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Eradication of *Staphylococcus aureus* Catheter-Related Biofilm Infections Using ML:8 and Citrox

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Staphylococci are a leading cause of catheter-related infections (CRIs) due to biofilm formation. CRIs are typically managed by either device removal or systemic antibiotics, often in combination with catheter lock solutions (CLSs). CLSs provide high concentrations of the antimicrobial agent at the site of infection. However, the most effective CLSs against staphylococcal biofilm-associated infections have yet to be determined. The purpose of this study was to evaluate the efficacy and suitability of two newly described antimicrobial agents, ML:8 and Citrox, as CLSs against *Staphylococcus aureus* biofilms. ML:8 (1% [vol/vol]) and Citrox (1% [vol/vol]), containing caprylic acid and flavonoids, respectively, were used to treat *S. aureus* biofilms grown *in vitro* using newly described static and flow biofilm assays. Both agents reduced biofilm viability >97% after 24 h of treatment. Using a rat model of CRI, ML:8 was shown to inactivate early-stage *S. aureus* biofilms *in vivo*, while Citrox inactivated established, mature *in vivo* biofilms. Cytotoxicity and hemolytic activity of ML:8 and Citrox were equivalent to those of other commercially available CLSs. Neither ML:8 nor Citrox induced a cytokine response in human whole blood, and exposure of *S. aureus* to either agent for 90 days was not associated with any increase in resistance. Taken together, these data reveal the therapeutic potential of these agents for the treatment of *S. aureus* catheter-related biofilm infections.

The effective treatment of staphylococcal catheter-related infections (CRIs) represents a significant clinical challenge, mainly due to the ability of staphylococci to form biofilms (1). The surfaces of intravascular catheters (IVCs) can rapidly become coated with host matrix proteins (2). Staphylococci express numerous surface proteins that can bind to these proteins (3). Current treatment for staphylococcal IVC infection involves device removal, but this is not always possible due to clinical circumstances. The Infectious Disease Society of America (IDSA) guidelines on the management of CRIs recommend the use of catheter lock solutions (CLSs) for the attempted salvage of an IVC associated with a CRI (4). However, there is no consensus on the most appropriate agent for use as a CLS in the treatment of staphylococcal CRIs. Several commonly used antibiotics and antiseptics were recently shown to be ineffective for the treatment of *Staphylococcus aureus* infections involving biofilms (5). Because current options are limited, the need for novel therapeutic agents for use as CLSs or as antistaphylococcal biofilm treatment options for other device-related infections is of great clinical importance (6).

We examined the antimicrobial effects and suitability for *in vivo* use of two newly described antimicrobial agents, namely, ML:8 and Citrox. ML:8 includes components previously approved for parenteral nutrition; the fatty acid caprylic acid is the main component and has been shown to be effective in the treatment of periodontal pathogens (7). The efficacy of caprylic acid against biofilms was also reported previously (8). Citrox is an antimicrobial formulation composed of flavonoids. Flavonoids are secondary metabolites that are found in plants, and their antimicrobial activities have been confirmed against a wide variety of bacterial species (9). The flavonoids in Citrox are neoeriocitrin, isonaringin, naringin, hesperidin, neohesperidin, neodiosmin, naringenin, poncirin, and rhiofolin (10). The efficacy of Citrox against biofilm-producing oral microorganisms has been reported previously (11).

The aim of this study was to examine if these two newly described antimicrobial agents can potentially be used for the treatment of *S. aureus*-mediated CRIs. We therefore investigated the effectiveness of ML:8 and Citrox to eradicate biofilms produced by methicillin-susceptible (MSSA) and -resistant (MRSA) *S. aureus* strains, using clinically relevant *in vitro* and *in vivo*-like models of CRIs, and examined their potential clinical use as CLSs.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The main bacterial strains and clinical isolates used in this study are described in Table 1. *S. aureus* strains were grown with aeration at 37°C in either Mueller-Hinton (MH) broth (Sigma) or RPMI 1640 medium (Gibco). MH broth was used for MIC testing and resistance testing, while RPMI 1640 medium was used for all biofilm-related experiments.

MIC testing. MICs for planktonic cells were established using a broth microdilution method. Serial dilutions of test agents (0.007 to 2% [vol/vol]) were prepared and exposed to bacterial strains grown in MH broth. Each strain was prepared to a 0.5 McFarland standard. Solutions were incubated for 24 h at 37°C before the results were examined to determine the MICs.

