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Eradication of *Staphylococcus aureus* biofilm infections using synthetic antimicrobial peptides.

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Running title: AMPs eradicate *S. aureus* biofilm infections

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ABSTRACT

Staphylococcus aureus biofilm accumulation on intravascular catheters is highly recalcitrant partially due to a shield of host-derived fibrin as part of bacterial biofilm, which is associated with increased resistance to host immune defences and antimicrobial agents. Here, we demonstrate that antimicrobial peptides (AMPs) are an effective anti-biofilm treatment when applied as catheter lock solutions (CLSs) in the treatment of *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 MRSA and MSSA isolates from patients with device-related infections grown under *in vivo*-relevant biofilm conditions, with or without venous shear. The cytotoxic and haemolytic activities of the AMPs against human cells and their immunomodulatory potential in human blood were also characterised. 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 antimicrobial drugs, in the treatment of *S. aureus* intravenous catheter infections.

Key words: *Staphylococcus aureus*, biofilm, antimicrobial peptides (AMPs), catheter lock solution (CLS).

INTRODUCTION

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 patient morbidity and mortality. Biofilms formed by *Staphylococcus aureus*, are one of the most frequent causes of CRI [1]. These densely populated bacterial structures are stabilised by homophilic interactions of surface proteins, such as the fibronectin binding proteins (FnBPs) [2, 3], as well as the extracellular matrix consisting 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, metabolically inactive and of decreased susceptibility to many existing antimicrobial agents. Therefore, systemic antibiotics administered to treat IVC infections frequently fail to sterilize the IVC, leaving the patient at a continuing risk of complications or recurrence and frequently leads to the need for device removal [7]. Identification of effective anti-biofilm agents is limited by unavailability of an appropriate treatment model, as 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) 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]. As current treatment options are limited, the need for novel therapeutic 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 their 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]. Due to their multimodal action, they are less likely to promote the development of antimicrobial resistance [13-15]. Nevertheless resistance to killing by AMPs have been reported, including secretion of bacterial proteases, alteration of surface charge to decrease binding of cationic AMPs or expression of efflux pumps [16]. *S. 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 insertion of positively charged phospholipids in their membrane [17-20].

Susceptibility of *S. aureus* to AMPs has to date been mostly assessed within the free-floating state and studies of their activity against *S. aureus* cells within a biofilm have been limited [21-24]. Here, we investigated the bactericidal and therapeutic potential of highly potent synthetic AMPs using a number of *in vitro* assays, ^{I)} Biofilm killing by 7 potent AMPs determined against coagulase-mediated biofilms of MRSA and MSSA, formed in the presence of human plasma and venous shear, ^{II)} Cytotoxicity, haemolytic and immunomodulatory potentials of the AMPs, ^{III)} The relevance of previously reported resistance mechanisms during biofilm killing. 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.

RESULTS

***S. aureus* planktonic cells are susceptible to AMPs.** The AMPs used in this study were selected based on previously reported anti-staphylococcal 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 with the exception of L-Polyphemusin, a cyclic peptide stabilised by 2 disulfide bonds. D-isoforms have been previously shown as more effective against bacteria, including *S. aureus* [33]. The D-Isoleucines in the original sequences of Bac8c, WMR and P18 were replaced with D-Leucines due to considerably higher cost of the former. Similar bactericidal activity has been previously shown for the D-Leu variants (Table 1)[34]. D-Omiganan has been used in this original sequence containing an Isoleucine-1.

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

AMPs inactivate *S. 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; ca. 2.2×10^8 CFU per mL for the MRSA isolate BH1CC and $2.6 \times 10^8 - 3 \times 10^8$ CFU per mL for the MSSA strain SH1000 (Fig. S1) [6].

