic treatment and multiple surgical debridement procedures, which can have significant detrimental effects for the patients.[3] In addition, methicillin-resistant *Staphylococcus aureus* (MRSA), which is resistant to most β-lactams, including oxacillin and has increasing global prevalence, can also cause chronic infection.[4] Currently, although various precautionary measures have been established to reduce the incidence of infections, including performing surgeries under laminar flow air conditions and administering antibiotics prophylactically, the occurrence of postoperative infections remains high.[5] Moreover, several factors have been reported as being associated with the treatment failure of implant-related

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infections. First, implant surfaces can provide shelter for bacteria to survive. In fact, fewer bacterial cells are required to cause infections following implant-related surgeries compared to those not involving placement of indwelling devices. Second, the formation of biofilms on the surface of implants serves to protect bacteria, altering their growth state while reducing antibiotic penetration. Hence, biofilm formation can facilitate the development of drug-resistant bacteria by limiting their contact with antimicrobial substances. Third, the prevalence of MRSA, which exhibits broad-spectrum resistance to β-lactams, poses a significant threat to patients as effective antibiotics are rarely available. Fourth, the presence of medical implants, and the associated tissue damage that occurs during surgery, can interfere with normal immune responses. Finally, postoperative pain is also recognized as a risk factor for infections as it can reduce wound perfusion and decrease tissue oxygen partial pressure, thus impairing the ability of immune cells to induce oxidative killing. Taken together, these factors enable various opportunistic pathogens to replicate rapidly causing postoperative infections.

Recently, drug repurposing and synergistic drug screening have become promising approaches to combat infections caused by multidrug-resistant pathogens. Compared to the development of novel therapeutic agents, this strategy is considered more efficient for the treatment of severe infections as it has the potential to overcome challenges associated with weak activity elicited by individual drugs. For example, statins have been shown to disassemble bacterial membrane microdomains, thereby decreasing antibiotic resistance and resensitizing MRSA to antibiotic therapies. Similarly, norgestim has been reported to inhibit staphylococcal biofilm formation and resensitize MRSA to β-lactams. Although these drugs can help to control MRSA replication, associated side effects have also been reported for some patients. Besides, the efficacy of these drugs for preventing implant infections remains unclear. It is, therefore, necessary to identify a promising therapeutic regimen to target drug-resistant bacteria that can be readily administered in clinical settings.

Nonsteroidal anti-inflammatory drugs (NSAIDs) represent a group of widely used analgesic and antiplatelet agents. In the United States alone, over 111 million NSAID prescriptions are administered with approximately $2 billion spent on over-the-counter NSAIDs each year. Diclofenac is a commonly used NSAID that inhibits the synthesis of prostaglandins by inactivating cyclooxygenase. As it is a well-tolerated NSAID, with few reported side effects, diclofenac is considered one of the few “first choice” drugs for the treatment of painful conditions such as postoperative pain, and inflammatory conditions. Additionally, several researchers suggest that diclofenac could inhibit the proliferation of a broad spectrum of microorganisms, including Escherichia coli, S. aureus, Candida albicans, Listeria monocytogenes, and Mycobacterium tuberculosis. Moreover, antibiotic effects have also been noted following administration of diclofenac in combination with β-lactams, which are often used during the perioperative period. However, the mechanism by which diclofenac resensitizes MRSA to β-lactams has not been systematically reported.

During the perioperative period, β-lactams are generally recommended as the first-line drug to prevent implant infections. However, its application does not effectively prevent drug-resistant infections. Meanwhile, NSAIDs are often used intraoperatively in combination with analgesic agents for local infiltration to relieve postoperative pain following joint arthroplasty. Hence, these two perioperative drugs may function synergistically to protect against the colonization and replication of drug-resistant bacteria.

In this study, we conducted a series of in vitro and in vivo experiments to explore whether diclofenac can inhibit the growth of multidrug-resistant staphylococci; to determine whether low dose diclofenac can resensitize MRSA to β-lactams, such as oxacillin and if so, to elucidate the underlying mechanism; and to determine whether low dose diclofenac can be used in combination with β-lactams to reduce implant-related infections.

2. Results

2.1. Diclofenac Inhibits the Growth of Methicillin-Resistant Staphylococci

The chemical structure of diclofenac is shown in Figure 2A. To examine the antibacterial effect of diclofenac against drug-resistant Staphylococcus, we performed a minimum inhibitory concentration (MIC) assay, time kill assay, and Live/Dead bacteria staining. The MIC value of diclofenac (125 µg mL⁻¹) was found to be the same for MRSA, methicillin-sensitive Staphylococcus aureus (MSSA), and methicillin-resistant Staphylococcus epidermidis (MRSE). Moreover, bacterial viability was found to significantly decline after treatment with diclofenac at the MIC (Figure 2B). Additionally, it is important that antibiotics possess a low resistance frequency. In this regard, diclofenac was found to induce lower levels of resistance compared to daptomycin and vancomycin (Figure 2C), which is commonly administered as a primary agent for the treatment of MRSA infection. Moreover, compared with the control group, the number of viable bacteria in the diclofenac treatment group was lower (Figure 2D).

2.2. Diclofenac Inhibits the Expression of Genes Associated with β-Lactam Resistance and Biofilm Formation

The differentially expressed genes (DEGs) of MRSA were statistically analyzed to elucidate the antibacterial mechanism of diclofenac. We first applied a quantile normalization to the fragment per kilobase per million mapped reads (FPKM) values (Student’s t-test at a p value = 0.05), followed by selection of candidate genes displaying differential expression of at least two-fold compared to that in the control group. A total of 996 DEGs (678 upregulated and 318 downregulated) with highly significant expression patterns before and after diclofenac treatment were identified (Figure 3A). Enrichment analysis of KEGG pathways showed that DEGs associated with β-lactam resistance and energy metabolism were significantly downregulated by diclofenac (Figure 3B). All the sequencing data generated in this study have been submitted to the NCBI Sequence Read Archive with accession number SRP297471.