Static biofilm formation and treatment. *S. aureus* biofilms were formed as previously described (5, 12). Platelet-poor plasma was obtained from healthy volunteers. Plasma was diluted to 20% (vol/vol) in carbon-

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TABLE 1 Bacterial strains used in this study

S. aureus strain	Characteristics	Reference
SH1000	MSSA reference strain; functional *rsbU* derivative of 8325-4 *rsbU*$^+$; serotype (ST) 8, CC8	34
BH48 (04)	MSSA clinical isolate; ST 8, CC8	35
BH1CC	MRSA clinical isolate; SCC*mec* type II, ST 8, CC8	35
USA300 JE2	MRSA strain USA300 derivative lacking plasmids P01 and P03; JE2 LAC ST 8, CC 8	36
USA300 *lux*	MRSA strain USA300 LAC constitutively expressing luciferase (*lux*) from *Photorhabdus luminescens*; CC8	37

ate buffer (pH 9.6) and used to precondition the wells of a 96-well microtiter plate (Nunc, Denmark) at 37°C for 2 h (13). The plasma solution was removed, and an overnight culture of the test organism in RPMI 1640 was diluted 1:1, to an optical density at 600 nm (OD_{600}) of 0.7, in RPMI 1640. From this suspension, 100-μl aliquots were inoculated into the microtiter plate wells and incubated at 37°C for 24 h, 3 days, or 5 days, as indicated. The RPMI 1640 medium was changed daily for mature biofilms. Following the initial incubation and washing, 100 μl of the test solution (ML:8 or Citrox [0.025 to 1% {vol/vol}] diluted in RPMI 1640) was added to each test well at 37°C for 24 h and then washed twice with sterile distilled water. RPMI 1640 medium without bacteria was used as a negative control and was held in the well during incubations; washing steps were carried out as described above.

Resazurin conversion assay. One hundred microliters of a redox indicator dye, either alamarBlue (Biosource, Invitrogen, United Kingdom) (20% [vol/vol] alamarBlue in RPMI 1640 medium) or resazurin (88 μM resazurin in water), was added to each well as described previously (14). Plates were incubated for a further 60 min at 37°C in the dark to determine biofilm viability after antimicrobial treatment. Biofilm viability was determined using a fluorimeter with an excitation wavelength of 544 nm and an emission wavelength of 590 nm. The fluorescence produced was proportional to the number of living cells present. Each experiment was performed using three technical and biological replicates; results represent biofilm OD values (means ± standard deviations [SD]).

Measurement of antibiofilm activity of antimicrobial agents under flow conditions. A flow cell and pump were used to create a microfluidic model (Cellix Ltd., Ireland). Vena8 Fluoro+ flow chambers were coated with 100% plasma for 2 h at 37°C. Exponentially growing *S. aureus* cultures were adjusted to an OD_{600} of 0.2 in RPMI 1640 and injected into each chamber to attach for 1 h at 37°C on an inverted microscope. The pump was activated, and RPMI 1640 at a shear rate of 6.25 dynes (200 μl/min) was infused through the chambers of the chip for 24 h. Treatment agents (1% [vol/vol]) were then injected into each chamber and allowed to treat the biofilm statically for a further 24 h. Chambers were analyzed using bright-field and confocal microscopy.

Confocal microscopy. Biofilm structure and treatment efficacy were analyzed using an inverted confocal microscope (LSM 510 Meta; Zeiss) and LSM510 image capture software. Bacterial cells were visualized using Syto 9 green (3.34 mM) and propidium iodide (20 mM). Optimized lasers (argon [488 nm] and HeNe [632.8 nm]) were used to excite the dyes and to capture the fluorescence emitted from the cells, under a magnification of ×40. ImageJ software was used to calculate the fluorescence intensity for each chamber.

Cell culture. HaCaT cells, a human keratinocyte cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) (Bio-Sciences, Ireland) with 2 mM L-glutamine supplemented with 10% (vol/vol) fetal bovine serum (FBS). THP-1 cells, a human acute monocyte leukemic cell line, were cultured in RPMI 1640 (Bio-Sciences, Ireland) supplemented with 10% (vol/vol) FBS.

MTT assay for assessing cytotoxicity. Cells were seeded into the wells of 96-well microtiter plates (Nunc, Denmark) at a density of 1×10^5 cells/ml. THP-1 and HaCaT cells were incubated in serum-free medium containing increasing concentrations of test agent for 24 h. Control wells were treated with Triton X-100 (1%) or medium free of test agents. MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (500 μg/ml) was then added to each well, incubated for 4 h, and washed with phosphate-buffered saline (PBS). MTT fixative solution (isopropanol) was added to each well, and the plates were fixed for 4 min with shaking. The absorbance was measured at 595 nm in a Thermo Multiskan Ex plate reader (Thermo Fisher, United Kingdom). All incubations were done at 37°C in a 5% CO_2 humidified incubator. Three technical replicates as well as three biological replicates were tested.

Determining the hemolytic activity of ML:8 and Citrox. Potential hemolytic activity of ML:8 and Citrox was determined using fresh human erythrocytes from healthy donors in a modified version of the method originally described by Cantisani et al. (15). Blood was drawn and centrifuged, plasma was removed, and the remaining erythrocytes were washed three times with PBS. Erythrocytes were diluted in 40 ml PBS, and 50-μl aliquots were added to a 96-well plate (Nunc, Denmark). Test agents (0.007 to 2% [vol/vol]) were prepared in RPMI 1640, and 50 μl of each solution was added to erythrocytes. Plates were incubated at 37°C for 24 h. Supernatant was then withdrawn from the top of each well and placed in a new plate. The release of hemoglobin was monitored by measuring the absorbance of the supernatant at 490 nm. Negative and positive controls consisted of erythrocytes suspended in RPMI 1640 and erythrocytes in RPMI 1640 with 1% Triton X-100, respectively.

