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Eradication of *Staphylococcus aureus* Biofilm Infections Using Synthetic Antimicrobial Peptides

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Here, we demonstrate that antimicrobial peptides (AMPs) are an effective antibiofilm treatment when applied as catheter lock solutions (CLSs) against *S. aureus* biofilm infections. The activity of synthetic AMPs (Bac8c, HB43, P18, Omiganan, WMR, Ranalexin, and Polyphemusin) was measured against early and mature biofilms produced by methicillin-resistant *S. aureus* and methicillin-susceptible *S. aureus* isolates from patients with device-related infections grown under in vivo–relevant biofilm conditions. The cytotoxic and hemolytic activities of the AMPs against human cells and their immunomodulatory potential in human blood were also characterized. The D-Bac8c^{2,5Leu} variant emerged as the most effective AMP during in vitro studies and was also highly effective in eradicating *S. aureus* biofilm infection when used in a CLS rat central venous catheter infection model. These data support the potential use of D-Bac8c^{2,5Leu}, alone or in combination with other AMPs, in the treatment of *S. aureus* intravenous catheter infections.

Keywords. *Staphylococcus aureus*; biofilm; antimicrobial peptides (AMPs); catheter lock solution (CLS).

Implantable medical devices such as intravascular catheters (IVCs) have revolutionized modern healthcare; however, colonization of these devices by surface-adhering bacteria results in biofilm formation and subsequent catheter-related infection (CRI) associated with significant morbidity and mortality. Those formed by *Staphylococcus aureus* are among the most frequent causes of CRI [1]. Biofilms are stabilized by homophilic interactions of surface proteins, such as the fibronectin binding proteins (FnBPs) [2, 3], as well as the extracellular matrix, which consists of polysaccharide intracellular adhesin (PIA) and extracellular DNA [4]. Moreover, biofilms formed in vivo are shielded by insoluble fibrin [5, 6], which makes them impenetrable to antibodies and phagocytic cells. Externally exposed bacterial cells grow and disseminate, whereas those within deeper tissue layers may form dormant niches, which are metabolically inactive and of decreased susceptibility to many existing antimicrobial agents. Therefore, systemic antibiotics administered to treat IVC infections frequently fail, leaving the patient at a continuing risk of complications or recurrence and frequently leading to the need for device removal [7]. Identification of effective antibiofilm agents is limited by unavailability of an appropriate treatment model because biofilm susceptibility further depends on local environment. The Infectious Disease Society of America (IDSA) guidelines on the management of CRIs recommend the use of catheter-locking solutions (CLSs), in combination with systemic antibiotics, for the salvage of an IVC associated with CRI [8]. However, there is as of yet, no consensus on an appropriate agent, and several commonly used antibiotics have recently been shown to be ineffective [9]. Because current treatment options are limited, the need for novel agents for either use as CLSs, or indeed as anti-staphylococcal biofilm treatments, is of great importance.

Antimicrobial peptides (AMPs) represent a promising therapeutic option against biofilm infections [10]. Their membrane-permeabilizing properties make them uniquely effective in the rapid killing of multidrug-resistant bacteria. Moreover, they are effective against both dormant and growing cells, irrespective of the cells' metabolic state. Their charge promotes interaction with negatively charged bacterial surfaces and thus determines their antibacterial character [11]. Their activity may also involve interference with metabolic processes or with intracellular targets that may result in inhibition of cell-wall synthesis, nucleic-acid synthesis, and protein production [12]. Because of multimodal action, they are less likely to promote the development of antimicrobial resistance [13–15]. Nevertheless resistance to killing by AMPs has been reported, including secretion of bacterial proteases, alteration of surface charge to decrease binding of cationic AMPs, or expression of efflux pumps [16]. *Staphylococcus aureus* has been shown to alter the anionic charge of its surface by modification of teichoic acids with D-alanyl groups, as well as enhanced

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insertion of positively charged phospholipids in its membrane [17–20].

Susceptibility of *S. aureus* to AMPs has to date been mostly assessed within the free-floating state, and studies of the activity of AMPs against *S. aureus* cells within a biofilm have been limited [21–24]. Here, we investigated the therapeutic potential of highly potent synthetic AMPs using a number of in vitro assays, including: (1) biofilm killing by 7 potent AMPs determined against coagulase-mediated biofilms of *S. aureus* variants formed in the presence of human plasma and venous shear; (2) cytotoxicity studies as well as measurement of hemolytic and immunomodulatory potentials of the AMPs; (3) Finally, the activity of the AMPs selected as having the highest resistance mechanisms and their relevance in biofilm killing by AMPs. The activity of the AMP selected as having the highest therapeutic potential, Bac8c^{2,5Leu}, was further tested as a CLS in an animal model of catheter-related infection.

MATERIALS AND METHODS

Peptide Synthesis

D-Bac8c^{2,5Leu}, D-HB43, D-P18^{8Leu}, D-WMR^{3,6Leu}, and D-Ranalexin were synthesized as previously shown [25, 26]. Formation of the disulfide bridge in Ranalexin was accomplished by air oxidation of a 0.75-mM solution of the peptide in ammonium acetate buffer (pH = 8; 50 mM). L-Polyphemusin was synthesized and supplied by Almac.

Bacterial Strains, Growth Conditions, and Susceptibility Testing

Methicillin-resistant *S. aureus* strains; USA300, USA300*lux* [27], and BH1CC [2] and MSSA strains; SH1000 [28] and BH48 [2] were cultured in Müeller-Hinton for susceptibility testing or in Roswell Park Memorial Institute 1640 medium (RPMI-1640) for biofilm formation. Minimal inhibitory concentrations (MICs) were determined by standard microdilution method (Clinical and Laboratory Standards Institute).