Generally, penicillin-binding protein 2a (PBP2a) shows low β-lactam affinity and remains active to allow peptidoglycan biosynthesis at normally lethal β-lactam concentrations.
Figure 1. Scheme illustration of diclofenac used in combination with oxacillin to alleviate implant infections caused by MRSA. The proposed mechanism suggests that diclofenac can enhance the bactericidal activity of oxacillin by inhibiting the expression of β-lactamase and PBP2a.

Figure 3C, *mecA*, which is the primary resistance gene encoding PBP2a, was reduced by 3.07-fold upon treatment with diclofenac compared to that in the control group. Its inactivation not only results in a poorly crosslinked peptidoglycan and β-lactam hyper-susceptibility but is also associated with increased susceptibility to other antibiotics.\(^{[26]}\) Besides, diclofenac induces a significant 2.29- and 2.02-fold reduction in the levels of VraS and VraR, respectively, which are central to peptidoglycan synthesis.\(^{[27]}\) In addition, the fem factors (essential for methicillin resistance),\(^{[28]}\) *femA* and *femB*, were also reduced by 1.31- and 1.65-fold, respectively, in the diclofenac-treated group compared to the control group.

Another important pathway inhibited by diclofenac is quorum sensing via the accessory gene regulator (agr) system. The *agr* phenotype may affect several aspects of biofilm behavior, including attachment to surfaces and biofilm dispersal and may contribute to the chronicity of biofilm-associated infections.\(^{[29]}\) The RNA-seq results indicated that *agrA*, *B*, and *C* were significantly reduced by 5.50-, 3.87-, and 5.22-fold, respectively, in the diclofenac-treated group compared to the control group. Meanwhile, *hld*, whose 3’ end encodes delta toxin, was also reduced by 7.44-fold.

qRT-PCR assay was further conducted to validate transcriptional changes induced by diclofenac. Genes associated with β-lactam resistance, peptidoglycan biosynthesis, biofilm formation, and quorum sensing were carefully examined. A relatively high consistency between qRT-PCR and transcriptomic data was observed in terms of expression patterns (Figure 3D). Although the absolute fold change for each gene differed between the qRT-PCR and transcriptomic data, similar trends were observed.

**2.3. Diclofenac Restores β-Lactams Efficacy toward MRSA**

Combined drug therapy to combat MRSA infections is often effective for reducing the associated side effects of individual agents.\(^{[12]}\) Therefore, the synergistic effects of drugs are important considerations. As shown in Figure 4A and Table S2 in the Supporting Information, low dose diclofenac significantly enhanced the antibacterial effect of several β-lactams. Specifically, 1/4 MIC of diclofenac (31.25 µg mL\(^{-1}\)) and 1/4 MIC of oxacillin (250 µg mL\(^{-1}\)) effectively inhibited MRSA growth. Meanwhile, the fractional inhibitory concentration index (FICI) was 0.5 for oxacillin, indicating that diclofenac is an efficient compound to offset drug resistance. Additionally, the time kill assay results demonstrated that a combination of 1/4 MIC diclofenac and 1/4 MIC oxacillin could significantly inhibit the growth of MRSA (Figure 4B). However, 1/4 MIC oxacillin or 1/4 MIC diclofenac alone was not effective in inhibiting MRSA growth.

**2.4. Diclofenac Combined with Oxacillin Destroys the Ultrastructure of Bacteria**

Electron micrographs obtained for the control group showed regular, intact surfaces, as expected (Figure 4C). In contrast, cells treated with low dose of diclofenac and oxacillin displayed severe cellular damage, including irregular and collapsed surfaces. These results were then confirmed using transmission electron microscopy (TEM), which demonstrated that untreated cells exhibited a circular and smooth shape, surrounded by a well-defined cell wall, with a prominent septal midline within the nascent septum (Figure 4D). In contrast, diclofenac-treated
Figure 2. Diclofenac inhibits the growth of methicillin-resistant staphylococci. A) Chemical structure of diclofenac. B) Time-dependent killing of methicillin-sensitive *Staphylococcus aureus* (MSSA) ATCC 25923, MRSA ATCC 43300 and methicillin-resistant *Staphylococcus epidermidis* (MRSE) ATCC 35984 by diclofenac at the minimal inhibitory concentration (MIC). The detection limit of the experiment was $\geq 10^2$ CFU mL$^{-1}$. CON, Control; Dic, Diclofenac. Each point represents the average value of three independent trials. Error bars are not presented for clarity. C) Resistance development during serial passaging in the presence of sub-MIC concentrations of antimicrobials. Daptomycin and vancomycin served as the positive control. Each point represents the average value of three independent trials. D) Representative confocal laser scanning microscopy (CLSM) images of MSSA, MRSA, and MRSE treated with 1x MIC diclofenac and stained for detecting bacterial viability.

bacteria presented irregular thickening and an increase in the occurrence of “fuzzy” cell walls.