Investigation of immune response to ML:8 and Citrox. Whole blood was exposed to either ML:8, Citrox, or an untreated medium control for 2 h at 37°C in 96-well microtiter plates (Nunc, Denmark). Exposed blood was collected into Eppendorf tubes and centrifuged at 150 × *g* for 10 min. After centrifugation, serum was collected and used immediately or stored at −20°C. A Bio-Plex Pro human cytokine 8-plex assay plate containing eight cytokines was used; these included both proinflammatory and anti-inflammatory cytokines and chemokines (interleukin-2 [IL-2], IL-4, IL-6, IL-8, IL-10, granulocyte macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor alpha [TNF-α], and gamma interferon [IFN-γ]). Serum samples were diluted 1:4, and the plate was analyzed according to the manufacturer's instructions. The plate was read to assess fluorescence using a Bio-Plex 200 plate reader. DuoSet IL-8 and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were used to validate the cytokine results. ELISAs were carried out in accordance with the manufacturer's instructions. The plate was read to determine the optical density of each well immediately after the addition of the stop solution; plates were read at 450 nm using a Thermo Multiskan-Ex plate reader (Thermo Fisher, United Kingdom).

Investigation of potential of ML:8 and Citrox to induce antimicrobial resistance. Antimicrobial resistance testing was performed using Mueller-Hinton (MH) agar plates supplemented with antimicrobial agents at concentrations of 0.125 and 0.0625% (vol/vol) for ML:8 and 0.015 and 0.0075% (vol/vol) for Citrox. MSSA strain SH1000 and MRSA strain BH1CC were adjusted to a 0.5 McFarland standard; 100-μl aliquots were plated in triplicate on plates containing test agents. Plates were incubated for 48 h at 37°C and then transferred to 4°C for 48 h to allow bacterial cells to recover from the challenge. Bacterial cells were harvested, adjusted to a 0.5 McFarland standard, and cultured on fresh agar plates with test agents added. This process was repeated in triplicate twice weekly for 12 weeks.

Animal studies. (i) Husbandry and care. Male Sprague-Dawley rats with preimplanted jugular vein central venous catheters (polyurethane, 0.025-in. internal diameter, and 40-μl volume) were obtained from

TABLE 2 MICs of ML:8 and Citrox for *Staphylococcus aureus* strains used in this study

Agent	Concn range tested (% [vol/vol])	MIC (% [vol/vol]) for MSSA strain		MIC (% [vol/vol]) for MRSA strain	
		SH1000	BH48	BH1CC	USA300 JE2
ML:8	0.007–2	0.125	0.125	0.125	0.125
Citrox	0.007–2	0.015	0.015	0.015	0.015

Charles River Labs (Kent, United Kingdom). Initial weights were 200 to 250 g per rat. Rats received water and Agway rodent chow *ad libitum* throughout the experiments. All work was performed in accordance with statutory instrument no. 543 (2012) issued by the Irish government. Animals were held in quarantine for 1 week on arrival to monitor health status; animals were examined for signs of illness, pains, and infection at the end of the week. Catheters were flushed with heparin saline (500 IU) every second day of the quarantine week to maintain patency.

(ii) Rat intravascular catheter model of infection. A rat IVC model of infection was developed as described previously by Ulphani and Rupp (16), van Praagh et al. (17), and Chauhan et al. (18, 19), with the following modifications. Briefly, rats were anesthetized with isoflurane (5% for induction and 2% for maintenance) for all catheter manipulations. Jugular vein central venous catheters were inoculated by instillation of 0.2 ml of saline containing 1×10^6 CFU/ml (high inoculum) or 1×10^4 CFU/ml (low inoculum) of MRSA strain USA300 *lux*. The bacteria remained in the lumen of the catheter for 1 or 5 days. After 1 or 5 days of infection (early or mature biofilm, respectively), 50 μl of treatment agent (ML:8 or Citrox) was instilled into the lumen of the catheter daily for a further 4 days. Subcutaneous injections of vancomycin (50 mg/kg of body weight) were administered every 12 h for the duration of the experiment. Three rats were used per treatment group. Bioluminescence intensity at the site of infection was monitored daily using a PerkinElmer IVIS imaging system (exposure, 20 s; binning, level 4, setting f1).

(iii) Terminal harvest and analysis of results. Rats were anesthetized, blood was collected via cardiac puncture and serially diluted, and CFU counts were determined. Rats were euthanized by cervical dislocation. Catheters were removed, and their bioluminescence intensities were analyzed via an IVIS imaging system. Catheters were then transferred to sterile tubes. Biofilms were harvested from the lumens of the catheters by using TrpLE (1×; Gibco). Bacterial numbers were determined using a broth dilution and spread plate method on tryptone soy agar (TSA) (Fannin, LIP, Ireland).

Ethical approvals. Blood donations were obtained from healthy adult donors. Written, informed consent was obtained from participants at the time of collection. Ethics approval for collection and use of blood was granted by the Ethics Committee of the Royal College of Surgeons in Ireland (REC820). Animal experiments were conducted under Health Products Regulatory Authority Ireland guidelines, with ethical approval from the RCSI Ethics Committee (REC931).

