Title: Nitroxide functionalized antibiotics are promising eradication agents against Staphylococcus aureus biofilms.

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Running title: Nitroxide-mediated dispersal of S. aureus biofilms.

Keywords: Anti-biofilm, Antibiotics, Biofilm Eradication, Biofilm Dispersal, Nitroxide, Staphylococcus aureus, Infection, Calgary Biofilm Device, fluorescent probes, fluoroquinolones.

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ABSTRACT

Treatment of *Staphylococcus aureus* biofilm-related infections represents an important medical challenge worldwide, as biofilms, even of drug-susceptible *S. aureus* strains, are highly refractory to conventional antibiotic therapy. Nitroxides were recently shown to induce dispersal of Gram-negative biofilms *in vitro*, but their action against Gram-positive bacterial biofilms remains unknown. Here we demonstrate that the biofilm dispersal activity of nitroxides extends to *S. aureus*, a clinically important Gram-positive pathogen. Co-administration of the nitroxide CTEMPO with ciprofloxacin significantly improved the antibiotic's biofilm-eradication activity against *S. aureus*. Moreover, covalently linking the nitroxide to the antibiotic moiety further reduced ciprofloxacin's minimal biofilm eradication concentration. Microscopy analysis revealed that fluorescent nitroxide-antibiotic hybrids could penetrate *S. aureus* biofilms and enter into cells localising at the surface and base of the biofilm structure. No toxicity was observed for the nitroxide CTEMPO and the nitroxide-antibiotic hybrids against human cells. Taken together, our results show that nitroxides can mediate dispersal of Gram-positive biofilms and that dual-acting biofilm-eradication antibiotics could provide broad-spectrum therapies for the treatment of biofilm-related infections.

INTRODUCTION

*Staphylococcus aureus* is a Gram-positive commensal and opportunistic human pathogen, which is a major cause of nosocomial and community-acquired infections (1). The inherent ability of *S. aureus* to attach to medical devices and host tissues and establish biofilms is a major driver of failing antibiotic therapies and the persistence of chronic infections (2-4).
Thus, there is an urgent need for novel strategies for the treatment and eradication of *S. aureus* biofilms.

Current antimicrobial strategies, which are effective against planktonic bacteria, often have little or no effect when administered to biofilms (5, 6). During infection, bacteria reside mostly within biofilms but can revert to the planktonic lifestyle by modulating the expression of specific genes (7). Consequently, the development of small molecules with the ability to trigger a cell change from biofilm to the planktonic state has become a promising area of anti-biofilm research (8, 9).

One of the most promising small molecules with anti-biofilm activity is nitric oxide (NO), which has been shown to inhibit biofilm formation and trigger biofilm dispersal in a dose dependent manner (10-12) in a variety of biofilm-forming bacteria (13). In *Pseudomonas aeruginosa*, for example, treating a mature biofilm with sub-lethal NO concentrations (pM to nM range) triggers a transition from the sessile (biofilm) to the motile (planktonic) state, providing a means of effective antibiotic therapy (11, 13). The anti-biofilm properties of NO in *P. aeruginosa* are mediated by regulation of intracellular levels of the secondary messenger cyclic dimeric guanosine monophosphate (cyclic di-GMP), which plays a pivotal role in biofilm development; high levels facilitate biofilm formation, while low levels prompt biofilm dispersal (7, 14). However, the targeted and controlled delivery of NO to biological systems as therapies is a challenge due to the reactive nature and short half-life (0.1 – 5 seconds) (15) of the gaseous molecule. Consequently, methods for circumventing this challenge have been the focus of intensive research over the past several years, with the use of NO-donors in biofilm dispersal now comprehensively documented (16, 17). A variety of NO-donor compounds with anti-biofilm activity have already been described.
However, donor molecules are often themselves inherently unstable (18), necessitating an alternative approach.

Nitroxides are long-lived, stable free radical species, which contain a disubstituted nitrogen atom linked to a univalent oxygen atom (19). Nitroxides are considered sterically hindered, structural mimics of NO, as both compounds contain an unpaired electron, which is delocalized over the nitrogen-oxygen bond. The biological activity of nitroxides is often attributed to their NO-mimetic properties, with both being efficient scavengers of protein-derived radicals (20). Nitroxides, however, are mostly air-stable crystalline solids, in contrast to NO, which is unstable and gaseous at room temperature. This significant difference makes nitroxides ideal candidates for circumventing the handling and delivery issues associated with NO.

We have previously demonstrated the ability of nitroxides to inhibit and disperse bacterial biofilms of *P. aeruginosa* and *E. coli* (21, 22). Nitroxides were also shown to enhance biofilm antibiotic susceptibility when co-administered as a combined treatment (23). Furthermore, we have shown that by covalently tethering a nitroxide to the antibiotic ciprofloxacin (Figure 1, Cipro-PROXYL, Cipro-TEMPO, and Cipro-TMIO) and thus delivering the antibiotic at the site of nitroxide mediated dispersal, we could achieve effective eradication of mature *P. aeruginosa* biofilms (24, 25). Furthermore, we have also recently developed several profluorescent fluoroquinolone-nitroxide probes (switch on fluorescent probes) (Figure 1, Fluoroquinolone-TEMPO) for investigating the interactions of nitroxide functionalised antibiotics with bacterial cells (26). While the dispersal and anti-biofilm properties of nitroxides have been successfully documented against Gram-negative bacteria, their anti-biofilm properties against Gram-positive bacteria remain unexplored.
Here we have employed a reproducible, high-throughput in vitro biofilm assay to comprehensively examine the full antimicrobial, anti-biofilm, and biofilm-eradication potential of nitroxides and antibiotic-nitroxide hybrids against *S. aureus*. Our findings demonstrate the anti-biofilm action of nitroxides against a clinically important Gram-positive pathogen extending this promising therapeutic strategy to a large number of *S. aureus* biofilm-related infections.