The membrane permeability was then measured using propidium iodide (PI), which is incorporated into cells with damaged membranes (Figure 4E). Low dose of diclofenac combined with oxacillin was found to significantly reduce the membrane integrity. Similar results were observed in the 5x MIC diclofenac group. However, similar phenomena were not observed in the 1x MIC diclofenac group and the 1x MIC vancomycin group. Membrane depolarization was determined using the potential-sensitive fluorescent dye 3,3-dipropylthiostilbocyanine diSC3(5), which is released into the medium following disruption of membrane potential. Low dose of diclofenac combined with oxacillin was found to significantly dissipate the membrane potential in the MRSA group compared to the control group (Figure 4F). Additionally, a cellular ATP level assay and efflux inhibition assay were conducted to validate the RNA-seq results. As shown in Figure S1A,B in the Supporting Information, low dose diclofenac combined with oxacillin significantly reduced the levels of cellular ATP compared to oxacillin. This inhibitory effect occurred in a dose-dependent manner. However, the efflux pump activity was not significantly affected by diclofenac treatment.
Figure 3. Changes in the transcriptome of MRSA ATCC 43300 treated with diclofenac (62.5 µg mL\(^{-1}\)) or DMSO (control). A) Volcano map for the distribution of differentially expressed genes (DEGs). B) DEGs enriched in the KEGG pathway. Entries with larger bubbles contain more DEGs. C) Cluster analysis of selected DEGs. Red indicates a highly expressed gene, and blue indicates a weakly expressed gene. Each group contains data for three independent samples. D) Validation of RNA-Seq data for selected genes by qRT-PCR. The expression of DEGs involved in \(\beta\)-lactam resistance, peptidoglycan biosynthesis, quorum sensing, and biofilm formation was evaluated. Data analysis was performed using the comparative CT method, with 16S rRNA serving as the comparator. The results are presented as fold-changes relative to the control, which was set to a value of 1. Data are expressed as the mean ± SD; \(p\)-values are calculated using one-way ANOVA with Dunnett correction. \(n=3\); \(* * \ p < 0.01; \ *** \ p < 0.001.

2.5. Diclofenac Combined with Oxacillin Prevents Biofilm Formation

Bacterial adherence on the surface of Ti6Al4V disks was observed by CLSM (Figure 5A). All control group samples were well coated with biofilm. In contrast, the diclofenac treatment groups exhibited less colonization of bacteria. Next, the morphology of biofilms was observed with scanning electron microscopy (SEM). The biofilm in the control group was composed of several compact bacterial colonies (Figure 5B). However, there was less adherence of bacteria on the surface of Ti6Al4V disks following treatment with low doses of diclofenac and oxacillin. Next, crystal violet staining indicated that high dose of diclofenac, or the combination of low dose of diclofenac and oxacillin, significantly reduced the biofilm mass (Figure 5C).

2.6. Diclofenac Leads to Proteomic Alterations in MRSA

To further validate the antibacterial mechanism of diclofenac, SWATH-MS and parallel reaction monitoring (PRM) were performed to investigate proteomic alterations in MRSA.
Figure 4. Diclofenac combined with oxacillin destroys the ultrastructure of bacteria. A) Synergism between diclofenac and oxacillin, evaluated against MRSA ATCC 43300 using the fractional inhibitory concentration index (FICI) microdilution checkerboard method. OD\textsubscript{600} nm was measured after 18-h incubation at 37 °C. Experiments were independently repeated twice. Synergy, FICI \leq 0.5; no interaction, 0.5 < \text{FICI} \leq 4; antagonism, \text{FICI} > 4. B) Time-dependent killing of MRSA by combination of 1/4 MIC diclofenac (Dc) and 1/4 MIC oxacillin (Ox). The detection limit of the experiment was \geq 10^2 CFU mL\textsuperscript{-1}. Each point represents the average value of three independent trials. Error bars are not presented for clarity. C) Scanning electron microscopy images of bacteria incubated with 1x MIC diclofenac (125 µg mL\textsuperscript{-1}), 1/4xMIC diclofenac (Dc), and 1/4x MIC oxacillin (Ox) for 24 h. D) Transmission electron microscopy images of bacteria incubated with 1x MIC diclofenac, 1/4 MIC diclofenac (Dc), and 1/4 MIC oxacillin (Ox) for 8 h. White arrowheads indicate bacteria with damaged membranes and walls. The experiment was repeated three times. E) Membrane permeabilization analysis. Uptake of propidium iodide by MRSA treated with diclofenac (Dc), oxacillin (Ox) or vancomycin (Van). Each point represents the average value of three independent trials. Error bars are not presented for clarity. F) Membrane depolarization assay. Changes in fluorescence intensity of the membrane potential-sensitive dye diSC\textsubscript{3}(5) within 30 min after treatment of MRSA with diclofenac, oxacillin or vancomycin. Each point represents the average value of three independent trials. Error bars are not presented for clarity.
Figure 5. Diclofenac combined with oxacillin prevents biofilm formation. A) Confocal laser scanning microscopy images of biofilms treated with 1x MIC diclofenac (Dc) alone or 1/4 MIC diclofenac (Dc) in combination with 1/4 MIC oxacillin (Ox) for 24 h and stained by using a bacterial viability kit. B) Scanning electron microscopy images of biofilms treated with 1x MIC diclofenac (Dc) alone or 1/4 MIC diclofenac (Dc) in combination with 1/4 MIC oxacillin (Ox) for 24 h. C) Biofilms treated with 1x MIC diclofenac (Dc) alone or 1/4 MIC diclofenac (Dc) in combination with 1/4 MIC oxacillin (Ox) for 24 h and stained with 1% crystal violet. Biofilm formation was quantified by measuring sample absorbance at 595 nm using a microtiter plate reader. Data are expressed as the mean ± SD; n = 5; p-values are calculated using one-way ANOVA with Dunnett correction; *** p < 0.001.