Statistical analysis. Two-tailed, paired Student's *t* tests were used to determine the statistical significance of data by using Microsoft Excel. Significance was defined as having a P value of ≤ 0.05. For the animal experiments, descriptive statistics (including means and standard deviations) were calculated for each test agent. Log_{10} transformation of CFU data from catheters was used to ensure a normal distribution. A two-sample *t* test was conducted to compare the log-transformed CFU values.

RESULTS

ML:8 and Citrox inactivate early and mature *in vitro S. aureus* biofilms. The MICs of ML:8 and Citrox against planktonic bacteria are shown in Table 2. The results indicate an MIC of 0.125% (vol/vol) for ML:8 and one of 0.015% (vol/vol) for Citrox for all strains listed in Table 1. No difference in susceptibility was observed between the MRSA and MSSA strains examined. Initial *in vitro* biofilm testing was carried out against *S. aureus* coagulase-mediated biofilms grown on surfaces coated with human plasma (5, 12). Twenty-four-hour treatments with ML:8 and Citrox at 1% (vol/vol) were both effective for the inactivation of biofilms that had been developed over 1, 3, and 5 days as described in Materials and Methods (Fig. 1). No significant difference ($P \leq 0.05$) in bacterial susceptibility to ML:8 or Citrox was observed between strains or stages of biofilm maturity. The effects of ML:8 and Citrox were further investigated on biofilms formed on plasma-coated surfaces under shear force by using live/dead staining and confocal microscopy. Experiments under shear force were carried out using the four lead bacterial strains, and no variation between strains was observed. Results shown are for MSSA strain SH1000. Upon visual inspection, 24-h treatment with ML:8 (Fig. 2A, panel ii) and Citrox (Fig. 2A, panel iii) resulted in predominantly red cells, indicating cell death. Quantitative analysis revealed that treatment with ML:8 (1% [vol/vol]) and Citrox (1% [vol/vol]) resulted in 97% and 98% reductions in cell viability, respectively (Fig. 2B). To ensure that our treatment times and doses were not limited to a small number of *S. aureus* strains, a larger group of biofilm-positive isolates representing various genetic backgrounds was also included in the study. These strains and isolates represent other clonal complexes (CCs) commonly associated with *S. aureus* infection, namely, CC5, CC8, CC15, CC22, CC30, CC45, CC122, and CC398 (20, 21). Significant variation between clonal complexes was not observed (see Fig. S1 in the supplemental material). The optimum dose against biofilms (ODB) formed by *S. aureus* was determined to be 1% (vol/vol) for both ML:8 and Citrox.

ML:8 and Citrox demonstrate compatibility with host cells equivalent to that for comparative agents in *in vitro* testing. The cytotoxicity of ML:8 and Citrox and two existing CLSs in clinical use, namely, Duralock-C and ethanol, was investigated using monocyte and keratinocyte cell lines, THP-1 and HaCaT cells, respectively. The 50% inhibitory concentration (IC_{50}) of each agent was determined using an MTT assay as described in Materials and Methods, and the results are presented in Table 3. While cytotoxicity was observed for ML:8 and Citrox at concentrations below that seen to be effective against biofilms, similar results were also observed for the CLSs ethanol and Duralock-C, which are currently in clinical use. Cytotoxicity of ML:8 was evident at concentrations between 6- and 17-fold below the ODB; results for Citrox showed IC_{50}s 24- and 26-fold lower than the ODB. Ethanol exposure resulted in cytotoxicity at concentrations 12.5- and 21-fold below the ODB, while Duralock-C resulted in IC_{50}s 97- and 202-fold below the optimum concentrations for use against bacterial biofilms. The hemolytic properties of ML:8, Citrox, and comparative agents were also assessed in human whole blood. Hemolysis was evident after exposure to high concentrations of ML:8 (≥0.25% [vol/vol]) and Citrox (≥0.125% [vol/vol]); hemolysis was also observed for the comparative agents Duralock-C and ethanol, at concentrations of ≥11.6% and ≥3.75% (vol/vol), respectively. Continuous subculturing with concentrations of ML:8 and Citrox 1-fold below the MICs shown in Table 2 was carried out over 90 days to assess the potential to induce resistance. No increase in resistance was detected after exposure of the MSSA strain SH1000 and the MRSA strain BH1CC over this period. An investigation into the host immune response to ML:8 and Citrox