Most of investigated AMPs reduced biofilm viability in a dose-dependent manner within 6h (Fig. 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; while D-WMR and L-Polyphemusin killed biofilms at 256 µg/mL (Fig. 1). Up to 2048 µg/mL of D-P18^{8Leu} only reduced viability but did not eradicate the biofilms (Fig. 1). Half-maximal effective (EC50) concentrations were determined against statically grown biofilms (Fig. 1). The EC50s for D-Bac8c^{2,5Leu}, D-HB43 and D-Ranalexin were similar (ca. 7-8µM) ranging from 9.6-17.1 µg/mL. The EC50 values for D-Omiganan, L-Polyphemusin, and D-WMR were higher (>10 µM) from 18.6 up to 38.5 µg/mL (Fig. 1). These results reveal that D-Bac8c^{2,5Leu}, D-HB43 and D-Ranalexin were most effective in biofilm killing.

AMPs were similarly effective against fibrin-embedded biofilms pre-formed under venous shear (Fig. S2). Incubation with 128µg/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 (Fig. S2, 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 AMPs' variants with the counter-ions TFA or hydrochloride (HCl). Biofilms of USA300 were equally susceptible to treatment with D-HB43 variants (Fig.S3). Similar results were obtained for the D-Bac8c^{2,5Leu} and D-WMR variants (data not shown).

Biofilm tolerance to AMPs. To investigate the relevance of mechanisms previously shown as important in providing *S. aureus* resistance to antimicrobial peptides 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 wild type, *aps*- and *mprf*-deficient mutants were compared in their susceptibility to AMPs killing (Fig. 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 three strains required the same MBC (Fig. 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 human umbilical vein endothelial cells (HUVEC) and keratinocytes (HaCaT) (Table 2). D-P18^{8Leu} and D-HB43 were the most cytotoxic with IC₅₀ at respectively; 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. D-Ranalexin, with an IC₅₀ at 63µg/mL (30 µM) against HaCaT cells and 60µg/mL (28.5 µM) against HUVEC culture, were less cytotoxic. L-Polyphemusin and D-Omiganan had approximately 4-fold higher IC₅₀. The D-Bac8c^{2,5Leu} and D-WMR^{3,6Leu} were least cytotoxic at concentrations shown to be sufficient in biofilm eradication (Table 2).

Haemolytic activity was determined against human red blood cells (hRBCs). Concentrations required for haemolysis of 50% of hRBCs for 24h are listed in Table 2, indicating that D-HB43 and Ranalexin were the most haemolytic while D-Omiganan, L-Polyphemusin and D-WMR^{3,6Leu} were the least haemolytic among the candidates.

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

Immunogenic potential of AMPs. Production of cytokines in human blood was measured following exposure to AMPs (Fig. 4). The concentrations of human interleukins (hIL) 2, 4, 6, 8 and 10, as well as interferon γ (INF γ), tumour necrosis receptor- α (TNF α) and human granulocyte-macrophage colony-stimulating factor in human blood were quantified following 2h exposure to AMPs, biofilms of MRSA USA300 or the biofilms pre-exposed to AMPs. Unlike, AMPs alone, biofilm increased the levels of IL-8 and TNF α (Fig. 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 while D-Bac8c^{2,5Leu} may interact with the inflammatory signalling pathway of IL-8 (Fig. 4).

Treatment of an *in vivo* *S. aureus* biofilm infection with D-Bac8c^{2,5Leu}. Anti-biofilm activity of D-Bac8c^{2,5Leu} was further investigated in the presence of human plasma (Fig. S4). Dose-dependent killing of *S. aureus* biofilm was measured after 1h incubation with D-Bac8c^{2,5Leu}, suggesting that the biofilm bactericidal activity of the AMP was uninhibited in