**RESULTS**

The nitroxide CTEMPO can disperse established *S. aureus* biofilms.

Established *S. aureus* ATCC 29213 biofilms were co-treated with CTEMPO (160 – 2.5 µM) and ciprofloxacin (6 µM) or ciprofloxacin alone (6 µM) for 24 hours. Viable bacteria remaining in the treated biofilms were recovered in media without nitroxide or antibiotics and monitored for growth, with lag time recorded as a direct measure of the initial colony forming units (CFU) present in each well. Lag time was found to be inversely proportional to the number of bacterial cells residing in the biofilm that survived antimicrobial treatment (short lag time indicates many cells survived antimicrobial treatment, while longer lag times indicate fewer cells survived antimicrobial treatment). Significant increase in lag time, indicative of a significant reduction in biofilm-associated bacteria recovered post-treatment, was observed at CTEMPO concentrations in the range of 10 to 80 µM (*p* = 0.0003, Kruskal-Wallis test), compared to control biofilms treated with ciprofloxacin alone (Figure 2). As no antimicrobial activity was observed for CTEMPO alone at those concentrations against planktonic or biofilm-residing *S. aureus* ATCC 29213 cells (Table 1), the nitroxide was most likely inducing cell dispersal in biofilms, not bacterial killing. CTEMPO alone (no
Nitroxide co-administration improves the efficacy of ciprofloxacin against *S. aureus* biofilms.

As nitroxides appear to induce *S. aureus* biofilm dispersal, we sought to examine whether this could improve the efficacy of ciprofloxacin against established *S. aureus* biofilms. Ciprofloxacin is a commonly prescribed fluoroquinolone antibiotic with potent antimicrobial activity against planktonic *S. aureus* ATCC 29213 (27). We confirmed the MIC of ciprofloxacin for this strain to be within the previously published range (1.5 - 0.4 μM; Table 1) (27). Despite the low MIC, established *S. aureus* ATCC 29213 biofilms required 4096 μM of ciprofloxacin for complete (99.9%) eradication (concentration range tested: 4096 - 32 μM), demonstrating the biofilm’s extremely high antibiotic tolerance. The minimum biofilm eradication concentration (MBEC) of ciprofloxacin against established *S. aureus* ATCC 29213 biofilms was thus determined to be 4096 μg/mL (Table 1).

Co-administration of CTEMPO (at 2.5, 8, 10, 20, 40, or 80 μM) with ciprofloxacin (at a concentration range of 4096 - 32 μM) against established *S. aureus* ATCC 29213 biofilms resulted in a significant reduction in the MBEC value of ciprofloxacin at CTEMPO concentrations between 8 - 80 μM (Table 1). Ciprofloxacin potentiation was greatest with 8 μM CTEMPO co-administration, which reduced ciprofloxacin’s MBEC value from 4096 μM to 256 μM, representing a 16-fold improvement in the drug’s efficacy against Gram-positive *S. aureus* ATCC 29213 biofilms (Table 1). To confirm the synergistic effects of nitroxides and ciprofloxacin against *S. aureus* biofilms, we performed co-treatment with a different nitroxide, 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO). CTMIO was also able to...
potentiate the action of ciprofloxacin against established *S. aureus* ATCC 29213 biofilms (4-136 fold improvement), without possessing antimicrobial activity alone against planktonic or biofilm-residing cells (Table 1). Taken together, these results demonstrate the ability of nitroxides to significantly improve the efficacy of antibiotic therapy against *S. aureus* biofilms.

**Ciprofloxacin-nitroxide hybrids are potent *S. aureus* biofilm-eradication agents.**

Considering the synergistic effect of ciprofloxacin-nitroxide co-administration on *S. aureus* biofilm eradication, we evaluated whether combining the nitroxide and antibiotic within one molecule would offer greater improvement. We rationalized that by localising the antibiotic directly at the site of nitroxide-mediated dispersal, it could more effectively eradicate dispersed cells than in combination treatment. To test this hypothesis, we utilized several ciprofloxacin-nitroxide hybrids that we have previously generated (Figure 1) (25, 26). Cipro-PROXYL, cipro-TEMPO, and cipro-TMIO (Figure 1) were first screened in MIC assays for activity against planktonic *S. aureus* ATCC 29213 (Table 1). All compounds were determined to possess potent *S. aureus* ATCC 29213 activity (MIC range 1.5 - 12.5 µM) with cipro-TMIO being the most active (MIC 1.5 µM, same MIC as ciprofloxacin). Subsequent minimum bactericidal concentration (MBC) analysis of cipro-TMIO and ciprofloxacin revealed that cipro-TMIO was at least twice as bactericidal as ciprofloxacin against *S. aureus* ATCC 29213 cells (cipro-TMIO MBC: 1.5 µM vs. ciprofloxacin MBC: 3.0). Then, established *S. aureus* ATCC 29213 biofilms were treated with cipro-PROXYL, cipro-TEMPO, cipro-TMIO, fluoroquinolone-TEMPO, or fluoroquinolone-TEMPOme (fluoroquinolone-TEMPO derivative with free radical removed). Cipro-PROXYL, cipro-TMIO, and fluoroquinolone-TEMPO all exhibited potent biofilm-eradication activity (Table 1). However, cipro-TMIO was by far the most potent
agent with an MBEC value of 64 µM. This made cipro-TMIO at least 16-fold more potent than ciprofloxacin/CTMIO co-treatment (MBEC 1024 µM) and at least 64-fold more potent than ciprofloxacin alone (MBEC 4096 µM) against *S. aureus* biofilms. These results demonstrate the advantage of covalently linking a nitroxide moiety to an antibiotic to enhance its activity against Gram-positive biofilms. Furthermore, ciprofloxacin-nitroxide hybrids were found to be stable in air at room temperature over a 12-month period with no loss in activity in MIC assays (data not shown).