Biofilm Formation and Treatment in Microtiter Wells

Coagulase-mediated biofilms were prepared as described previously for either 24 hours or 5 days (daily media replacement) [6].

Biofilm treatments with AMPs or ethanol at 40% (v/v), used as a positive control, were performed in biofilm-containing wells. The viability of the biofilms was measured using a resazurin-conversion assay, live/dead staining, and/or viable count.

Resazurin-Conversion Assay

Adenosine triphosphate (ATP)-dependent conversion of the nonfluorescent resazurin into the fluorescent resorufin as a measure of bacterial viability was performed as previously described [6]. Briefly, the resazurin at 44 µM in RPMI-1640 was added 1:1 (v/v) to biofilms for 1 hour at 37°C, protected from light. Fluorescence intensity was detected at excitation of 544 nm and emission of 590 nm.

Biofilm Formation Under Shear Flow Using Microfluidic System

Vena8 Fluoro+ flow chambers were inoculated with human plasma and incubated at 37°C for 30 minutes. *Staphylococcus aureus* suspensions were injected into the chambers and incubated for 4 hours following initiation of the pump supplying RPMI-1640 at the shear of 200 µL/min (6.25 dynes/cm^2) for 24 hours at 37°C.

Live/Dead Staining of Biofilms Formed Under Flow

Biofilms were stained with 150 µM of SYTO9 green (Molecular Probes) and 1 mM propidium iodide (Molecular Probes) for 1 hour in room temperature (RT), protected from light, followed by washing. Confocal microscopy (Inverted Zeiss LSM 510 META) was used to visualize the green/red fluorescence. Four representative images were obtained per sample group per experiment. Each experiment was performed at least 3 times. Quantification of the fluorescence intensity was performed using Image J 1 software [29].

MTT Assay

Keratinocytes (aneuploid immortal keratinocyte cell line, HaCaT) cells were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum, and primary human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 Bulletkit media (Lonza). Seeded at 3 × 10^5 cells/mL for 24 hours at 37°C, cells were incubated with a range of AMPs (up to 4 mg/mL) or Triton X 1% (v/v) in appropriate culture media. Media were removed, and the cells were washed and incubated with 500 mg/L of MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma) for 4 hours, protected from light. Dimethyl sulfoxide was used to solubilize formed crystals. Absorbance was recorded at 560 nm.

Hemolysis Assay

Human blood was drawn (ethylenediaminetetraacetic acid [EDTA]; 1.6 mg/mL), and red blood cells (RBCs) were separated by centrifugation at 1000 × g for 5 minutes at 18°C. Upon 2 washes with phosphate-buffered saline, the RBCs were further diluted by 2-fold (v/v) into phosphate-buffered saline and incubated with AMPs or Triton X at 0.5% control. Absorbance at 570 mM was used to determine the level of hemolysis.

Cytokine Release Assay

Human blood from healthy donors was drawn into syringes containing EDTA (1.6 mg/mL) and each added to (1) wells containing MRSA biofilm, (2) AMPs alone (D-Bac8c^{2,5Leu}, D-Omiganan, D-WMR, all at 60 µM), or (3) biofilm preexposed to 60 µM of either of the above AMPs for 2 hours at 37 ˚C, following separation of the plasma by centrifugation

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(1000 × g) for 10 minutes. Quantification of human cytokines from plasma was performed using Bio-Plex 200 (Bio-Rad). The concentrations of human of human interleukin 2 (IL-2), interleukin 4 (Il-4), interleukin 6 (IL-6), interleukin 8 (IL-8), and interleukin 10 (IL-10), interferon γ (INFγ), tumour necrosis receptor α (TNFα), and human granulocyte-macrophage colony-stimulating factor in human blood were quantified. The assays were performed using wash stations with the magnetic plate to minimize operator-related variations. A standard curve was used to maximize sensitivity for samples containing very low levels of analytes. At least 3 heathy donors' samples were used to determine the mean concentration of each cytokine.

Rat Jugular Vein Catheter Infection Model

Sprague-Dawley rats with preimplanted jugular vein catheters were supplied by Charles River UK. Catheters were infected with 40 µL of USA300*lux* [30] (10^4 CFUs/mL) for 1 day. Subcutaneous administration of vancomycin (50 mg/kg) twice daily was performed to prevent systemic infection; it was not used within the CLS and did not influence the localized treatment of established biofilm within the lumen of the catheter. Biofilm development was confirmed by imaging (Perkin Elmer IVIS Spectrum). Therapy with D-Bac8c^{2,5Leu} (256 µg/mL), in sodium chloride 0.1% (w/v), was administered daily for 5 days as a CLS and was "locked" within the lumen of the catheter for 24 hours. Catheters were removed from sacrificed animals and subjected to imaging (Perkin Elmer IVIS Spectrum: exposure, 20s; binning: 4, f1). Furthermore, bacterial numbers at the catheter were harvested (TrypLE Express treatment and vigorous washing) and subjected to CFU count.

Ethics Approval

The Ethics Committee of the Royal College of Surgeons in Ireland granted ethics approvals for blood collection and use (REC820 and REC951). Animal experiments were approved by and performed under Irish Government Department of Health and the RCSI Ethics Committee (REC931).

RESULTS

Staphylococcus aureus Susceptibility to Antimicrobial Peptides

The AMPs used in this study were selected based on previously reported antistaphylococcal activity (Table 1). The AMPs were synthesized as either native peptides or improved activity derivatives. To prevent the likely deactivation of AMPs by proteases, candidates were synthesized as D-enantiomers apart from L-Polyphemusin, a cyclic peptide stabilized by 2 disulfide bonds. D-isoforms have been previously shown to be more effective against bacteria, including *S. aureus* [39]. The D-isoleucines in the original sequences were replaced with D-leucines due to considerably higher cost of the former and similar bactericidal activity previously shown for the variants (Table 1) [40].