the presence of plasma (Fig. S3). An *in vivo* jugular vein catheter infection rat model in which catheters were colonised with USA300*lux* (a USA300 derivative expressing luciferase), was used to investigate the effectiveness of Bac8c^{2,5Leu} as a catheter lock solution (CLS). Biofilms of USA300*lux* formed on the catheters grown *in vivo* were exposed to a Bac8c^{2,5Leu} CLS at a concentration of 256µg/mL for a period of 5 days (CLS changed daily) (Fig. 5). No visible luminescence remained on the catheters in which the biofilm had been treated with Bac8c^{2,5Leu} (Fig. 5). Consistent with this Bac8c^{2,5Leu} treatment was accompanied by a log₁₀ of 9 reduction in the number of CFU recovered from catheter tips (Fig. 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, which although generally effective in treating bloodstream infections, frequently fail to sterilize the IVC requiring device removal. Catheter lock solutions, in association with systemic antibiotics, are used and are recommended treatment in an attempt to eradicate the biofilm within an IVC and allow salvage of the IVC in patients with a *S. aureus* CRI. The range of CLSs with demonstrated efficacy against a CRI is, however, limited due to antibiotic resistance and the recalcitrant nature of the biofilm infections [35]. 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 biofilm formation by *S. aureus*.

The properties of AMPs to act as a localized biofilm treatment, such as in CLS therapy, are favourable. AMPs utilize the 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 anti-staphylococcal AMPs, including native and synthetic candidates, in order 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 we investigated were effective in dose-dependent biofilm killing with D-Bac8c^{2,5Leu}, D-HB43 and D-Ranalexin being the most effective AMPs. Even though controlled CLS treatment minimises the risk of cytotoxic effect by antimicrobial agents, and has resulted in the use of relatively cytotoxic CLS, 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 RBC, HUVEC and keratinocytes; Bac8c^{2,5Leu}, D-Omiganan & D-

WMR^{3,6Leu} were the least cytotoxic and haemolytic and amongst the most effective biofilm eradicators.

Importantly, the biofilms used in the study were cultured in minimal RPMI-1640 medium in the presence of human plasma promoting formation of coagulase-mediated structures such as those formed *in vivo*, as within intravascular catheters, mediated by the activity of bacterial coagulase which cleaves plasma fibrinogen which is converted into insoluble fibrin promoting production of a fibrin-embedded scaffold [4, 6, 36]. The relevance of the treatment environment, biofilm complexity and potential contribution of plasma binding factors to the biofilm's susceptibility to AMPs was reflected by the lack of correlation between the AMPs MICs (Table 1) and their bactericidal activity against biofilms (Fig. 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, 35]. Similarly, in this study, application of mature biofilms resulted in two-fold higher (D-Bac8c^{2,5Leu}) or even four-fold higher (D-Omiganan & D-WMR^{3,6Leu})- doses being required for unrecoverable killing of biofilms. Moreover, a trend was observed where AMPs of lower net positive charge (ca. 4) were more effective in biofilm killing despite a lack of correlation between charge and EC50.

We investigated if 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, 37]. In CA-MRSA these resistance factors such as *dlt* operon [18], multiple peptide resistance factor (MprF) [38] and VraFG transporter are controlled by the Aps sensor/regulator protein [39]. Although there was a statistically significant increase in resistance of biofilms formed by strains of functional Aps or MprF, the dose required for eradication of either of the biofilms was indifferent suggesting a minor level

of tolerance to AMPs provided by these factors in biofilm. While the development of an AMP-based CLS requires the study of individual candidates, ultimately, the most potent 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 reaction may occur upon systemic leak of an agent. 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 systemic leak we measured the ability of most potent AMPs to activate cytokine release or modulate the inflammatory reaction to *S. aureus* biofilm during treatment. Neither D-Bac8c^{2,5Leu}, D-Omiganan nor D-WMR^{3,6Leu} triggered release of cytokines in whole human blood, suggesting unlikely immunogenic potential upon exposure to human host tissue. D-Bac8c^{2,5Leu} however had a role in decreasing the elevated levels of IL-8, but not TNF α , due to biofilm exposure indicating it may interfere with the the IL-8 release as a result 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 μ g/mL, was highly effective in eradicating MRSA biofilm infection while untreated catheters resulted in harvesting log₁₀ 9 CFU per mL.