**Fluoroquinolone-nitroxide hybrids can penetrate *S. aureus* biofilms and enter into surface and base-residing cells.**

To investigate the enhanced activity of nitroxide-antibiotic hybrids against established *S. aureus* biofilms at the cellular level, we utilized fluoroquinolone-TEMPO, a previously reported profluorescent fluoroquinolone-nitroxide that exploits the inherent fluorescence of fluoroquinolones and the fluorescence quenching properties of the nitroxide free radical (26). We have previously shown that fluoroquinolone-CTEMPO was active against *S. aureus* planktonic cells and acted as a switch on fluorescent probe emitting bright fluorescence upon cell entry (26). These properties make fluoroquinolone-TEMPO a valuable tool for visualising *S. aureus* biofilm eradication by nitroxide-antibiotic hybrids. We first established the MBEC value for fluoroquinolone-TEMPO (MBEC 720 µM; Table 1) and found it to be at least 5-times more potent than ciprofloxacin treatment of *S. aureus* ATCC 29213 biofilms, further supporting the increased potency of tethered nitroxide-antibiotic hybrids. Importantly we showed that this potentiation effect was specific to the presence of the free radical nitroxide as the methoxyamine derivative fluoroquinolone-TEMPOMe, which lacks
the nitroxide free radical, had lost its antibacterial activity (MIC >1200 µM and MBEC >1480 µM; Table 1).

We then treated established S. aureus biofilms with sub-MBEC concentrations of fluoroquinolone-TEMPO or the fluoroquinolone-TEMPOMe control, stained the treated biofilms for live/dead cells, and examined them using confocal laser scanning microscopy (CLSM). Fluoroquinolone-TEMPO, which acts as a switch on fluorescent probe becoming fluorescent upon cell entry, was found to penetrate the S. aureus ATCC 29213 biofilms, entering both surface and base residing cells (see Figure 3, image A; horizontal and vertical side panels). Upon cell entry, the fluorescence of fluoroquinolone-TEMPO was no longer quenched by the free radical nitroxide and its presence was clearly visible inside biofilm-residing cells (Figure 3, image F). Intriguingly, fluoroquinolone-TEMPOMe, which is constitutively fluorescent due to lacking the free radical nitroxide (conversion to methoxyamine), did not appear to enter biofilm-residing cells, instead, it appeared to be confined to the intercellular space of the biofilm where EPS is typically found (Figure 4, image E). Taken together, these results suggest that the nitroxide free radical likely facilitates the antibiotic’s entry into cells, explaining its enhanced potency against S. aureus biofilms.

**Fluoroquinolone-TEMPO and CTEMPO are non-toxic to human cells**

Our findings suggest that antibiotic-nitroxide hybrids such as fluoroquinolone-TEMPO have potential in future clinical applications. As part of their early-stage preclinical evaluation, general toxicity against human epithelial cells was investigated for fluoroquinolone-TEMPO and CTEMPO. No cytotoxicity was observed against human epithelial T24 cells in lactate.
dehydrogenase (LDH) assays following 24-hour cell exposure to fluoroquinolone-TEMPO and CTEMPO at concentrations ranging from 20 µM to 720 µM (IC₅₀ >720 µM).

**DISCUSSION**

Currently, treatment options for *S. aureus* biofilm-related infections are limited, with conventional antibiotics often exhibiting little to no therapeutic effect against biofilm-residing bacteria (5, 6). Consequently, treatment for biofilm infections usually involves prolonged high doses of antibiotics and/or surgical removal of infected tissue or implanted medical devices (28). Accordingly, methods for improving the biofilm-eradication activity of conventional antibiotics are urgently needed. Recent studies have demonstrated that nitroxides can mediate biofilm dispersal which can increase the efficacy of the commonly prescribed antibiotic ciprofloxacin against bacterial biofilms (23-25). However, until now, the anti-biofilm properties of nitroxides have only been demonstrated against two Gram-negative pathogens *P. aeruginosa* and *E. coli* (21-25). In this study, we showed that biofilms of the clinically important Gram-positive pathogen *S. aureus* are also susceptible to the biofilm-dispersing properties of nitroxides.