Minimum inhibitory concentrations were determined against *S. aureus* isolates (Table 1). D-HB43 was the most effective, having the lowest MIC at 4 mg/L (2.75 µM), whereas D-P18^{8Leu} was least effective at 64 mg/L (27.8 µM). D-WMR^{3,6Leu}, D-Omiganan, and D-Ranalexin were equally effective in growth inhibition, with MICs at 8 mg/L or approximately 4 µM, whereas D-Bac8c^{2,5Leu} and L-Polyphemusin inhibited *S. aureus* growth at 8–16 mg/L (>6 µM).

Antimicrobial Peptides Inactivate *Staphylococcus aureus* Biofilms

All biofilms were cultured in the presence of human plasma to form fibrin-embedded structures, thereby mimicking the in vivo environment [6]. Viable counts revealed that the densities of the biofilms were approximately 2.2 × 10^8 colony-forming units (CFUs) per milliliter for the MRSA isolate fBH1CC and 2.6 × 10^8–3 × 10^8 CFUs per milliliter for the MSSA strain SH1000 (Supplementary Figure 1) [6].

Most of the investigated AMPs reduced biofilm viability in a dose-dependent manner within 6 hours (Figure 1). There was no further reduction of viability after this treatment time. Complete inactivation of biofilms was achieved with 128 µg/mL of D-Bac8c^{2,5Leu}, D-HB43, or D-Ranalexin; whereas D-WMR and L-Polyphemusin killed biofilms at 256 µg/mL (Figure 1). Up to 2048 µg/mL of D-P18^{8Leu} only reduced

Table 1. Characteristics of Studied Antimicrobial Peptides

Antimicrobial peptide	Amino-acid sequence	Average mass (g/mol)	Disulphide bridge	Net charge	MIC, µg/mL (µM)
D-Bac8c^{2,5Leu} [31]	rlwvlwrr	1183.47	…	+4	8 (6.75)
D-HB43 [31]	fakllaklakkll	1455.94	…	+5	4 (2.75)
D-P18^{8Leu} [33, 34]	kwklfkklpkflhlakkf	2299.97	…	+8.8	64 (27.8)
D-WMR^{3,6Leu} [35]	wglrrllkygkrs	1631.99	…	+6	8 (4.9)
D-Omiganan [36]	ilrwpwwpwrrk	1821.21	…	+4	8 (4.4)
D-Ranalexin [37]	flgglikivpamicavtkkc	2105.73	14–20	+4	8 (3.8)
L-Polyphemusin [38]	RRWCFRVCYRGFCYRKCR	2457.981	4–17, 8–13	+7	16 (6.5)

Minimal inhibitory concentrations were determined against *Staphylococcus aureus* BH1CC, USA300, BH48, SH100 and SH1000.
Abbreviation: MIC, minimal inhibitory concentration.

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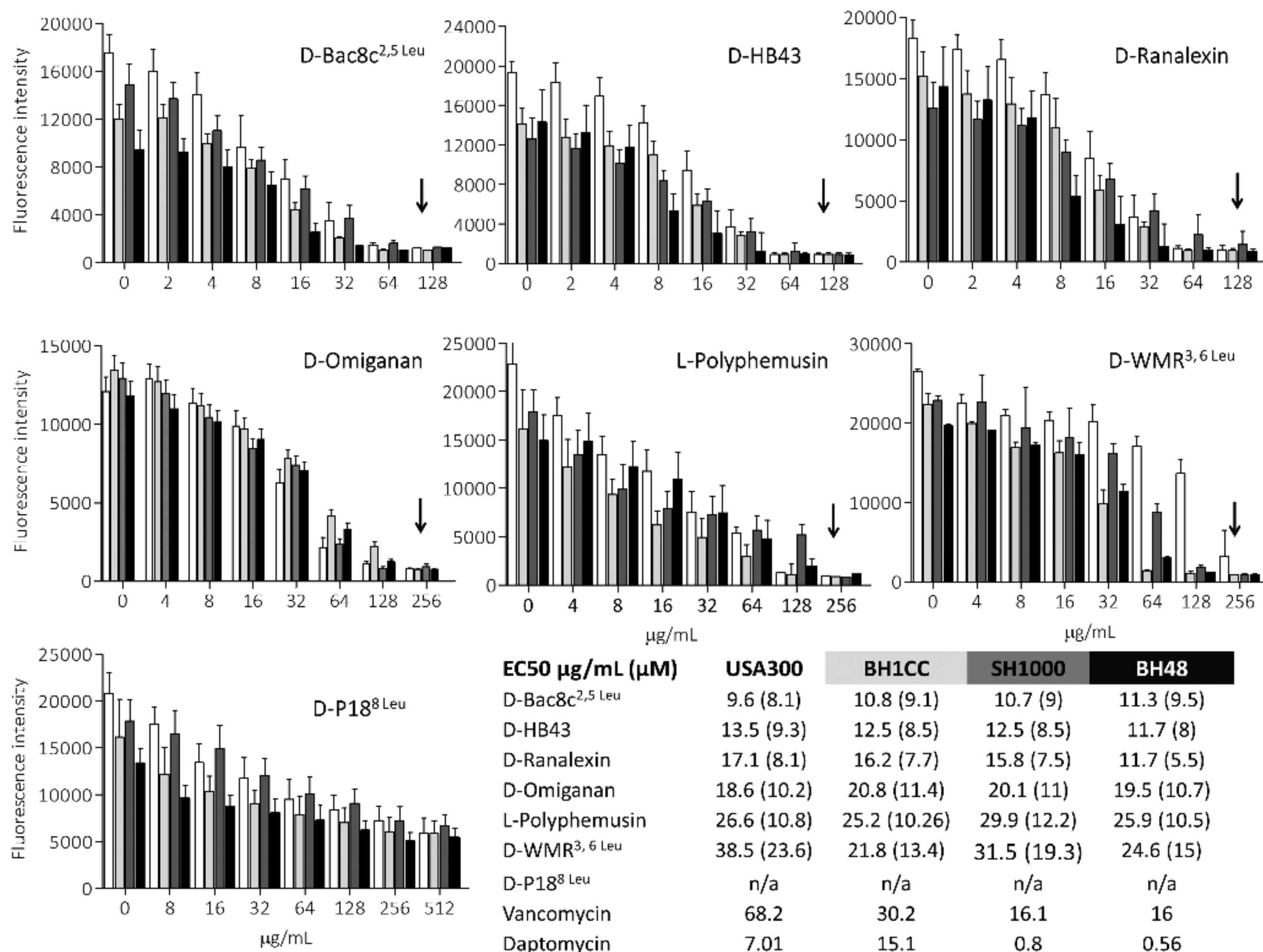