SWATH-MS is a recently developed, quantitative proteomics-based MS method that combines data independent acquisition (DIA) and targeted data analysis. A total of 425 differentially expressed proteins (DEPs) were identified between the diclofenac treatment group and control group, among which 214 were downregulated and 211 were upregulated (Figure 6A). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium with the dataset identifier PXD023055. Next, the functions of these DEPs were further examined by KEGG pathway enrichment analysis (Figure 6B). The results show that diclofenac significantly affected pathways associated with β-lactam resistance, peptidoglycan biosynthesis, and energy metabolism, which is consistent with the RNA-seq and qRT-PCR results. Then, the alterations of several DEPs were visualized using a heat map (Figure 6C). After diclofenac treatment, DEPs involved in β-lactam resistance, such as mecR and blaZ, were significantly downregulated. Next, 40 proteins were selected as target proteins for PRM analysis to validate the authenticity and accuracy of the SWATH quantitative results. We found that the expression levels of all target proteins determined by PRM showed consistent trends with the SWATH quantitative results (Figure 6D). The results based on the quantitative methods such as RNA-seq, qRT-PCR, SWATH-MS, and PRM have good consistency, which strongly indicated the reliability of
Figure 6. Diclofenac causes MRSA proteomic alterations. A) Volcano plot for screening of differentially expressed proteins (DEPs) between the control group and diclofenac-treated groups. B) DEPs enriched in the KEGG pathway. The ordinate is the top 20 pathways with significant enrichment. C) Heat map of hierarchical clustering analysis of the selected DEPs between the control groups and diclofenac-treated groups. D) Expression changes of selected DEPs were further validated using parallel reaction monitoring, which showed consistent trends with the SWATH quantitative results. E) Structural model of PBP2α protein complexed with diclofenac compound. In the close-up view, the hydrogen bonds formed between the compound (magenta) and the protein are depicted as dashed black lines, and the residues involved in the hydrogen bond formation include Thr165 and Glu239. F) Schematic diagram of the possible mechanism employed by diclofenac to resensitize MRSA to β-lactams. Diclofenac reduced the expression of β-lactamase as well as peptidoglycan synthesis and PBP2α activity.

diclofenac to resensitize MRSA to β-lactams. Next, molecular docking was performed to explore whether diclofenac could directly interact with PBP2α to inhibit its activity. Computer-simulated docking, using a grid box surrounding the entire crystal structure of PBP2, predicted that diclofenac could fit into pockets near Glu239 and Thr165 (Figure 6E), which was found to be important for PBP2α enzyme activity. Taken together, the possible mechanism by which diclofenac may resensitize MRSA to β-lactams is presented in Figure 6F.
2.7. Diclofenac at its MIC Demonstrates Acceptable Safety in Mammalian Cells

The viability of the cultured cells was evaluated using the CCK-8 assay and LIVE-DEAD staining. The CCK-8 assay demonstrated a dose-dependent toxic effect with increasing concentrations of diclofenac on cells (Figure S2A in the Supporting Information). As we can see, cell viability was retained above 80% at all concentrations below 125 µg mL⁻¹. However, significant toxicity was observed when cells treated with diclofenac at concentrations above 250 µg mL⁻¹. Moreover, most cells in the treated groups exhibited similar viability levels as the control groups (Figure S2B in the Supporting Information). This low cytotoxicity of diclofenac at MIC value agrees with the result from the CCK-8 cytotoxicity test. Besides, compared with the untreated group, cells did not show significant morphological changes characteristic of cell death, including cell shrinkage and few cellular extensions (Figure S2C in the Supporting Information).

2.8. Diclofenac Combined with Oxacillin Ameliorates Murine Skin and Soft Tissue Infections

Diclofenac efficacy was analyzed by multiple parameters, including dermonecrotic area, abscess volume, CFU density in situ, and histopathology. As shown in Figure 7A,B, combination of low dose diclofenac and oxacillin significantly restricted the dermonecrosis area and abscess volume compared with that in the vehicle group after 7 days post-infection with MRSA ATCC 43300 strain. In addition, a single dose of diclofenac resulted in a smaller skin lesion area compared to that in the vehicle group, whereas oxacillin-treated mice exhibited morphology similar to that in the vehicle group. Furthermore, the number of CFUs within the abscesses was significantly reduced in mice treated with diclofenac compared with that in the vehicle groups (Figure 7C). Lastly, hematoxylin and eosin (H&E) and Gram staining revealed multiple sites of MRSA abscess formation in the vehicle group (Figure 7D). However, mice treated with low dose diclofenac and oxacillin had substantially fewer MRSA micro abscesses and minimal detectable invasion.

2.9. Diclofenac Combined with Oxacillin Ameliorates Murine Implant Infections

The biofilm-associated infections were examined by CLSM, spread plate methods, and histopathology. As shown in Figure 8A,B, diclofenac alone or in combination with oxacillin could significantly reduce biofilm formation on Ti6Al4V disks 7 days post-infection with the MRSA ATCC 43300 strain. Furthermore, the combination of diclofenac and oxacillin exhibited superior antibacterial effects against biofilm-associated infections. Additionally, the number of CFUs per site was significantly reduced in mice treated with diclofenac compared with that in the oxacillin groups (Figure 8C). Lastly, H&E and Gram staining indicated that mice treated with diclofenac alone, or in combination with oxacillin, had fewer MRSA micro abscesses than mice in the vehicle group (Figure 8D). Taken together, the combination of diclofenac and oxacillin not only reduced the required dose of a single drug but also enhanced the antibacterial effect of β-lactams in the treatment of implant infections.

3. Discussion

Approximately one fifth of all annual deaths occur as a result of infectious diseases[13]. Moreover, the prevalence of drug-resistant bacteria and the shortage of available antibiotics have complicated anti-infective therapy.[12] Therefore, drug repurposing screens and synergistic drug combinations have become an effective substitute for the treatment of infectious diseases.[13] Compared with administration of single antibiotics, that of synergistic combinations of two known compounds have several advantages. For example, they can reduce the concentrations required for each individual drug, thereby reducing the risk of drug toxicity and medical expenses. Besides, lower concentrations of antibiotics are less likely to induce drug resistance mutations in sensitive bacteria. In this study, we demonstrated that diclofenac, a widely used NSAID, could resensitize MRSA to β-lactams both in vitro and in vivo. In addition, even after continuous exposure to high concentrations of diclofenac, MRSA strains did not develop resistance. Moreover, RNA-seq and SWATH-MS results indicated that diclofenac effectively reduced the expression of genes and proteins associated with β-lactam resistance, peptidoglycan biosynthesis, biofilm formation, and bacterial virulence. As a result, the antibacterial efficacy of oxacillin was significantly improved, which might greatly reduce the current dependence on glycopeptide antibiotics such as vancomycin and teicoplanin.