Our findings demonstrate that nitroxide-mediated biofilm dispersal of the Gram-positive pathogen *S. aureus* occurs over a µM concentration range (5 - 80 µM), which is similar to our previous findings for Gram-negative pathogens *P. aeruginosa* and *E. coli* (20 µM) (21, 23). While the precise biofilm dispersal mechanism by nitroxides is currently unknown, nitroxides are considered NO mimics and as such their biofilm-dispersal action potentially involves inhibition of intracellular levels of cyclic di-GMP, which has been directly linked to NO-mediated biofilm dispersal in several Gram-negative and Gram-positive bacterial species (14, 29, 30). The signalling nucleotide cyclic di-GMP has been shown to play
a pivotal role in biofilm formation and control, with higher levels facilitating biofilm formation and lower levels triggering biofilm dispersal (31-33). Interestingly, our finding that the optimal nitroxide concentration for biofilm dispersal is similar between Gram-positive and Gram-negative species may suggest that the mechanism by which nitroxide-mediated dispersal occurs is similar between these species.

Our study demonstrates that the nitroxide CTEMPO potentiates the activity of ciprofloxacin against *S. aureus* biofilms, significantly improving its eradication efficacy (at least 16-fold MBEC improvement). This is consistent with our previous results using nitroxide/ciprofloxacin combination treatment of established *P. aeruginosa* and *E. coli* biofilms (23). Interestingly, other studies which have utilized NO (instead of nitroxides) in combination with antimicrobials have reported similar improvements in antimicrobial activity (e.g. 20-fold increase in chlorine’s activity against multi-species biofilms) (13, 17) further supporting a similarity between the mechanism of NO- and nitroxide-mediated biofilm dispersal. Importantly, the ability of nitroxides to enhance the biofilm-eradication activity of antimicrobials against biofilms is also comparable to other leading methods such as the use of D-amino acids (8-fold improvement over antibiotic alone) (34) and *Cis*-2-decenoic acid (at least 4-fold improvement over antibiotic alone) (35). Taken together these results support the use of nitroxides as effective enhancers of antibiotic biofilm-eradication activity.

As an improvement to co-treatment, a currently emerging alternative has been the development of dual-acting hybrid compounds. Such compounds combine the anti-biofilm activity of a dispersal agent with an antimicrobial agent to produce a hybrid compound which can both disperse and eradicate biofilm-residing cells. We have previously utilized this
strategy to produce ciprofloxacin-nitroxide hybrids which exhibited potent *P. aeruginosa* and *E. coli* biofilm-eradication activity (24, 25, 36). In this study, we showed that ciprofloxacin-nitroxide hybrids are also potent *S. aureus* biofilm-eradication agents. Importantly, cipro-TMIO was able to completely eradicate (99.9%) established *S. aureus* biofilms at a concentration of only 64 µM (MBEC 64 µM), which is very similar to its *P. aeruginosa* biofilm-eradication activity (94% eradication at 20 µM) (25). Furthermore, cipro-TMIO’s biofilm-eradication activity is comparable to other promising compounds currently developed as *S. aureus* biofilm-eradication agents, such as halogenated phenazines (MBEC 10 µM) (37) and quaternary ammonium compounds (MBEC 25 µM) (38).

Interestingly, in the case of cipro-TMIO the addition of the nitroxide moiety to ciprofloxacin’s core structure does not negatively impact the hybrid’s activity against planktonic *S. aureus* cells (MIC 1.5 µM, same as ciprofloxacin), and even appears to improve bactericidal activity (MBC 1.5 µM, 2-fold improvement over ciprofloxacin). However, this trend was not evident in our previous study where cipro-TMIO was administered to *P. aeruginosa* planktonic cells (MIC 160 µM, at least 100-fold increase over ciprofloxacin) (25). These results suggest that the presence of the nitroxide at the secondary amine of ciprofloxacin does not interfere with, and may even facilitate, the fluoroquinolone’s mode of action (inhibition of DNA gyrase and/or topoisomerase IV) in *S. aureus* cells. Thus, it appears that ciprofloxacin-nitroxide hybrids produced via functionalization at the secondary amine of ciprofloxacin may be more suitable for the treatment of *S. aureus* as opposed to *P. aeruginosa* infections. Also, cipro-TMIO’s activity against both planktonic and biofilm-residing *S. aureus* cells, along with its lack of toxicity to human cells (25), makes it an ideal candidate for the treatment of a large number of *S. aureus*-related infections.
While no general toxicity to human cells was observed for either CTEMPO or any of the nitroxide functionalised antibiotics tested so far, future animal studies could explore specific physiological effects linked to nitroxide administration. In particular, some nitroxides have been shown to exhibit vasodilation properties (39) and their potential use as antihypertension drugs has been extensively explored (40-45). One of the most potent nitoxide-based vasodilators, and hence the most promising antihypertensive drug candidate, is 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) (Figure 1). This nitroxide which is structurally similar to CTEMPO (piperidine based nitroxide) is currently the lead antihypertensive candidate having shown efficacy in several animal based studies (40-45). Interestingly, it appears the vasodilation properties of TEMPOL may not extend to other tetra substituted nitroxides, more specifically non-piperidine based nitroxides, such as 3-carbamoyl-2,2,5,5-tetramethylpyrrolidin-1-ylxylo (pyrrolidine based nitroxide), a result which suggests that the six-membered heterocyclic ring of TEMPOL may be imperative to the vasodilation properties of this nitroxide (39). In addition, the vasodilation properties of TEMPOL were shown to be highly concentration dependent, with the minimum dose required to elicit a vasodilation response in a pig model being 145 µM (39). However, effective antihypertensive activity required much higher doses (≥ 1 mM) (40-42). Considering that in our study, (i) only 8 µM of CTEMPO could effectively potentiate the action of ciprofloxacin against S. aureus biofilms, (ii) cipro-TMIO is not a piperidine based nitoxide and (iii) it has an active concentration of 64 µM, it is reasonable to assume that our compounds would be unlikely to induce vasodilation at concentrations used for the treatment of biofilm-related infections. Nevertheless, their vasodilation/hypertension properties would be worth exploring in future.
This study has demonstrated that both co-treatment (nitroxide/ciprofloxacin) and ciprofloxacin-nitroxide hybrids (cipro-TMIO) exhibit potent planktonic and biofilm-eradication activity against *S. aureus* ATCC 29213 (methicillin-susceptible *S. aureus*). However, ciprofloxacin and other fluoroquinolones are no longer effective against resistant *S. aureus* (46). Instead, other antibiotics, such as vancomycin and rifampicin, have become the preferred treatments for MRSA infections (46, 47). While this study has focused on restoring the activity of the fluoroquinolone class of antibiotics, we are hopeful that a similar approach might also be effective for other antibiotic classes.