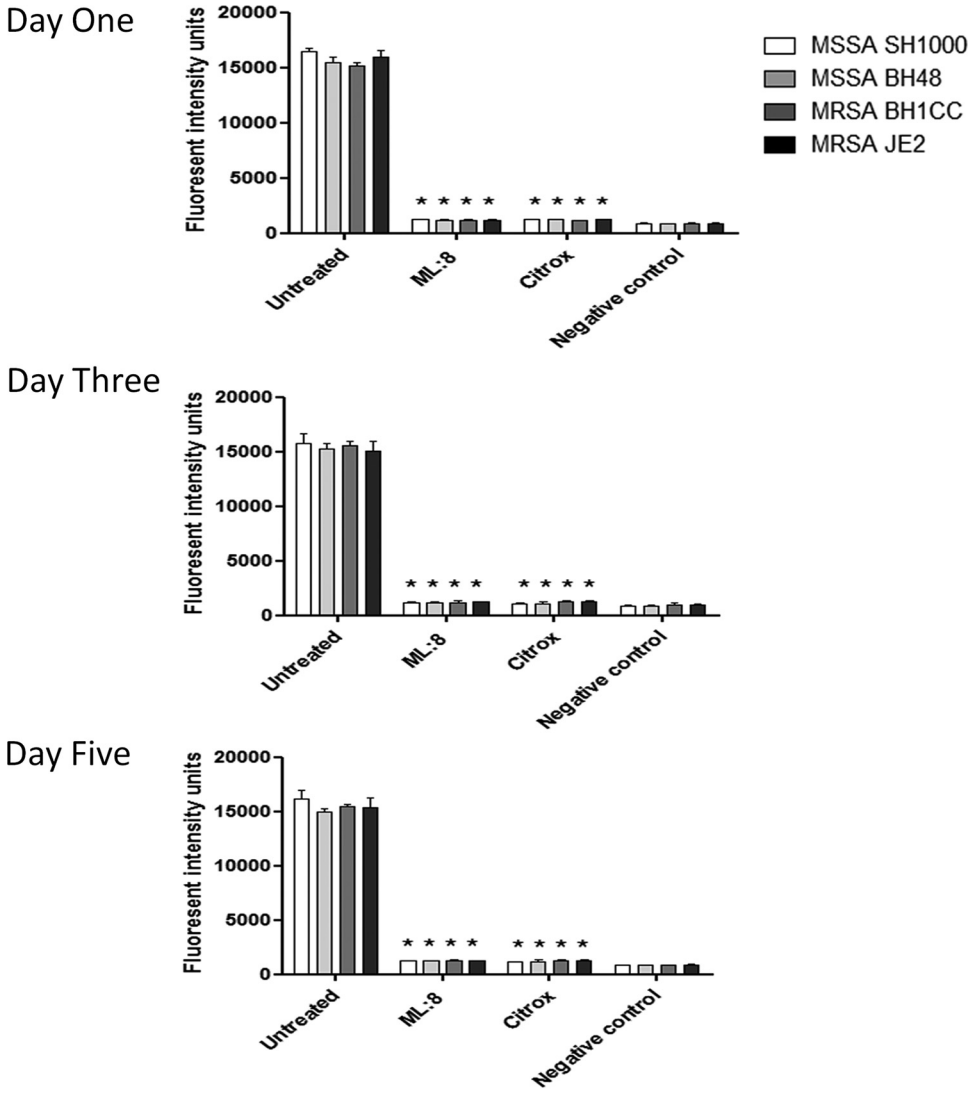

FIG 1 Susceptibility of mature *S. aureus* biofilms to ML:8 and Citrox. Biofilms formed by MSSA strains SH1000 and BH48 and MRSA strains BH1CC and JE2 were grown in RPMI 1640 for 1, 3, or 5 days and then treated with ML:8 (1% [vol/vol]) or Citrox (1% [vol/vol]) at 37°C for 24 h. RPMI 1640 without bacteria was used as a negative control. Viability of biofilms was measured using the resazurin conversion assay. Assays were performed in triplicate, and data represent mean fluorescence intensities and SD. Statistically significant results are indicated (*, $P \leq 0.05$; two-tailed Student *t* tests).

revealed no significant induction of the cytokines tested compared to a medium control, indicating that ML:8 and Citrox did not trigger an immune response in the exposure time examined (2 h) (see Fig. S2 in the supplemental material). Results for IL-8 and TNF-α were confirmed by ELISA.

ML:8 and Citrox are effective for treatment of *in vivo S. aureus* biofilm infection. Sprague-Dawley rats with implanted IVCs were used for *in vivo* testing. Infection was established in the IVC, and the treatment agents ML:8 and Citrox were examined as CLSs in this model. Figure 3A shows a representative image of a Sprague-Dawley rat 5 days after infection of the IVC with MRSA strain USA300 *lux*. This image was captured prior to the initiation of CLS treatment, and high levels of luminescence are shown within the implanted catheter. Subcutaneous injections of vancomycin prevented systemic infection; this was confirmed by the negative blood cultures obtained at the experimental endpoint (Fig. 3B).

Images of explanted catheters were captured using IVIS imaging, and representative images are shown in Fig. 4A for the untreated (i) as well as ML:8 (ii)- and Citrox (iii)-treated catheters. While luminescence remained high (maximum value, 1×10^7) in catheters treated with ML:8 (1% [vol/vol]), it was shown to be lower than the level for the untreated control (maximum value, 4×10^7). No detectable luminescence was observed in the Citrox-treated catheters. Bacterial biofilms harvested from the explanted catheters resulted in a CFU count of 8.73×10^{10} CFU/catheter for the untreated control, with an SD of 0.89, and 8.69×10^{10} CFU/catheter for the ML:8 (1% [vol/vol])-treated catheters, with an SD of 1.04 (Fig. 4B). Catheters treated with Citrox (1% [vol/vol]) did not contain any detectable viable bacteria (Fig. 4B).