EC50 μg/mL (μM)	USA300	BH1CC	SH1000	BH48
D-Bac8c$^{2,5\ Leu}$	9.6 (8.1)	10.8 (9.1)	10.7 (9)	11.3 (9.5)
D-HB43	13.5 (9.3)	12.5 (8.5)	12.5 (8.5)	11.7 (8)
D-Ranalexin	17.1 (8.1)	16.2 (7.7)	15.8 (7.5)	11.7 (5.5)
D-Omiganan	18.6 (10.2)	20.8 (11.4)	20.1 (11)	19.5 (10.7)
L-Polyphemusin	26.6 (10.8)	25.2 (10.26)	29.9 (12.2)	25.9 (10.5)
D-WMR$^{3,\ 6\ Leu}$	38.5 (23.6)	21.8 (13.4)	31.5 (19.3)	24.6 (15)
D-P18$^{8\ Leu}$	n/a	n/a	n/a	n/a
Vancomycin	68.2	30.2	16.1	16
Daptomycin	7.01	15.1	0.8	0.56

Figure 1. Susceptibility of *Staphylococcus aureus* biofilms to killing by antimicrobial peptides (AMPs). Biofilms of *S. aureus* USA300 (white bar), BH1CC (light gray), SH1000 (dark gray), and BH48 (black) were formed in microtiter wells. The AMPs D-Bac8c^{2,5Leu}, D-HB43, D-P18^{8Leu}, D-WMR^{3,6Leu}, D-Ranalexin, D-Omiganan and L-Polyphemusin were incubated with biofilms for 6 hours at 37°C following measurement of the viability using a resazurin-conversion assay. Results are means ± SD of fluorescence intensity obtained in 3 independent experiments. Upon treatment and media replacement, biofilm recovery was measured after 24 hours. Concentrations where no recovery was observed are marked with arrows. Concentrations that were half effective in reducing biofilm viability were calculated. Abbreviations: EC50, half effective; NA, not applicable.

viability but did not eradicate the biofilms (Figure 1). Half-maximal effective (EC50) concentrations were determined against statically grown biofilms (Figure 1). The EC50s for D-Bac8c^{2,5Leu}, D-HB43, and D-Ranalexin were similar (approximately 7–8 µM), ranging 9.6–17.1 µg/mL. The EC50 values for D-Omiganan, L-Polyphemusin, and D-WMR were higher (>10 µM), from 18.6 to 38.5 µg/mL (Figure 1). These results reveal that D-Bac8c^{2,5Leu}, D-HB43, and D-Ranalexin were most effective in biofilm killing.

Antimicrobial peptides were similarly effective against fibrin-embedded biofilms preformed under venous shear (Supplementary Figure 2). Incubation with 128 µg/mL of D-Bac8c^{2,5Leu}, D-HB43, D-Ranalexin, or D-Omiganan significantly increased the ratio of the dead to live cells. D-P18^{8Leu} demonstrated lower potency in terms of the live/dead ratio. Although D-WMR was less effective against the USA300 biofilm at 128 µg/mL, it remained very effective against other strains at concentrations >64 µg/mL (Supplementary Figure 2; data not shown).

Moreover, we determined that the contribution of the counter-ion trifluoroacetate (TFA) to the biofilm bactericidal effects was negligible based on comparison of activity for AMP variants with the counter-ions TFA or hydrochloride (HCl). Biofilms of USA300 were equally susceptible to treatment with D-HB43 variants (Supplementary Figure 3). Similar results were obtained for the D-Bac8c^{2,5Leu} and D-WMR variants (data not shown).

Biofilm Tolerance to Antimicrobial Peptides

To investigate the relevance of mechanisms previously shown as important in providing *S. aureus* resistance to AMPs produced as part of human immunity resistance-defective mutants of multiple peptide resistance factor (MprF) and the Aps sensor/regulator of *dlt*, VraFG and MprF were used. Biofilms of *S. aureus* USA300

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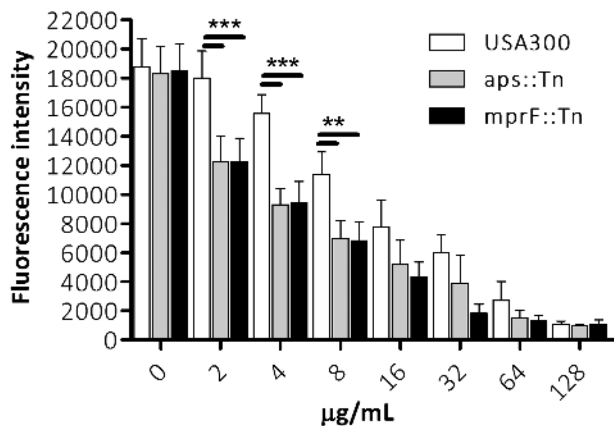