The profluorescent nitroxide fluoroquinolone-TEMPO is a biofilm-eradication agent with the same anti-biofilm mechanism as cipro-TMIO. Thus, by utilizing the probe properties of fluoroquinolone-TEMPO, we demonstrated that the state of the nitroxide (free radical) remains unchanged while interacting with the EPS of the biofilm (i.e., the fluorescence of fluoroquinolone-TEMPO is not ‘switched on’ while in the EPS or prior to cell entry). However, as fluoroquinolone-TEMPO enters the intracellular space, its fluorescence is quickly quenched, indicating that the free radical nitroxide has undergone a chemical change. Furthermore, as our results indicate that the presence of the free radical nitroxide is fundamental to the biofilm-eradication activity of fluoroquinolone-TEMPO, it can be inferred that (a) the free radical must be involved in the anti-biofilm activity of nitroxides and (b) the anti-biofilm role of nitroxides must occur via interference and/or regulation of an intracellular process. These findings are in support of the hypothesis that nitroxides like NO likely regulate the intracellular levels of cyclic di-GMP.

Hence, the results presented here support the hypothesis that much like co-treatment (nitroxide/antibiotic), the biofilm-eradication activity of nitroxide functionalized...
antibiotics, such as cipro-TMIO and fluoroquinolone-TEMPO, may also occur via a dual-action mechanism, were the nitroxide moiety triggers biofilm dispersal, and the antibiotic moiety subsequently eradicates the no longer antibiotic tolerant dispersed cells (Figure 5). However, this alone does not appear to explain the additional efficacy that nitroxide functionalized antibiotics exhibit over co-treatment. Interestingly, our results also indicate that fluoroquinolone-TEMPO can penetrate S. aureus biofilms and enter both surface and base-residing cells. Thus, it is conceivable that this additional penetration combined with the dual-action (dispersal and eradication) are both contributing to the biofilm-eradication activity of cipro-TMIO.

In conclusion, we have demonstrated that the biofilm dispersal activity of nitroxides is not limited to Gram-negative pathogens but also extends to important Gram-positive pathogens. This work also highlights the ability of nitroxides to restore the antibacterial activity of ciprofloxacin against S. aureus biofilms, either by administering them as a combinational treatment or, more potently, as nitroxide functionalized ciprofloxacin derivatives. While this study has focused on fluoroquinolones, a class of antibiotics widely used against many common infections, the methodology presented is easily adaptable to other antibiotic and antimicrobial classes, widely expanding the possible repertoire of anti-biofilm agents that are so urgently needed. This work serves as an early preclinical evaluation of nitroxide functionalized antibiotics as new antimicrobials for the treatment of S. aureus biofilms and showcases a promising therapeutic strategy with broad-spectrum antibiofilm potential.

MATERIALS AND METHODS

Bacterial and human cell culture media and conditions.
Staphylococcus aureus strain ATCC 29213 was used in this study. Bacteria were grown routinely in Lysogeny broth (LB) medium with shaking (200 rpm) at 37 °C. MIC assays were conducted in Mueller Hinton (MH) medium (OXOID, Thermo Fisher, Australia), biofilms were grown in LB medium, and biofilm challenges (dispersal and antimicrobial susceptibility testing) were performed in MH medium or M9 minimal medium (pH 7.0) containing 90 mM Na$_2$HPO$_4$, 22 mM KH$_2$PO$_4$, 9 mM NaCl, 19 mM NH$_4$Cl, 2 mM MgSO$_4$, 100 µM CaCl$_2$, and glucose at 22 mM. Human T24 bladder epithelial cells (obtained from ATCC® HTB-4™) were cultured in McCoy’s 5A modified medium (Thermo, Australia) supplemented with 10% heat-inactivated fetal bovine serum (Thermo, Australia). Confluent monolayers were formed by cell culture at 37 °C in a humidified atmosphere of 5% CO$_2$.

Nitroxide and antibiotic stock solutions.