Biofilm formation on the surfaces of implants is another significant challenge associated with treating device-related infections. Once a biofilm forms on the surface of indwelling devices, it eventually leads to chronic infection.[8-14] Even if antibiotics are used at concentrations 100 to 1000 times higher than the normal dose, it is difficult to completely eradicate the biofilm.[13] Here, we demonstrated that, the combination of low-dose diclofenac and oxacillin could effectively inhibit the adherence of bacteria to the surface of titanium disks both in vitro and in vivo. Therefore, this synergistic effect might greatly reduce the probability of implant infections caused by MRSA. RNA-seq and SWATH-MS results further indicated that diclofenac not only inhibited the expression of surface proteins associated with biofilm formation but also reduced the production of virulence proteins such as sarR. Therefore, the combination of low dose diclofenac with β-lactams might represent a promising regime for the prevention of biofilm-associated infections during the perioperative period.

In the last few decades, commercial launching of new bacterial agents has decreased due to difficulties associated with drug discovery and development.[10,14] Therefore, drug repurposing and combination screens may provide alternative approaches at a fraction of the time and cost required for traditional methods of drug development.[12] Moreover, clinical trials will require less time to complete as the corresponding pharmacological and safety profiles have already been established for each of the individual drugs. To date, many FDA approved drugs, such as statin and metformin, have been found to have additional antibacterial properties to treat severe infections.[14,16] Diclofenac, one of the most common anti-inflammatory and analgesic drugs, is often used to relieve various kinds of pain, such as postoperative pain. According to research, periarticular injection of large doses of
Figure 7. Murine skin and soft tissue infections treated with diclofenac (80 mg kg$^{-1}$, s.c.), oxacillin (200 mg kg$^{-1}$, i.p.), or diclofenac (80 mg kg$^{-1}$, s.c.) combined with oxacillin (200 mg kg$^{-1}$, i.p.). A) Restriction of the abscess area on day 7 in the infection model is shown. Each image shows dermonecrotic lesions from a representative mouse for each group. B) Dermonecrosis areas and abscess volume were measured at selected time points on day 7 in the infection model. Data are expressed as the mean ± SD; n = 10; p-values are calculated using one-way ANOVA with Dunnett correction, ns, not significant; * p < 0.05; *** p < 0.001. C) Distribution of CFU density in the abscesses was determined by quantitative culturing from day 7 of the infection. Data are expressed as the means ± SD; n = 10; p-values are calculated using one-way ANOVA with Dunnett correction, ns, not significant; * p < 0.05; ** p < 0.01, *** p < 0.001. D) Abscess histopathology on day 7 in the infection model. Gram staining as well as hematoxylin and eosin (H&E) staining revealed the location of abscesses and bacteria. In gram-stained tissues, MRSA cells are dyed violet.
Figure 8. Murine implant infections treated with diclofenac (80 mg kg$^{-1}$, s.c.), oxacillin (200 mg kg$^{-1}$, i.p.), or diclofenac (80 mg kg$^{-1}$, s.c.) combined with oxacillin (200 mg kg$^{-1}$, i.p.). A) Confocal laser scanning microscopy and spread plate method used to observe biofilm formation on the surface of Ti6Al4V disks after 7 days of infection. B) Distribution of CFU density per disk was determined by quantitative culturing from day 7 of the infection. Data are expressed as the means ± SD; n = 10; p-values are calculated using one-way ANOVA with Dunnett correction, * p < 0.05; *** p < 0.001. C) Distribution of CFU density per site was determined by quantitative culturing from day 7 of the infection. Data are expressed as the means ± SD; n = 10; p-values are calculated using one-way ANOVA with Dunnett correction, ns, not significant; ** p < 0.01, *** p < 0.001. D) Histopathological evaluation on day 7 in the infection model. The location of abscesses and bacteria were examined by Gram and hematoxylin and eosin (H&E) staining. In gram-stained tissues, MRSA cells are dyed violet.
local anesthetics after total hip/knee arthroplasties provides better pain relief and more rapid rehabilitation.\cite{23b} Moreover, steroids, NSAIDs, and morphine are often administered in combination with local anesthetics.\cite{23a,37} Therefore, the addition of diclofenac to the intra-articular cocktail analgesic injection will not only provide effective pain relief but may also prevent biofilm formation during the early stages of implant infection (Figure 9). Furthermore, the synergistic combinations of diclofenac and $\beta$-lactams can increase therapeutic efficacy against MRSA, which could further reduce the need for glycopeptide antibiotics. Therefore, to improve the antibacterial efficiency of diclofenac while simultaneously reducing its side effects, the diclofenac and $\beta$-lactam mixture could be loaded on different kinds of biomaterials, such as nanoparticles, nanosheets, and others. (Figure 9).

Although many experiments have been performed to demonstrate the ability of diclofenac to resensitize MRSA to $\beta$-lactams, certain gaps in knowledge remain that require further investigation. First, it is important to ascertain the potential adverse side effects associated with consuming oral diclofenac for an extended period of time.\cite{38} Hence, it is also necessary to develop a suitable drug delivery system to improve the antibacterial effects while reducing its side effects. Second, investigation into the efficacy of combined therapy comprising diclofenac and other types of antibiotics, such as aminoglycosides, is required, which will be a focus of our future studies.