The effect of ML:8 on lower-inoculum (10^4 CFU/ml) biofilms formed over 1 day was also investigated. Results are shown in Fig. 4C. High-level luminescence was observed in the untreated catheter (Fig. 4C, panel i), while no detectable luminescence was ob-

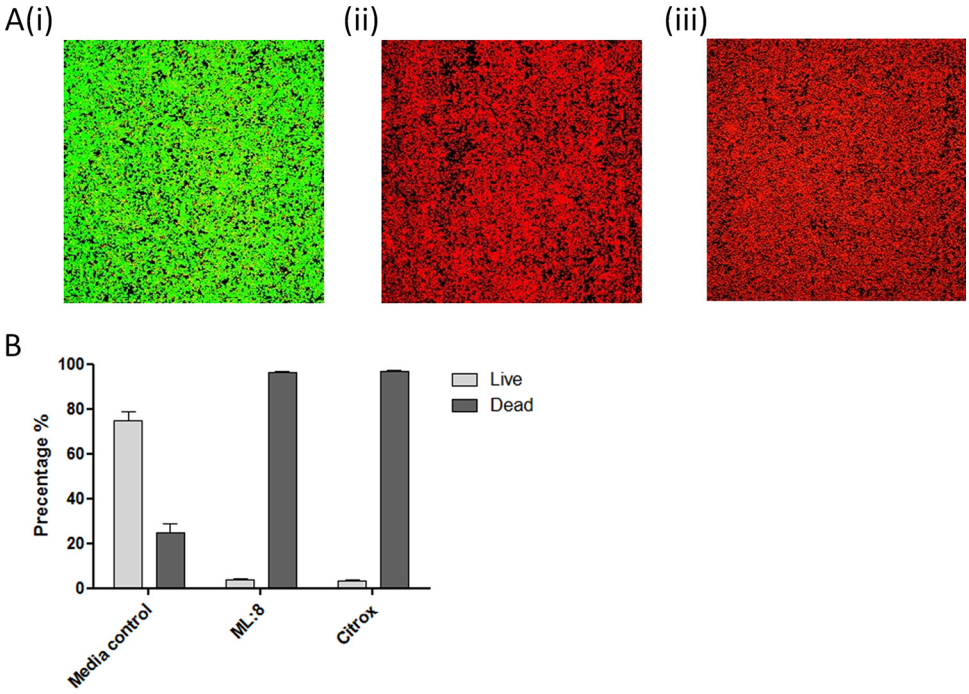

FIG 2 Effects of ML:8 and Citrox on biofilms formed under shear conditions. (A) MSSA strain SH1000 biofilms were formed under conditions of flow in RPMI 1640 and then treated statically with medium (i), ML:8 (1% [vol/vol]) (ii), or Citrox (1% [vol/vol]) (iii) for 24 h at 37°C. Live/dead staining was carried out and visualized using a confocal microscope. (B) ImageJ software was used to calculate the percentages of pixels representing live (green) and dead (red) bacteria. Data represent mean percentages and SD. Assays were performed in triplicate, and representative images are shown.

served for the ML:8-treated catheter (Fig. 4C, panel ii). Harvested bacterial biofilms resulted in a CFU count of 7.58×10^{10} CFU/catheter for the untreated control, with an SD of 0.95, and no bacteria were detected for the ML:8 treatment group (Fig. 4D).

DISCUSSION

Current treatment for staphylococcal CRIs often involves device removal. However, this is not always possible due to clinical circumstance and can also result in considerable patient morbidity and mortality (22). Systemic antibiotics are usually administered to treat CRIs, but although they are generally effective at eliminating circulating planktonic bacteria, they frequently fail to sterilize the device or IVC, leaving the patient at risk of complications or recurrence. One treatment approach involves the use of CLSs in combination with systemic antibiotics to eradicate the *S. aureus* biofilm within the IVC. However, recent research has highlighted that a large number of antibiotics and antiseptic agents are not effective as CLSs for the treatment of biofilm-mediated *S. aureus* infection (5). Therefore, there is an urgent need for novel and effective agents for the treatment of device-related infections involving staphylococcal biofilms (6, 23).

TABLE 3 Comparison of IC_{50}s of treatment agents against THP-1 monocytes and HaCaT keratinocytes

Treatment agent	IC_{50} (% [vol/vol])	
	THP1 cells	HaCaT cells
ML:8	0.058	0.149
Citrox	0.041	0.038
Ethanol	2.4	1.7
Duralock-C	0.48	0.21

In this study, two agents, ML:8 and Citrox, were investigated to determine their potential use as novel antistaphylococcal biofilm treatments. At concentrations of 0.125% (vol/vol) and 0.015% (vol/vol), respectively, ML:8 and Citrox were effective against planktonic cells. Investigations also found concentrations as low as 1% (vol/vol) for both ML:8 and Citrox to be optimum for the inactivation of bacterial cells in a sessile state under both static and flow conditions. This is significant because staphylococcal biofilms under conditions of flow have previously been shown to be more tolerant to treatment than those formed under static conditions, and treatment of patients with conventional antimicrobials against staphylococcal biofilm infections, such as CRI, frequently results in treatment failure due to the antimicrobial resistance of organisms within a biofilm and failure of antimicrobial agents to reach the desired MIC within this environment (24, 25). Both agents also killed biofilms formed over 1, 3, and 5 days, suggesting the potential for ML:8 and Citrox to be used in the treatment of established staphylococcal biofilm infections, such as those that occur clinically when CRIs are diagnosed after establishment of biofilm. Our findings are consistent with research published on the antimicrobial potential of caprylic acid (7, 8). A study examining the effect of ML:8-X10, a formulation similar to ML:8, and vancomycin showed equivalent activities of both agents against *S. aureus* (26). This is in contrast to our study, in which we found the activity of ML:8 against biofilm-forming *S. aureus* to be superior to those of many commonly used antimicrobials (5). The antistaphylococcal biofilm properties of Citrox are reported for the first time in this study. A previous study described the antimicro-