The nitroxides 4-carboxy-2,2,6,6-tetramethylpiperidine-1-oxyl (CTEMPO) (Sigma, Australia) and 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl (CTMIO) (48) were prepared in DMSO at a concentration of 325 mM (stock solution). The antibiotic ciprofloxacin (Sigma, Australia) was prepared in aqueous hydrochloric acid (0.1 M) at a concentration of 60.35 mM (stock solution). Cipro-PROXYL, cipro-TEMPO, cipro-TMIO, fluoroquinolone-TEMPO, and fluoroquinolone-TEMPOMe were synthesized in-house utilizing previously established procedures (25, 26), and stock solutions were prepared in DMSO (8 mM). All stock solutions were stored in the absence of light at -20 °C. Working solutions were prepared in either M9 minimal media or MH media and used the same day.

Nitroxide and antibiotic MIC and MBC assays.

The MIC for CTEMPO, CTMIO, ciprofloxacin, cipro-PROXYL, cipro-TEMPO, cipro-TMIO, fluoroquinolone-TEMPO, and fluoroquinolone-TEMPOMe were determined by the broth
microdilution method, in accordance with the 2015 (M07-A10) Clinical and Laboratory Standards Institute (CLSI). Specifically, in a 96-well plate, twelve two-fold serial dilutions of each compound were prepared to a final volume of 100 µL in MH medium. A positive control (bacteria only) and a negative control (medium only) were also included. At the initial time of inoculation, each well was inoculated with $5 \times 10^5$ CFU, which had been prepared from fresh overnight cultures. MICs were determined visually at the 18-hour end point. MIC was defined as the lowest concentration of a compound that prevented visible bacterial growth after 18 hours of static incubation at 37 °C. MBC values were determined by subculturing 20 µL from each well of the final MIC challenge plate (after 18 hours incubation) into 180 µL of fresh MH media in a 96 well plate, followed by static incubation for 24 hours at 37 °C. MBCs were determined visually at the 24-hour end point. MBC was defined as the lowest concentration of agent required to eradicate $\leq 99.9\%$ of cells (indicated by clear wells). CTEMPO and CTMIO were tested in the concentration range of 3200 to 1.56 µM, ciprofloxacin in the concentration range of 160 to 0.048 µM, and cipro-PROXYL, cipro-TEMPO, cipro-TMIO, fluoroquinolone-TEMPO, fluoroquinolone-TEMPOMe were all tested in the concentration range of 1200 to 0.3 µM. Negative controls containing DMSO (vehicle control for CTEMPO, CTMIO, cipro-PROXYL, cipro-TEMPO, cipro-TMIO, fluoroquinolone-TEMPO, and fluoroquinolone-TEMPOMe) were prepared and serially diluted as above. MIC and MBC testing was conducted in at least 2 independent experiments, each containing 3 culture replicates tested in duplicate technical repeats. The reported MIC and MBC values were determined as the lowest concentration in which all replicates exhibited no visible growth. No variation between replicates was observed.

Biofilm culture using the Calgary Biofilm Device (CBD).
Biofilms were grown and established in the CBD (MBEC™, Innovotech Inc., Canada) used unmodified. The device consists of a two-part reaction vessel. The top component contains 96 identical pegs protruding down from the lid, which fits into a standard flat bottom 96-well plate (bottom component). Biofilm culture was performed as previously described (49). Briefly, overnight LB bacterial cultures were diluted to $10^6$ CFU mL$^{-1}$ in LB and used to inoculate the enclosed flat bottom 96-well plate with $\sim 10^5$ bacterial cells (130 µL) in each well. The peg lid was inserted into the inoculated wells, and the complete CBD was incubated in a shaking incubator at 150 rpm, 37 °C, and 95% relative humidity for 24 hours.

**Nitroxide, antibiotic, co-treatment and hybrid compound MBEC assays.**

Biofilms were established as detailed above. For MBEC value determination, the CBD lid containing established biofilms was removed and rinsed for 10 seconds in PBS (96-well plate, 200 µL in each well), to remove loosely adherent bacteria. The rinsed CBD lid was then transferred to a new flat bottom 96-well plate (challenge plate), which contained the specific treatment. For nitroxides alone the challenge plate contained 2-fold serial dilutions with a concentration range of 1200 - 9.4 µM for CTMIO, and a concentration range of 2048 - 16 µM for CTEMPO. For ciprofloxacin alone the challenge plate contained 2-fold serial dilutions with a concentration range of 4096 - 32 µM. For co-treatment (ciprofloxacin/CTEMPO or CTMIO) experiments the challenge plate contained 2-fold serial dilutions of ciprofloxacin (concentration range 4096 - 32 µM) and CTMIO (tested at 8 µL only) at a consistent concentration in each well of either 80, 40, 20, 10, 8, or 2.5 µM in M9 minimal medium (total volume 200 µL per well). For hybrid compounds the challenge plate contained 2-fold serial dilutions with a concentration range of 1024 - 8 µM for cipro-PROXYL, cipro-TEMPO, cipro-TMIO, fluoroquinolone-TEMPO, or fluoroquinolone-TEMPOMe.
The complete CBD was then incubated at 37 °C, 120 rpm, 95% relative humidity for 24 hours. The lid was removed from the challenge plate and rinsed twice for 30 seconds in PBS (96-well plate, 200 µL in each well). The rinsed CBD lid, with attached pegs and treated biofilms, was transferred to a new 96-well plate containing fresh LB recovery media. To assist the transfer of any remaining viable cells to the recovery media, the device was sonicated for 30 minutes (<20 °C). The peg lid was then discarded, and the biofilm-recovered bacteria from each well of the recovery plate was serially diluted in PBS, and then triplicate 5 µL aliquots of each dilution were plated onto LB agar and incubated at 37 °C overnight for viable CFU enumeration. MBEC values were determined as the lowest concentration that resulted in a CFU value ≤ 99.9% of the untreated controls. MBEC values were obtained from at least 2 independent experiments, each consisting of 3 biological replicates each assessed in triplicate technical repeats.