4. Conclusion

In conclusion, diclofenac, one of the most commonly used NSAIDs, was found to effectively inhibit the growth of drug-resistant staphylococci both in vitro and in vivo. Moreover, high dose diclofenac treatment does not cause drug resistance mutations after continuous passage. Moreover, diclofenac can reduce the expression of genes and proteins associated with $\beta$-lactam resistance, peptidoglycan biosynthesis, and biofilm formation, which may help circumvent the development of drug resistance and improve the antibacterial efficacy of conventional antibiotics. Importantly, low dose diclofenac combined with oxacillin, also significantly alleviates MRSA infection in vivo. Hence, this combinational therapy may be a promising regime for the prevention of perioperative infections.

5. Experimental Section

**Bacterial Strains and Growth Conditions**: MSSA strain ATCC 25923, MRSA strain ATCC 43300, and MRSE strain ATCC 35984 were purchased from the American Type Culture Collection. Bacteria were grown in trypticase soy broth (TSB; BD Biosciences, Franklin Lakes, NJ, USA) overnight at 37 °C with shaking, diluted 1:10,000, and subsequently allowed to reach log phase growth.

**Figure 9.** Schematic representation of the potential applications of diclofenac-loaded biomaterials in the treatment of implant infection caused by MRSA.
**Antimicrobial Agents and Chemicals:**

Vancomycin and oxacillin were purchased from Sigma-Aldrich (St Louis, MO, USA). Diclofenac and daptomycin were purchased from Dalian Meilun Biotechnology Co., Ltd (Dalian, China). All drugs were made up to 10 mg mL\(^{-1}\) stocks in DMSO or ddH\(_2\)O. For assays with daptomycin, the medium or buffer was supplemented with 50 µg mL\(^{-1}\) Ca\(_2\)Cl\(_2\).

**Minimal Inhibitory Concentration (MIC) Assay:**

The MICs of the compounds used in this study were determined by the standard microdilution method according to the procedures outlined by the Clinical and Laboratory Standards Institute.\(^{[39]}\) Briefly, bacteria were grown to log phase and resuspended to a cell density of approximately 5 \times 10^5 CFU mL\(^{-1}\). Next, a two-fold serial dilution of the compound ranging from 500 µg mL\(^{-1}\) to 0.98 µg mL\(^{-1}\) was prepared in a final volume of 100 µL. All plates were covered and incubated at 37 °C for 24 h. The MIC was taken as the lowest concentration of the agent that completely inhibited visible bacterial growth. All MIC determinations were carried out in duplicate in three independent experiments.

**Time Kill Assay:**

The kinetics of diclofenac against MSSA ATCC 25923, MRSA ATCC 43300, and MRSE ATCC 35984 was tested as previously described.\(^{[40]}\) Briefly, 50 µL of diclofenac was serially diluted two-fold across a 96-well assay block. Next, 50 µL of bacterial inoculum (5 \times 10^5 CFU mL\(^{-1}\)) was added and incubated at 37 °C. At specific time points, serial dilutions of the cultures were plated and incubated overnight at 37 °C. The number of viable CFU mL\(^{-1}\) remaining in the original culture was calculated by bacterial colony counting.

**Antibiotic Resistance Assay:**

Antibiotic-resistant mutants were acquired by serial passaging over a 20-day period as described previously.\(^{[41]}\) Briefly, 100 µL of MRSA ATCC 43300 culture (10^5 CFU mL\(^{-1}\)) was added to a 96-well plate containing an extended concentration gradient of diclofenac, daptomycin or vancomycin. After incubating at 37 °C for 24 h, the OD 600 nm was measured with a spectrophotometer (BioTek, VT, USA). Bacterial growth was defined as OD 600 nm ≥ 0.1. For the following day’s MIC plate, 10 µL of the sub-lethal MIC MRSA cultures were diluted in 10 mL of TSB and incubated overnight at 37 °C. The remaining of the culture was stored in 20% glycerol at -80 °C.

**RNA Isolation and Quantitative RT-PCR (qrt-PCR):**

An RNeasy mini kit (Qiagen, Germany) was used to extract total RNA from MRSA ATCC 43300, according to the manufacturer’s instructions. A total of 1 µg of purified RNA was used for cDNA synthesis using the RT Master Kit (Takara, Shiga, Japan). Primers (Sangon Biotech, Shanghai, China) used for the qRT-PCR reaction are shown in Table S1 in the Supporting Information. A bulk PCR reaction mixture was prepared using the TB Green Premix Ex Taq™ Kit (Takara, Shiga, Japan), and the thermal cycling parameters were as follows: initial denaturation at 95 °C for 30 s; followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s, and 72 °C for 45 s. Quantitative RT-PCR was performed in triplicate and repeated in at least five separate experiments using ABI Prism 7500 (Applied Biosystems, Norwalk, CT, USA). Data were analyzed using the comparative CT method, with 16s rRNA expression serving as a normalization control. The results are presented as fold-changes relative to the control, which was set to a value of 1.

**RNA Sequencing:**

Gene expression analysis of the MRSA ATCC 43300 strain was analyzed after treatment with diclofenac (62.5 µg mL\(^{-1}\)) or 0.1% DMSO. Samples were collected after 8 h of treatment, and three independently prepared RNA samples from each condition were used for RNA Sequencing. Illumina sequencing was performed by Shanghai Majorbio Biopharm Technology Co., Ltd. (Shanghai, China) using the Illumina TruSeq RNA sample prep kit and HiSeq 4000 SBS kit (Illumina, Inc.). After sequencing, the data were analyzed using edgeR software, and statistical significance was defined at \(p < 0.05\). Only genes that were significantly differentially regulated \((p < 0.05)\) by at least two-fold compared to the control were considered.