Figure 2. Aps and MprF contribution to methicillin-resistant *Staphylococcus aureus* (MRSA) resistance against antimicrobial peptides (AMPs). Biofilms of *S. aureus* USA300 variants (wt and mutants with transposon, Tn insertions in either *aps* or *mprF* genes) were grown in microtiter wells for 24 hours at 37°C. Biofilms were treated with D-Bac8c^{2,5Leu} for 6 hours at 37°C following determination of bacterial viability by resazurin-conversion assay. Presented results are means of fluorescence intensity ± SD of fluorescence intensity obtained in 3 independent experiments. Two-way analysis of variance was performed to determine the statistical significance; ***$P < .001$, **$P < .01$.

wild-type as well as mutants of *aps* and *mprF* were compared in their susceptibility to killing by AMPs (Figure 2). Both *aps* and *mprF* mutant biofilms were more susceptible to D-Bac8c^{2,5Leu} and D-HB43 than USA300. Although the EC50 values were lower for the less-tolerant mutants, complete killing of biofilms formed by all 3 strains required the same minimal bactericidal concentration (MBC) (Figure 2; data not shown), suggesting that the protective role of Aps-controlled resistance factors contributes to, but is not critical for, biofilm tolerance to AMPs.

Cytotoxicity and Haemolysis

MTT assays were performed to determine cytotoxicity of the AMPs against HUVECs and HaCaTs (Table 2). D-P18^{8Leu} and D-HB43 were the most cytotoxic with half inhibitory concentrations (ICs$_{50}$) at 26.7µg/mL (11.3 µM) and 20.6 µg/mL (14 µM) against HaCaT and 16.4µg/mL (7 µM) and 11.1µg/mL (7.5 µM) against HUVECs, respectively. D-Ranalexin, with an IC$_{50}$ at 63µg/mL (30 µM) against HaCaT cells and 60µg/mL (28.5 µM) against HUVEC culture, was less cytotoxic. L-Polyphemusin and D-Omiganan had approximately 4-fold higher ICs$_{50}$. The D-Bac8c^{2,5Leu} and D-WMR^{3,6Leu} were least cytotoxic at concentrations shown to be sufficient in biofilm eradication (Table 2).

Hemolytic activity was determined against human RBCs. Concentrations required for hemolysis of 50% of human RBCs for 24 hours are listed in Table 2, indicating that D-HB43 and Ranalexin were the most hemolytic, whereas D-Omiganan, L-Polyphemusin, and D-WMR^{3,6Leu} were the least hemolytic among the candidates.

Antimicrobial Peptide Activity Against Mature Biofilms

Susceptibility of coagulase-mediated biofilms has been shown to be different for early and mature biofilm cultures [6, 9]. Antibiofilm properties of the D-Bac8c^{2,5Leu}, D-Omiganan, and D-WMR (of the highest therapeutic potential) were evaluated against 5-day-old biofilms (Figure 3). MBC for D-Bac8c^{2,5Leu} was determined as 256 µg/mL, whereas 4-fold higher concentration of the other 2 peptides was required to kill the mature biofilm, suggesting that D-Bac8c^{2,5Leu} most effectively retains its antibiofilm activity against older biofilms (Figure 3).

Immunogenic Potential of Antimicrobial Peptides

Production of cytokines in human blood was measured following exposure to AMPs (Figure 4). The concentrations of human IL-2, Il-4, IL-6, IL-8, and IL-10, as well as INFγ, TNFα, and human granulocyte-macrophage colony-stimulating factor in human blood, were quantified. The measurement was performed following 2 hours exposure to AMPs, biofilms or MRSA USA300 or biofilms pre-exposed to AMPs. Unlike AMPs alone, biofilm increased the levels of IL-8 and TNFα (Figure 4). The elevated concentration of IL-8 could be, however, inhibited by sub-bactericidal concentrations of D-Bac8c^{2,5Leu} but not the other AMPs. There was no increase of any tested cytokines upon exposure to the AMPs alone. These results suggest that Bac8c^{2,5Leu}, D-Omiganan, and D-WMR alone are unlikely to cause an increase in cytokine levels in human blood, whereas D-Bac8c^{2,5Leu} may interact with the inflammatory signalling pathway of IL-8 (Figure 4).

Treatment of an In Vivo *Staphylococcus aureus* Biofilm Infection With D-Bac8c^{2,5Leu}

Antibiofilm activity of D-Bac8c^{2,5Leu} was further investigated in the presence of human plasma (Figure S4). Dose-dependent

Table 2. Cytotoxicity and Hemolysis of Antimicrobial Peptides

IC$_{50}$ µg/mL (mM)	D-Bac8c^{2,5Leu}	D-HB43	D-Ranalexin	L-Polyphemusin	D-WMR^{3,6Leu}	P18^{8Leu}	D-Omiganan	Ethanol
HaCaT	426.7 (360)	20.6 (14)	63 (30)	226.4 (92)	1884 (1150)	26.7 (11.3)	302.8 (162.8)	1.2%
HUVEC	134 (113)	11.1 (7.5)	60 (28.5)	224 (91)	1755 (1087)	16.4 (7.)	NA	0.4%
Hemolysis (hRBC)	414 (350)	77 (4.8)	195 (92.6)	1885 (766.8)	>4000 (2450)	873 (380.5)	250 (137)	NA

Cytotoxicity was studied using an MTT assay against human keratinocytes and umbilical endothelial cells. Hemolysis assays were performed using red blood cells purified from human blood. Half inhibitory concentrations (IC$_{50}$) were determined as means of 3 independent experiments. Presented IC$_{50}$ values are either in µg/mL or (mM) concentrations.