Taken together, these *in vitro* studies and the *in vivo* potency towards biofilms of *S. aureus* determined for Bac8c^{2,5Leu} and potentially D-Omiganan & D-WMR^{3,6Leu} demonstrate their therapeutic potential as a viable treatment option for IVC infections within a CLS or a

266 similar localized treatment strategy against biofilm-related infection implanted medical
267 devices.

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 [40, 41]. Formation of the disulfide bridge in Ranalexin was accomplished by air oxidation of a 0.75mM solution of the peptide in ammonium acetate buffer (pH=8, 50 mM). L-Polyphemusin was synthesized and supplied by Almac, UK.

Bacterial strains, growth conditions and susceptibility testing.

MRSA strains; USA300, USA300*lux* [42] & BH1CC [2] and MSSA; strains SH1000 [43], BH48[2] were cultured in Mueller-Hinton for susceptibility testing or in RPMI-1640 (Gibco) 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 before for either 24h 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 (see below).

Resazurin-conversion assay. ATP-dependent conversion of the non-fluorescent resazurin into the fluorescent resorufin as a measure of bacterial viability was performed as previously described [6]. Briefly, the resazurin at 44 μ M in RPMI-1640 was added 1:1 (v/v) to biofilms

for 1h 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 (Kima, Cellix Ltd.).

Vena8 Fluoro+ flow chambers were inoculated with human plasma and incubated at 37°C for 30 min. *S. aureus* suspensions were injected into the chambers and incubated for 4h following initiation of the pump supplying RPMI-1640 at the shear of 200µl/min (6.25 dynes/cm²) for 24h at 37°C.

Live/dead staining of biofilms formed under flow. Biofilms (see above) were stained with 150 µM of SYTO9 green (Molecular Probes) and 1 mM propidium iodide (Molecular Probes) for 1h in RT, protected from light, followed by washing. Confocal microscopy (Inverted Zeiss LSM 510 META) was used to visualise the green/red fluorescence. Four representative images were obtained per sample group per experiment. Each experiment was performed at least three times. Quantification of the fluorescence intensity was performed using Image J 1 software [44].

MTT assay. HaCaT cells were cultured in DMEM (Gibco) supplemented with 10% FBS while primary HUVEC cells in EGMTM-2 Bulletkit media (Lonza). Seeding in microtiter plates at 3×10^5 cells/mL for 24 h 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, washed and incubated with 500 mg/L of MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-

diphenyl-2H-tetrazolium bromide] (Sigma) for 4 h, protected from light. DMSO was used to solubilize formed crystals. Absorbance was recorded at 560 nm.

Haemolysis assay. Human blood was drawn (EDTA, 1.6 mg/ml) and red blood cells (RBC) were separated by centrifugation at $1000 \times g$ for 5 min at 18°C. Upon two washes with PBS, the RBCs were further diluted by 2-fold (v/v) into PBS and incubated with AMPs or Triton X at 0.5% control. Absorbance at 570nm was used to determine the level of haemolysis.

Cytokine release assay. Human bloods were drawn into syringes containing EDTA (1.6 mg/ml) and each added to either: a) wells containing MRSA biofilm, b) AMPs alone (D-Bac8c^{2,5Leu}, D-Omiganan, D-WMR, all at 60 μ M), or c) biofilm pre-exposed to 60 μ M of either AMP for 2h at 37 °C, following separation of the plasma by centrifugation ($1000 \times g$) for 10 min. Quantification of human cytokines from plasma was performed using Bio-Plex 200 (Bio-Rad). The concentrations of human interleukins (hIL) 2, 4, 6, 8 and 10, interferon γ (INF γ), tumour necrosis receptor- α (TNF α) and human granulocyte-macrophage colony-stimulating factor in human blood were quantified. The assays were performed using wash station 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 three healthy donors' samples were used to determine the mean concentration of each cytokine.