**Antibiotic Synergy Test:**

The MIC of each compound (A and B) was calculated as follows: FICI = \((\text{MIC of compound A in combination/MIC of compound A alone}) + (\text{MIC of compound B in combination/MIC of compound B alone})\). The interaction between the two compounds was defined as follows: 1) FICI ≤ 0.5 indicates synergy; 2) 0.5 < FICI ≤ 4 indicates “no interaction;” 3) FICI > 4 indicates antagonism.\(^{[43]}\)

**Transmission Electron Microscopy:**

Preparation and examination of diclofenac-treated cells by TEM was performed as described previously.\(^{[44]}\) Briefly, bacterial cultures were exposed to diclofenac (125 µg mL\(^{-1}\)), a combination of low dose diclofenac (31.25 µg mL\(^{-1}\)) and oxacillin (250 µg mL\(^{-1}\)), or 0.1% DMSO (control) for 8 h at 37 °C. After centrifugation (5 000 rpm; 5 min), the pellets were resuspended in 1 mL of 2.5% glutaraldehyde (Solarbio, Beijing, China). Fixed cells were washed three times with 0.1 M sodium cacodylate buffer and post-fixed with 1% osmium tetroxide. The samples were stained with 2% uranyl acetate and infiltrated with Epon resin (ProSciTech, Townsville, Australia). Micrographs of the cells were examined by a JEM 1011 TEM (JEOL, Tokyo, Japan).

**Membrane Permeability Assay:**

The integrity of the cell membrane was evaluated as described previously.\(^{[45]}\) Briefly, black 384-well polystyrene plates (Corning, CLS573, NY, USA) were filled with 25 µL of PBS per well containing the indicated concentration of compounds. Log phase bacteria were washed three times with PBS and adjusted to 1 \times 10^8 CFU mL\(^{-1}\) with PBS. Then, PI was added to 10 mL of diluted bacterial suspension to a final concentration of 5 µg mL\(^{-1}\) and incubated at 37 °C for 30 min in the dark. A total 25 µL volume of the bacteria/PI mixture was added to each well of 384-well plates containing diclofenac. Lastly, fluorescence was measured using a spectrophotometer (Synergy H1, BioTek), with excitation and emission wavelengths of 535 and 620 nm, respectively. Data were corrected by subtraction of fluorescence signal from untreated cells in the presence of PI. All experiments were conducted in triplicate.

**Membrane Depolarization Assay:**

Bacterial cytoplasmic membrane depolarization was detected using the fluorescent dye 3,3-dipropyl-thiacycloborocaine dSiSC(S) (Sigma-Aldrich, Australia) as previously described.\(^{[46]}\) Briefly, log phase cells were pelleted, washed, and adjusted to an OD600 of 0.2 in assay buffer (10 \times 10^{-3} M HEPES, 50 µg mL\(^{-1}\) Ca\(_2\)Cl\(_2\), and 5 \times 10^{-3} M glucose). Next, dSiSC(S) was added to a final concentration of 1.5 µM and incubated in the dark to enable dye uptake and a stable reduction in fluorescence. After 30 min, cells were diluted in assay buffer, and 100 µL of bacterial suspension was added to a 96-well black-walled plate. Before adding test agents, background data were collected using a spectrophotometer (excitation/emission = 612/665 nm) to ensure fluorescence quenching. Each sample was tested in triplicate, and independent assays were performed three times, showing similar results.

**Cellular ATP Level Assay:**

The assay was performed using a BacTiter-Glo microplate assay kit (Promega, Madison, WI, USA) as described previously.\(^{[46]}\) Briefly, log-phased cells were diluted to 10^6 CFU mL\(^{-1}\) and subsequently incubated with diclofenac or oxacillin. TSB broth without added compounds served as the positive control. After 24 h incubation at 37 °C, bacteria medium was transferred into a black, 384-well microplate (Corning, CLS573, NY, USA), and each test sample was mixed with an equal volume of BacTiter-Glo reagent, which measures the cellular ATP level. The mixed samples were then incubated for 5 min at room temperature, and their luminescence intensities were determined by a Synergy H1 hybrid reader (BioTek, USA) at an integration time of 1 s per well. Relative luminescence units (RLU) values were subtracted from the background control of medium with bacteria.

**Ethidium Bromide Efflux Inhibition Assay:**

The efflux pump activity was examined by monitoring the fluorescence of ethidium bromide as previously described.\(^{[47]}\) Briefly, log-phased bacterial suspension was pelleted, washed, and resuspended in phosphate buffer saline to an OD600 of 0.2. Next, a sub-inhibitory concentration of ethidium bromide, the substrate of the efflux pump, was added to a final concentration of 2 µg mL\(^{-1}\). The cultures were then incubated for 30 min at 37 °C in the presence of 1/2 MIC daptomycin (62.5 µg mL\(^{-1}\)) or positive control reserpine (20 µg mL\(^{-1}\)). Subsequently, 100 µL of the suspensions were inoculated into a black-walled 96-well plate, and the fluorescence was monitored for 30 min with excitation and emission wavelengths of 530 and 600 nm, respectively, using a spectrophotometer.
Confocal Laser Scanning Microscopy: Cells exposed to diclofenac (125 µg mL\(^{-1}\)), a combination of low dose diclofenac (31.25 µg mL\(^{-1}\)) and oxacillin (250 µg mL\(^{-1}\)), or 0.1% DMSO were imaged by CLSM. Briefly, bacterial cultures were inoculated in sterile 24-well plates containing tested compounds. Sterile Ti6Al4V disks were then immersed into the medium as a substratum for biofilm growth and incubated at 37 °C for 24 h. Planktonic cells were removed by rinsing three times with PBS. Biofilm viability was determined using a bacterial viability kit (LIVE/DEAD BacLight, MA, USA). After incubating for 15 min in the dark, the stained disks were observed using a CLSM microscope (Leica TCS SP2; Heidelberg, Germany).