Abbreviations: HaCaT, human keratinocytes; HUVEC, human umbilical endothelial cells; IC$_{50}$, half inhibitory concentration; NA, not applicable.

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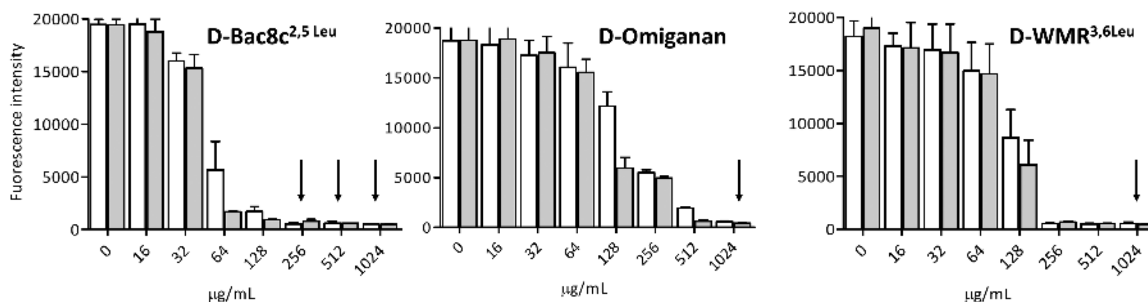

Figure 3. Efficacy of antimicrobial peptides (AMPs) against mature biofilms. Biofilms of USA300 (white bar) and SH1000 (gray bar), were formed in microtiter wells for 5 days. Antimicrobial peptides were incubated with biofilms for 24 hours at 37°C followed by measurement of the viability. Results are means ± SD of fluorescence intensity obtained in 3 independent experiments. Upon fluorescence reading, growth media were replaced, allowing bacterial recovery for 18 hours. Viability measurement was repeated. Concentrations with no growth are marked with arrows.

killing of *S. aureus* biofilm was measured after 1hour of incubation with D- Bac8c^{2,5Leu}, suggesting that the biofilm bactericidal activity of the AMP was uninhibited in the presence of plasma (Supplementary Figure 3). An in vivo jugular vein catheter infection rat model in which catheters were colonized with USA300*lux* (a USA300 derivative expressing luciferase) was used to investigate the effectiveness of Bac8c^{2,5Leu} as a CLS. Biofilms of USA300*lux* formed on the catheters grown in vivo were exposed to a Bac8c^{2,5Leu} CLS at a concentration of 256 µg/mL for a period of 5 days (CLS changed daily) (Figure 5). No visible luminescence remained on the catheters in which the biofilm had been treated with Bac8c^{2,5Leu} (Figure 5). Consistent

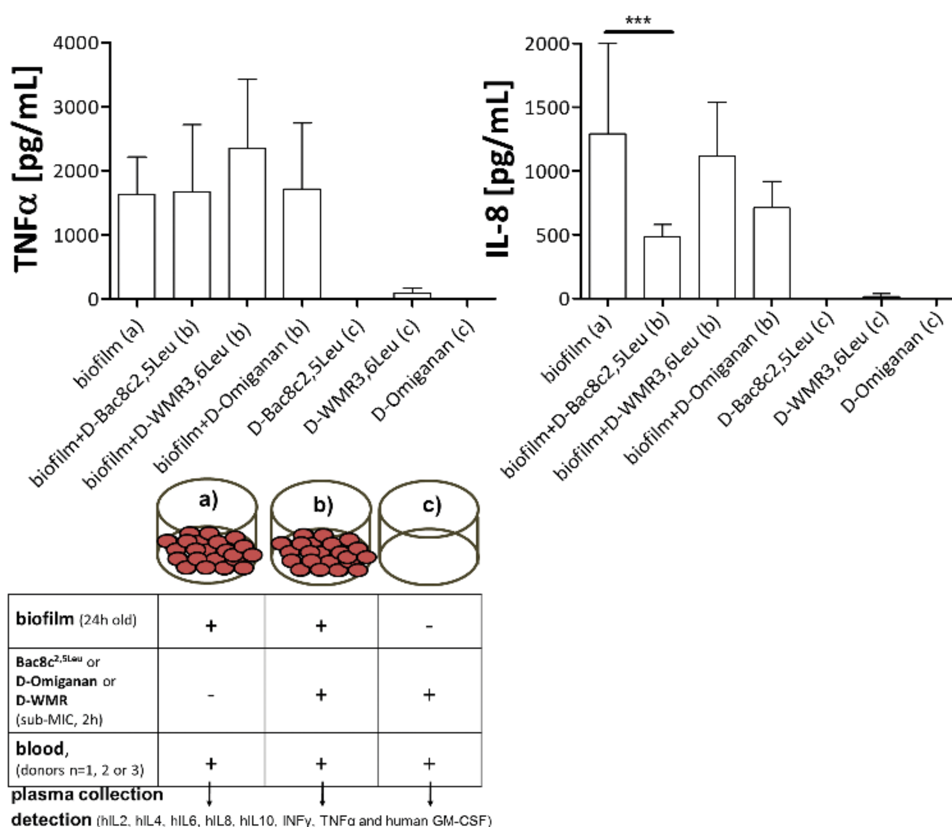

Figure 4. D-Bac8c^{2,5Leu} interferes with biofilm-induced interleukin 8 (IL-8) levels in human blood. Cytokines levels were quantified in bloods incubated with MRSA biofilm (†), antimicrobial peptides (AMPs) (‡), or MRSA biofilms preexposed with AMPs (§) (lower panel). Exposure to MRSA biofilms increased the levels of IL-8 and TNFα (†) (upper panels). Exposure to AMPs alone (Bac8c^{2,5Leu}, D-Omiganan, or D-WMR) did not elevate the cytokine levels in blood (upper panels). Each of 3 donor samples was measured in triplicate following the instruction manual. Statistically significant results are indicated: $^{***}P < .001$. Abbreviations: GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-2, interleukin 2; IL-4, interleukin 4; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; INFγ, interferon γ; MIC, minimum inhibitory concentration; TNFα, tumor necrosis factor α.