**Nitroxide-mediated biofilm dispersal assays.**

Biofilm dispersal assays were performed as described above for MBEC assays, with the following modification and additional steps. The challenge plate contained 2-fold serial dilutions of CTEMPO (concentration range 160 - 2.5 µM) or CTMIO (8 µM) and ciprofloxacin at a consistent concentration of 6 µM (included to eradicate dispersed cells) in M9 minimal medium (total volume 200 µL per well), control wells contained ciprofloxacin only at 6 µM. After sonication, the peg lid was discarded, and the recovery plate containing biofilm-recovered bacteria was covered with a breathable sealing membrane (Breathe-Easy® sealing membrane, Sigma, Australia), and incubated at 37 °C for 20 hours with shaking in microtiter plate reader (BMG, Australia). OD$_{600}$ measurements were obtained every 15 minutes over the 20 hour period. Growth curves for each well containing recovered biofilm cells were
then plotted and a non-linear regression function (solver) was applied to determine the lag time in each growth curve (lag time is directly proportional to the initial CFU count of each well, with wells initially containing a low CFU counts producing a longer lag time than wells with a higher initial CFU count) (50, 51). Lag times were determined for each replicate for each condition tested and group medians were compared by a Kruskal-Wallis test (in GraphPad Prism 7).

Confocal laser scanning microscopy of *S. aureus* biofilms

Biofilms were grown on the CBD as detailed above. Established biofilms were treated with fluoroquinolone-TEMPO or fluoroquinolone-TEMPOMe (150 µM) in M9 medium, incubated at 37 °C for 2 hours, rinsed in PBS for 5 seconds, and stained with LIVE/DEAD™ BacLight™ Bacterial Viability kit L7007 (Life Technologies, Australia) as per manufacturer’s protocol. Treated and stained biofilms were mounted using ProLong® Diamond Antifade Mountant (Life Technologies, Australia) and immediately analyzed by CLSM. CLSM was conducted on a Zeiss 780 NLO Point Scanning Confocal, equipped with a Mai-Tai deep see multi-photon laser (tunable between 690 and 1040 nm). For fluoroquinolone-TEMPO and fluoroquinolone-TEMPOMe the Mai-Tai laser was set at 720 nm (a wavelength which did not excite SYTO9 or propidium iodide), for SYTO9 the 488 nm laser was used, and the 561 nm laser was applied for propidium iodide. CLSM Z-slices were obtained every 0.5 microns throughout the entire thickness of each biofilm (approximately 10-20 µm). Images stacks were analyzed using the instrument software (Zen 2.3). All imaging experiments utilized a 100 × oil immersion objective.

LDH release cytotoxicity assay.
The cytotoxicity of fluoroquinolone-TEMPO and CTEMPO against human T24 urinary bladder epithelial cells was examined utilizing the Pierce™ LDH cytotoxicity assay kit (Life Technologies, Australia) as per manufacturer’s instructions. Briefly, triplicate confluent T24 cell monolayers were treated with fluoroquinolone-TEMPO or CTEMPO (concentrations between 720 - 20 μM) for 24 hours at 37 °C in a humidified atmosphere of 5% CO₂. Cells treated with DMSO/PBS (4.5% DMSO final concentration) or sterile water served as negative controls and cells treated with 10X lysis buffer (maximum LDH release) served as a positive control. After 24 hours incubation, 50 μL of the supernatant was transferred into a new 96-well plate, mixed with 50 μL of the reaction mixture (LDH assay kit) and incubated at room temperature (protected from light) for 30 minutes before the stop solution (50 μL) was added. The plate was then centrifuged (1000 x g) for 5 minutes to remove air bubbles, and the absorbance at 490 and 680 nm was measured in a spectrostar (BMG) plate reader.

ACKNOWLEDGMENTS

This work was supported by a Queensland University of Technology (QUT) grant (to M.T and K.E.F.-S.) and an Asian Office of Aerospace Research and Development Grant (FA2386-16-1-4094, R&D 16IOA094). AV is supported by an Australian Government Research Training Program (RTP) Scholarship; RD by a National Health and Medical Research Council grant (APP1144046 to MT); KEF-S by an Australian Research Council Future Fellowship (FT140100746); and MT by a QUT Vice-Chancellor’s Research Fellowship. The authors would also like to thank Professor Flavia Huygens, for the provision of S. aureus strain.

ABBREVIATIONS

CBD: Calgary Biofilm Device, cyclic di-GMP: bis-(3’-5’) -cyclic dimeric guanosine monophosphate, cipro-PROXYL: 1-Cyclopropyl-6-fluoro-7-(4-(2,2,5,5-tetramethyl-1-oxy-
pyrrolidine-3-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, cipro-TEMPO: 1-Cyclopropyl-6-fluoro-7-(4-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, cipro-TMIO: 1-Cyclopropyl-6-fluoro-7-(4-(1,1,3,3-tetramethylsoindolin-2-yloxy)-5-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, CFU: colony forming units, CTEMPO: 4-carboxy-2,2,6,6-tetramethylpiperidin-1-yloxy, CTMIO: 5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxyl, DMSO: dimethyl sulfoxide, EPS: extracellular polymeric substances, fluoro-TEMPO: 1-Cyclopropyl-6-fluoro-7-(2,2,6,6-tetramethyl-1-oxy-piperidine-4-yl)amino)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, fluoroquinolone-TEMPOMe: 1-Cyclopropyl-6-fluoro-7-[(1-methoxy-2,2,6,6-tetramethylpiperidine-4-yl)amino]-4-oxo-1,4-dihydroquinoline-3-carboxylic acid, MBC: minimal bactericidal concentration, MBEC: minimal biofilm eradication concentration, MIC: minimal inhibitory concentration, NO: nitric oxide.