Scanning Electron Microscopy: For SEM, the samples were processed as described previously.[48] Briefly, exponential-phase bacterial cultures were grown in 24-well plates in the presence of sterile Ti6Al4V disks. Then, diclofenac (125 µg mL\(^{-1}\)), a combination of low dose diclofenac (31.25 µg mL\(^{-1}\)) and oxacillin (250 µg mL\(^{-1}\)), or 0.1% DMSO was added to the TSB medium and incubated overnight at 37 °C for 24 h. Next, Ti6Al4V disks were removed and gently washed three times with PBS. The biofilms were fixed with 2.5% glutaraldehyde (Solarbio, Beijing, China) for 24 h at 4 °C. Subsequently, the biofilms were dehydrated for 10 min in an ethanol series (30%, 40%, 50%, 60%, 70%, 80%, and 90%) and for 20 min in absolute alcohol. The samples were observed using a scanning electron microscope (Hitachi S-4800, Japan).

Biofilm Inhibition Assay: The inhibitory effect of diclofenac on biofilm formation was examined using a previously described protocol with several modifications.[52] Briefly, eight-week-old female CD1 mice were treated with diclofenac (125 µg mL\(^{-1}\)) for 24 h. washed twice with PBS buffer, and treated with the viability/cytotoxicity reagent for 45 min at 37 °C. Labeled cells were analyzed by fluorescence microscopy (Nikon, Japan). Viable (live) cells were stained with calcine-AM (green), whereas dead cells were stained with ethidium homodimer-1 (EthD-1; red).

Murine Skin and Soft Tissue Infection Model: All in vivo animal experimental procedures were approved and conducted in accordance with the guidelines of the Animal Ethics Committee of Renji Hospital, China. We followed a previously described protocol to mimic skin and soft tissue infections with some modifications.[21] Briefly, eight-week-old female CD1 mice were infected with S. aureus (25–30 colonies were grown on blood agar, approximately 3×10\(^{5}\) CFU) of skin was inoculated with a 1:100 dilution of the FITC-labeled phallicin in 1 h in the dark. Nuclear DNA was counterstained with DAPI, and stained cells were visualized using a confocal laser scanning microscope (Leica TCS SP2; Heidelberg, Germany).

Dermonecrosis Area Measurement—Magnitude: After infection, dermonecrosis area and abscess volume were measured in each mouse flank. To quantify dermonecrosis area (cm\(^2\)), lesion size length (l) and width (w) were measured. Next, abscess volume (cm\(^3\)) was calculated according to the formula for a spherical ellipsoid: \(v = (4/3) \times l \times w^2\).

Abscess Evaluation—CFU Determination: Seven days post-infection, mice were sacrificed and processed for quantitative culture of abscesses. Each flank was aseptically dissected, and the abscess was removed and prepared for culture. After individually homogenizing and serially diluting the suspensions in sterile PBS, the dilutions were spread onto TSA plates.
The plates were incubated at 37 °C for 24 h and the resulting colonies counted as CFU per abscess.

**Abscess Evaluation—Histological Evaluation:** Seven days post-infection, tissue inflammation and bacterial quantity related to MRSA ATCC 43300 strain invasion were assessed in the skin abscesses. For histological staining, excised skin specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Next, sections of skin tissue were observed under a microscope after H&E, and Gram staining was performed.

**Murine Implant Infection Model:** Implant infection models were established as previously described,[53] with several modifications. Briefly, 8- to 10-week-old specific-pathogen-free (SPF) CD1 ICR female mice were obtained from Shanghai JieSiJe Laboratory Animal Co., Ltd. and randomly divided into five experimental groups: 1) vehicle; 2) vancomycin; 3) oxacillin; 4) diclofenac; 5) diclofenac + oxacillin. Each mouse was anesthetized with ketamine/xylazine; the flanks of mice were shaved, and an incision was made parallel to the spine. Next, sterilized Ti disks were placed into the subcutaneous pocket using aseptic technique and the incision sutured layer by layer. Then, 100 μL of exponential-phase MRSA ATCC 43300 suspension (10^5 CFU per site) was injected into the subcutaneous biomaterial bed. After 2 h of infection, a single dose of vancomycin (80 mg kg^-1, i.p.), oxacillin (200 mg kg^-1, i.p.), diclofenac (80 mg kg^-1, s.c.), diclofenac (80 mg kg^-1, s.c.) + oxacillin (200 mg kg^-1, i.p.), or saline was administered. Thereafter, treatment was administered every 24 h until 7 days post-implantation. To determine the bacterial load and biofilm formation, the Ti disks and soft tissue surrounding the implants were removed from each site and separately immersed in PBS. After individually homogenizing and serially diluting the suspensions, the dilutions were spread onto TSA plates. The plates were incubated at 37 °C for 24 h, and the number of bacteria on the implants or in the tissues were calculated. For histological evaluation, excised skin specimens were fixed in 4% paraformaldehyde and embedded in paraffin. Next, sections of skin tissue were observed microscopically after H&E and Gram staining.

**Statistical Analyses:** The results were analyzed using Prism 7 (GraphPad Software Inc., CA, USA), and the data are presented as the mean ± standard deviation (SD). Statistical significance was determined by unpaired two-tailed Student’s t tests where only two groups existed or by one-way ANOVA with Dunnett’s or Tukey’s post-test. Samples size (n) and preprocessing normalization of data was given in the corresponding figure legend. Differences between groups were considered significant at p < 0.05. (*p<0.05, **p<0.01, ***p<0.001).

**Supporting Information**

Supporting Information is available from the Wiley Online Library or from the author.

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**Conflict of Interest**

The authors declare no conflict of interest.