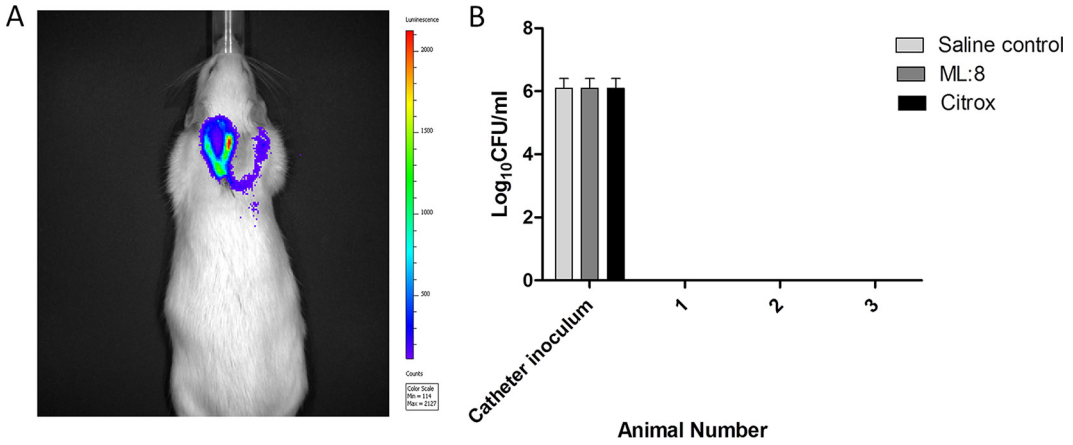

FIG 3 Monitoring infection development and dissemination. Optimized doses of high-inoculum (10^6 CFU/ml) MRSA USA300 *lux* in 40 μl were injected into catheters, and infection was allowed to develop for 5 days. (A) An IVIS imaging system was used to monitor the development of infection. Bioluminescence activity in a Sprague-Dawley rat was acquired using an IVIS 100 camera at 5 days postinfection. The color scale indicates the degree of luminescence, from high (red) to low (purple). A representative animal is shown ($n = 3$). (B) Catheter inoculation and dissemination into blood were assessed at the experiment endpoint. Animals were euthanized at day 9, blood was harvested, and $\log^{10}$ CFU counts were determined. Assays were performed in triplicate, and the data represent mean $\log_{10}$ CFU per milliliter and SD.

bial properties of two formulations of Citrox versus oral microorganisms; while it demonstrated broad antimicrobial properties against a range of pathogens, that study did not include staphylococci and was limited to artificial *in vitro* planktonic and biofilm assays, which did not replicate the *in vivo*-like environment described in this study (11).

To investigate further the potential therapeutic use of ML:8 and Citrox, the cytotoxic and hemolytic potentials of these agents were examined. Cytotoxicity to the HaCaT and THP-1 cell lines was evident at a concentration shown to be effective against bacterial biofilms (1% [vol/vol]). The effects of ML:8 and Citrox on erythrocytes were also examined to determine if either agent caused hemolysis of human blood. Hemolysis was observed after exposure to high concentrations (≥0.25% [vol/vol]) of the agents. However, compared to currently used CLSs, such as ethanol and sodium citrate solutions (Duralock-C), cytotoxicity and hemolytic levels were found to be no worse than those of the currently used treatments, which would therefore warrant further investigation of these agents as potential CLSs. For agents used as CLSs, if leakage from the IVC into the systemic circulation did occur, the