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Table 1: MIC and MBEC values for ciprofloxacin, CTEMPO, CTMIO, and various co-
treatments or antibiotic-nitroxide hybrids against *S. aureus* ATCC 29213.

| Treatment | MIC\(^1\) (µM) | MBEC\(^2\) (µM) |
|-----------|----------------|-----------------|
| **Controls** | | |
| Ciprofloxacin | 1.5 | 4096 |
| CTEMPO | >3200\(^a\) | >1200\(^a\) |
| CTMIO | >2048\(^a\) | >2048\(^a\) |
| **Co-treatment of *S. aureus* biofilms** | | |
| Ciprofloxacin & CTEMPO (2.5 µM) | nd | 4096 |
| Ciprofloxacin & CTEMPO (8 µM) | nd | 256 |
| Ciprofloxacin & CTEMPO (10 µM) | nd | 512 |
| Ciprofloxacin & CTEMPO (20 µM) | nd | 512 |
| Ciprofloxacin & CTEMPO (40 µM) | nd | 512 |
| Ciprofloxacin & CTMIO (80 µM) | nd | 2048 |
| Ciprofloxacin & CTMIO (8 µM) | nd | 1024 |
| **Hybrid treatment of *S. aureus* biofilms** | | |
| Cipro-PROXYL | 6.3 | 1024 |
| Cipro-TEMPO | 12.5 | >1024\(^a\) |
| Cipro-TMIO | 1.5 | 64 |
| Fluoroquinolone-TEMPO | 20 | 720 |
| Fluoroquinolone-TEMPOMe | >1200\(^a\) | >1480\(^a\) |

\(^a\)MICs were determined via the broth microdilution method in accordance with CLSI standard. \(^a\)MBECs were determined using the CBD. [a] Highest concentration tested. nd: Not done.
Figure 1: Chemical structures of TEMPOL, CTEMPO, ciprofloxacin, cipro-PROXYL, cipro-TEMPO, cipro-TEMPO, fluoroquinolone-TEMPO, and fluoroquinolone-TEMPOMe.
Figure 2: Nitroxide-mediated S. aureus ATCC 29213 biofilm dispersal. Established S. aureus biofilms were treated with the nitroxide CTEMPO (at a concentration range of 160 – 2.5 µM) and ciprofloxacin (6 µM) or with ciprofloxacin alone (6 µM) (Control) for 24 hours. Biofilm-associated bacteria were recovered after treatment and enumerated as described in methods. Lag time (min) from growth curves of recovered bacteria was calculated using nonlinear regression. Dot plots show data from treated and control biofilms obtained from 6 biological repeats. Lines show group medians. Group medians were compared using the Kruskal-Wallis test (p = 0.0003).
Figure 3: CLSM images of established *S. aureus* ATCC 29213 biofilms treated with fluoroquinolone-TEMPO (150 µM, sub-lethal concentration) for 2 hours, then stained with SYTO9, and PI (see methods). (A, E) Excited with 720 nm multi-photon laser (fluoroquinolone-TEMPO, pink); (B) Excited with 488 nm laser (SYTO9, live cells, green); (C) Excited with 561 nm laser (PI, dead cells, red); (D) Overlay of images A-C, light grey cells result from merging of green and pink; (F) Expanded image of panel E. The scale bars are 20 µm in length for panels A-E, and 5 µm for panel F. Micrographs show representative horizontal (xy) sections collected within each biofilm, with A-E also showing vertical sections representing the yz and xz planes, shown to the right and top of each individual panel, respectively, taken at the positions indicated by the lines.
Figure 4: CLSM images of established *S. aureus* ATCC 29213 treated with fluoroquinolone-TEMPOMe (150 µM) for 2 hours, then stained with SYTO9, and PI (see methods). (A) Excited with 720 nm multi-photon laser (Fluoroquinolone-TEMPOME, pink); (B) Excited with 488 nm laser (SYTO9, live, green); (C) Excited with 561 nm laser (PI, dead, red); (D) Overlay of images A-C; (E) Expanded image of D. The scale bars are 20 µm in length for images A-D, and 5 µm for image E. Images A-D also show the *xy*, *yz*, and *xz* dimensions.
Figure 5: Working model for the dual-action mechanism of fluoroquinolone-TEMPO against *S. aureus* biofilms. Upon administration, the functionalised antibiotic fluoroquinolone-TEMPO enters surface-residing biofilm cells and induces their dispersion from the biofilm in a concentration-specific manner. Dispersed cells are no longer tolerant to the antibiotic and are thus eradicated, exposing the next layer of biofilm-residing cells to the hybrid compound. Fluoroquinolone-TEMPO enters the newly exposed cells and the process is repeated until the biofilm is completely eradicated.