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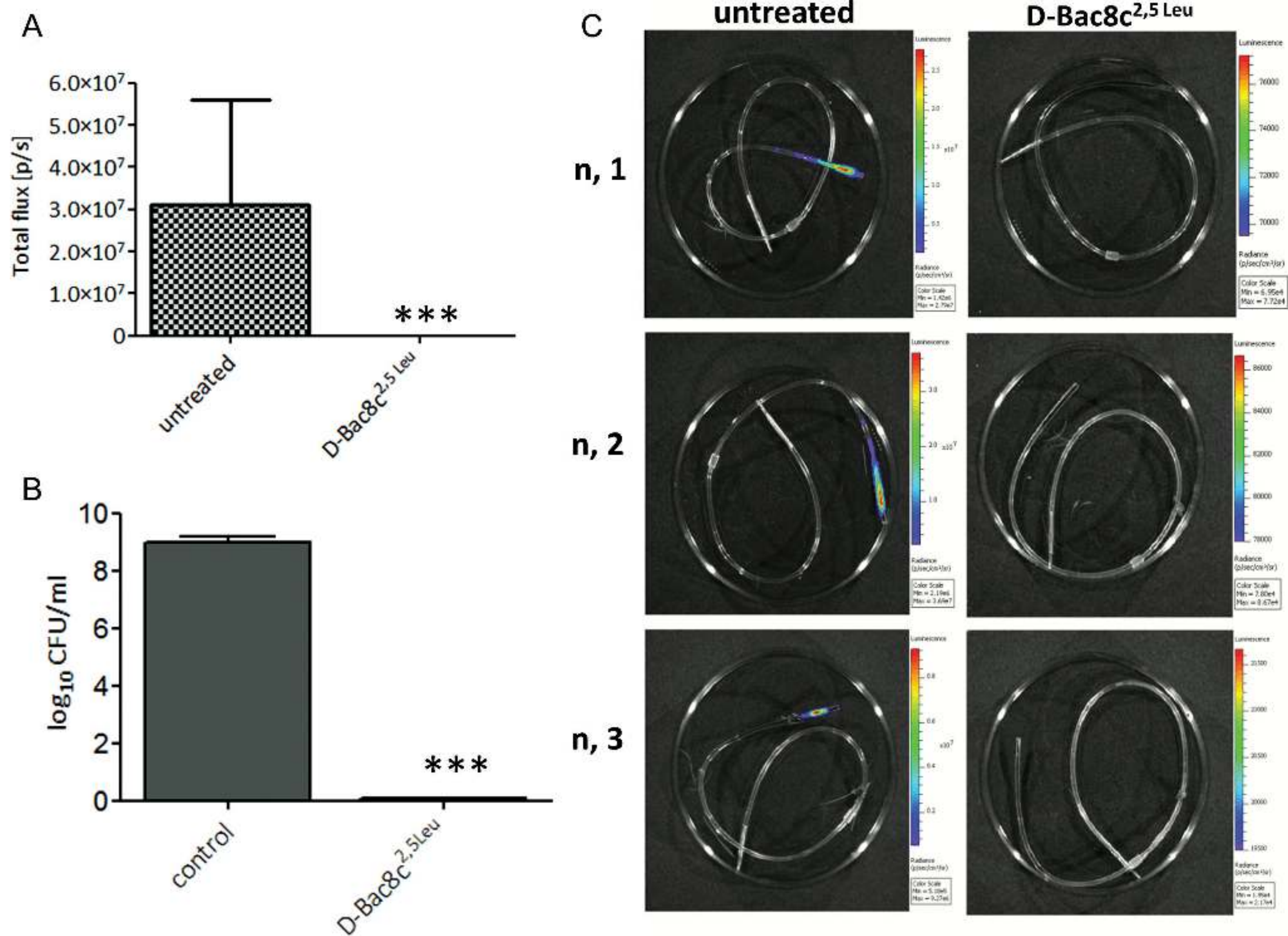

Figure 5. In vivo eradication of a methicillin-resistant *Staphylococcus aureus* (MRSA) biofilm catheter infection with catheter lock solution (CLS) consisting of D-Bac8c^{2,5Leu} (256 mg/L). The CLS was instilled into a jugular vein catheter of 3 rats (n; 1, 2, 3) to treat intravascular catheter (IVC)–associated *S. aureus* USA300*lux* biofilms. In the control group (3 rats, n; 1, 2, 3), the CLS was replaced with 0.9% sodium chloride. The CLS was renewed every 24 hours for 5 days. The day after the final treatment, animals were killed, and the catheters were removed and subjected to quantification of bioluminescence using IVIS (*A*, *C*). Bacterial cells were harvested using TrypLE^{T} reagent, serially diluted, and plated on tryptic soy agar for colony-forming unit (CFU) counting (*B*). Statistical significance is indicated: ****P* < .001.

with this, Bac8c^{2,5Leu} treatment was accompanied by a $\log_{10}$ of 9 reduction in the number of CFUs recovered from catheter tips (Figure 5). These data support the efficacy of Bac8c^{2,5Leu} as a CLS in the treatment of biofilm-associated infections.