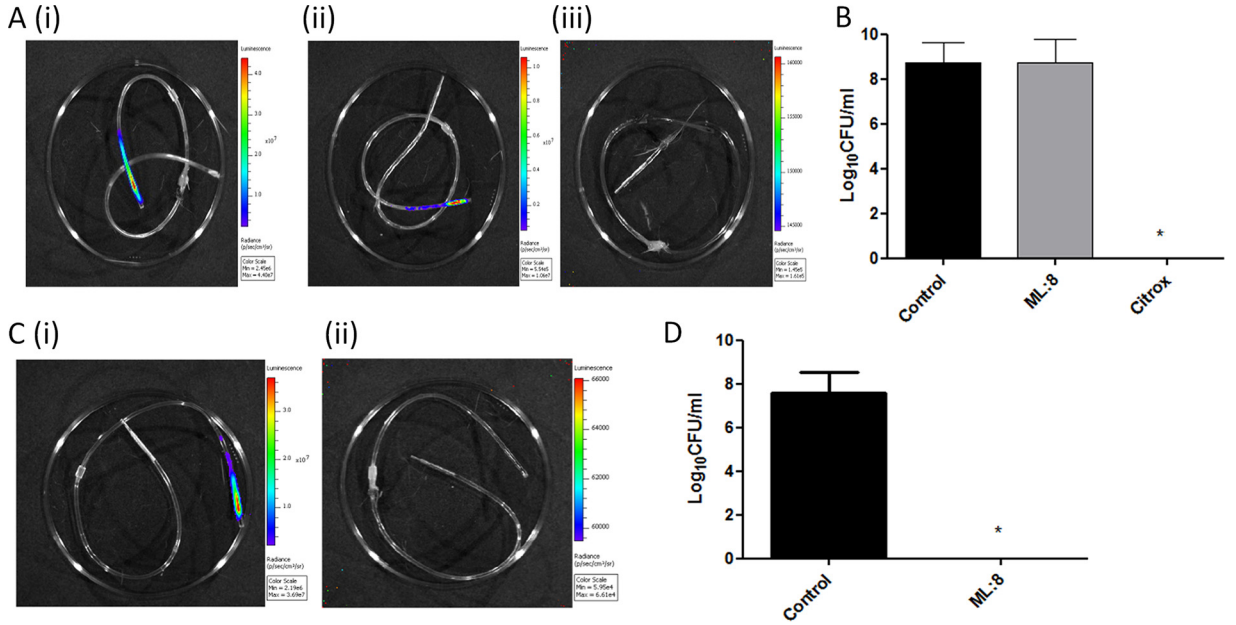

FIG 4 Effects of ML:8 and Citrox on *in vivo* biofilms formed over 5 days. Optimized doses of 10^6 CFU/ml (A and B) and 10^4 CFU/ml (C and D) MRSA USA300 *lux* in 40 μl were injected into catheters, and infection was allowed to develop for 1 (C and D) or 5 (A and B) days. This was followed by treatment for 4 days with saline (control), ML:8 (1%), or Citrox (1%). Animals were euthanized at day 9. Catheters were explanted, bioluminescence activity was acquired using an IVIS 100 camera (A and C), and $\log^{10}$ CFU counts were determined (B and D). Representative catheters are shown. Three animals were used per group, and data represent mean $\log_{10}$ CFU per milliliter and SD. Statistically significant results are indicated (*, $P \leq 0.05$; two-tailed Student *t* tests versus untreated animals).

solution would be diluted rapidly, dramatically minimizing the risk of toxicity described for the concentrations tested in this study. Taken together, this rapid dilution in conjunction with the small volume in a catheter reduces the risk of harm to a patient.

Short-chain fatty acids, including acetic, butyric, and propionic acids, have previously been linked to increased levels of IL-1β, IL-6, and IL-8, leading to a proinflammatory response in humans (27), while some flavonoids, including quercetin, have previously been shown to inhibit expression of the proinflammatory cytokine TNF-α (28, 29). The potential of ML:8 or Citrox to induce or inhibit an innate immune response involving a range of cytokines was therefore investigated. Reassuringly, no cytokine response was detected in fresh whole blood following exposure to either novel agent. Interestingly, no resistance to ML:8 or Citrox arose in the MSSA strain SH1000 or the MRSA strain BH1CC over a 90-day period of exposure to sublethal concentrations of either agent. In contrast, bacterial resistance to antibiotics has previously been reported after 2 to 5 days of exposure to sublethal concentrations (30). Resistance to other agents, such as tea tree oil, have also been shown to emerge within 90 days (31).

To ensure that our initial results were not confined to limited genetic backgrounds, we expanded our study to include many other *S. aureus* clonal complexes (CCs) commonly isolated from patients within hospitals. Previous studies have shown an association between *S. aureus* clonality, invasive disease, and antimicrobial resistance (32, 33). Significant variation in the susceptibility of biofilms produced by strains from various genetic lineages was not shown after treatment with 1% (vol/vol) ML:8 or Citrox.

A rat model of IVC infection assessed the effects of these novel agents *in vivo*. Citrox (1% [vol/vol]) was effective in the eradication of MRSA biofilms formed over 5 days in this model, using an initial inoculum of 10^6 CFU/ml. In comparison, ML:8 (1% [vol/vol]) did not result in a significant reduction in biofilm viability against biofilms formed under the same conditions. ML:8 eradicated biofilm viability in this model when biofilms were formed over 1 day, using a lower inoculum of 10^4 CFU/ml. Biofilm maturity may be an important contributing factor in the resistance of 5-day *in vivo* biofilms to ML:8 treatment. Increased resistance of mature 5-day biofilms was previously reported for a number of commonly used antimicrobials (5, 12).

Importantly, the findings with these novel agents could be investigated further in other *S. aureus* models of infection, such as prosthetic joint infections, surgical site infections, and wound infections; this is of particular significance and importance given the increasing use of medical devices, with their associated risk of infection, within modern health care. While many antimicrobial agents kill bacteria in a planktonic state, only a limited number are effective at treating device-related staphylococcal infections involving biofilms, and therefore these agents represent additional treatment options versus staphylococci. Taken together, our findings demonstrate that ML:8 and Citrox have the potential to be used therapeutically for the treatment of CRIs involving *S. aureus* biofilms.

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