DISCUSSION

Systemic antibiotics are usually administered to treat CRIs, although generally effective in treating bloodstream infections, frequently fail to sterilize the IVC, requiring device removal. Catheter lock solutions, used in association with systemic antibiotics, are recommended for salvage of infected IVCs in patients with *S. aureus* CRI. The range of CLSs with demonstrated efficacy against a CRI is, however, limited due to antibiotic tolerance and the recalcitrant nature of the biofilm infections [41]. The CLSs currently in clinical use include antibiotics such as vancomycin or gentamicin and antimicrobials such as ethanol or sodium citrate. Therefore, there is a real clinical need for novel effective agents for the treatment of IVC infections due to *S. aureus*.

The properties of AMPs to act as a localized biofilm treatment, such as in CLS therapy, are favorable. AMPs use effective membrane-targeting, multimodal activity against biofilm and have fast bactericidal action, low immunogenicity, low cytotoxicity, and low risk of resistance. Here we selected potent antistaphylococcal AMPs, including native and synthetic candidates, to characterize them in respect to their biofilm-bactericidal properties and therapeutic potential for the treatment of *S. aureus* CRI.

As determined in this study, most AMPs were effective in dose-dependent biofilm killing, with D-Bac8c^{2,5Leu}, D-HB43, and D-Ranalexin being the most effective. Even though controlled CLS treatment minimizes the risk of cytotoxic effect by antimicrobial agents and has resulted in the use of relatively

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cytotoxic CLSs, such as solutions of >20% (v/v) ethanol, we considered cytotoxicity to be a critical factor in selection of potential AMPs for the treatment of a CRI. As determined against a range of human cells—including RBCs, HUVECs and HaCaTs—Bac8c^{2,5Leu}, D-Omiganan, D-WMR^{3,6Leu} were the least cytotoxic and hemolytic and among the most effective biofilm eradicators.

Importantly, in this study, biofilms were cultured in minimal RPMI-1640 in the presence of human plasma, promoting formation of coagulase-mediated, fibrin-embeded biofilms such as those formed within intravascular catheters in vivo [4, 6, 42]. Bacterial coagulase cleaves plasma fibrinogen to generate insoluble fibrin that promotes production of a fibrin-embedded scaffold [4, 6, 42]. The relevance of the treatment environment, biofilm complexity, and potential contribution of plasma binding factors to the biofilm's susceptibility to AMPs were reflected by the lack of correlation between the AMPs' MICs (Table 1) and their bactericidal activity against biofilms (Figure 1). Also, the susceptibility of fibrin-embedded biofilms has been reported to decrease upon maturation, possibly due to increasing bacterial density, the growing ratio of dormant to multiplying bacteria, or the increasing barrier formed by extracellular matrix [6, 30, 41]. Although the mechanism(s) of AMP-mediated biofilm killing remain unclear, in this study, mature biofilms also needed 2-fold (D-Bac8c^{2,5Leu}) or even 4-fold higher (D-Omiganan & D-WMR^{3,6Leu}) doses for unrecoverable killing of biofilms. Moreover, a trend was observed where AMPs of lower net positive charge (approximately 4) were more effective in biofilm killing despite a lack of correlation between charge and EC50.

We investigated whether the resistance mechanisms described previously as protective for *S. aureus* against AMPs and produced as part of the innate immune are of significance in biofilm susceptibility to AMPs [20, 43]. In community-acquired (CA-MRSA), these resistance factors, such as the *dlt* operon [18], multiple peptide resistance factor (MprF) [44], and VraFG transporter, are controlled by the Aps sensor/regulator protein [45]. Although biofilms formed by strains with functional Aps or MprF were more tolerant of AMP treatment than *aps*- and *mprF*-mutant biofilms, the doses required for eradication of *aps*, *mprF,* and wild-type biofilms was the same, suggesting only a minor level for these factors in biofilm tolerance. Although the development of an AMP-based CLS requires the study of individual candidates, ultimately, lock solution could be a synergistic combination of these AMPs, an approach which would also prevent the emergence of cross-resistance against peptides from the innate immune system.

Even though controlled CLS administration is restricted to the catheter lumen, adverse reactions may occur upon unintended leakage of a CLS into the systemic circulation. AMPs have been previously reported to possess immunomodulatory properties triggering pro- or anti-inflammatory effects during infection. To evaluate the potential of an immunomodulatory reaction—for example, due to a leak into the systemic circulation—we measured the ability of our most potent AMPs to activate cytokine release or modulate the inflammatory reaction to *S. aureus* biofilm during treatment. D-Bac8c^{2,5Leu}, D-Omiganan, and D-WMR^{3,6Leu} did not trigger release of cytokines in whole human blood, suggesting unlikely immunogenic potential of these AMPs upon exposure to human host tissue. However, D-Bac8c^{2,5Leu} had a role in decreasing the elevated levels of IL-8, but not TNFα, due to biofilm exposure, indicating it may interfere with the IL-8 release because of biofilm exposure. However, more studies are needed to determine the mechanism of this potentially anti-inflammatory effect.

To broaden our in vitro findings, gained under in vivo–mimicking conditions, a rat model of IVC infection was used to validate the efficacy of D-Bac8c^{2,5Leu} in vivo. D-Bac8c^{2,5Leu} at a concentration of 256 µg/mL was highly effective in eradicating MRSA biofilm infection from the catheter lumen, whereas untreated catheters resulted in harvest of $\log_{10}$ 9 CFUs/mL.

Taken together, these in vitro studies and the in vivo potency toward biofilms of *S. aureus* determined for Bac8c^{2,5Leu} and potentially D-Omiganan and D-WMR^{3,6Leu} demonstrate their therapeutic potential as a viable treatment option for IVC infections within a CLS or a similar localized treatment strategy against biofilm-related infection of implanted medical devices.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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