Rat jugular vein catheter infection model. Sprague-Dawley rats with pre-implanted jugular vein catheters were supplied by Charles River UK. Catheters were infected with 40 μ L of USA300*lux* [42] (10^4 CFU/mL) for 1 day. Subcutaneous administration of vancomycin (50

mg/kg) twice daily was performed to prevent systemic infection. Biofilm development was confirmed by imaging (Perkin Elmer IVIS Spectrum). Antibiotic lock therapy with D-Bac8c^{2,5 Leu} (256 µg/mL) in sodium chloride 0.1% (w/v) was administered daily (for 24h), for 5 days. Catheters were removed from sacrificed animals, subjected to imaging (Perkin Elmer IVIS Spectrum: exposure, 20s; binning: 4, f1). Furthermore, bacterial numbers at the catheter were harvested (TrypLETM 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).

347

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353

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FIGURE LEGENDS AND TABLES

Figure 1. Susceptibility of *S. aureus* biofilms to killing by antimicrobial peptides AMPs killing. Biofilms of *S. aureus* USA300 (white bar), BH1CC (light grey), SH1000 (dark grey) and BH48 (black) were formed in microtiter wells. AMPs; D-Bac8c^{2,5 Leu}(A), D-HB43 (B), D-P18^{8Leu} (C), D-WMR^{3,6Leu} (D), D-Ranalexin (E) and L-Polyphemusin (F) were incubated with biofilms for 6h at 37°C following measurement of the viability using a resazurin-conversion assay. Results are means \pm SD of fluorescence intensity obtained in three independent experiments. Upon treatment and media replacement biofilm recovery was measured after 24h. Concentrations where no recovery was observed are marked with arrows. Concentrations that were half effective (EC50) in reducing biofilm viability were calculated.

Figure 2. Aps and MprF contribution to MRSA resistance against AMPs. Biofilms of *S. aureus* USA300 variants were grown in microtiter wells for 24h at 37°C. Biofilms were treated with D-Bac8c^{2,5 Leu} for 6h 37°C following determination of bacterial viability by resazurin-conversion assay. Presented results are means of fluorescence intensity \pm SD of fluorescence intensity obtained in three independent experiments. Two-way ANOVA was performed to determine the statistical significance; *** $P < 0.001$, ** $P < 0.01$.

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

Figure 4. D-Bac8c^{2,5Leu} interferes with biofilm-induced IL-8 levels in human blood. Cytokines levels were quantified (Bio-Plex, BioRad) in bloods incubated with; (a) MRSA biofilm, (b) AMPs or (c) MRSA biofilms pre-exposed with AMPs (lower panel). Exposure to MRSA biofilms (a) increased the levels of INF γ and TNF α (upper panels). Exposure to AMPs (Bac8c^{2,5Leu}, D-Omiganan or D-WMR) did not elevate the cytokines levels in blood (upper panels). Each of three donor samples was measured in triplicate using directions following the instruction manual. Statistically significant results are indicated, (*** $P < 0.001$)

Figure 5. *In vivo* eradication of an MRSA biofilm catheter infection with CLS consisting of D-Bac8c^{2,5 Leu} (256 mg/L). CLS was instilled into a jugular vein catheter of 3 rats to treat IVC-associated *S. aureus* USA300lux biofilms. In control group (n=3) the CLS was replaced with 0.9% sodium chloride. CLS was renewed every 24 h for 5 days. The day after the final treatment, animals were sacrificed, catheters removed and subjected to quantification of bioluminescence using IVIS (AC). Bacterial cells were harvested using TrypLE™ reagent, serially diluted, and plated on tryptic soy agar for colony-forming unit (CFU) counting (B). Statistical significance is indicated, (*** $P < 0.001$).

Table 1. Studied AMPs; amino-acid sequence, physicochemical properties, minimal inhibitory concentrations (MICs) and references. MICs were determined against *S. aureus* BH1CC, USA300, BH48 and SH100.

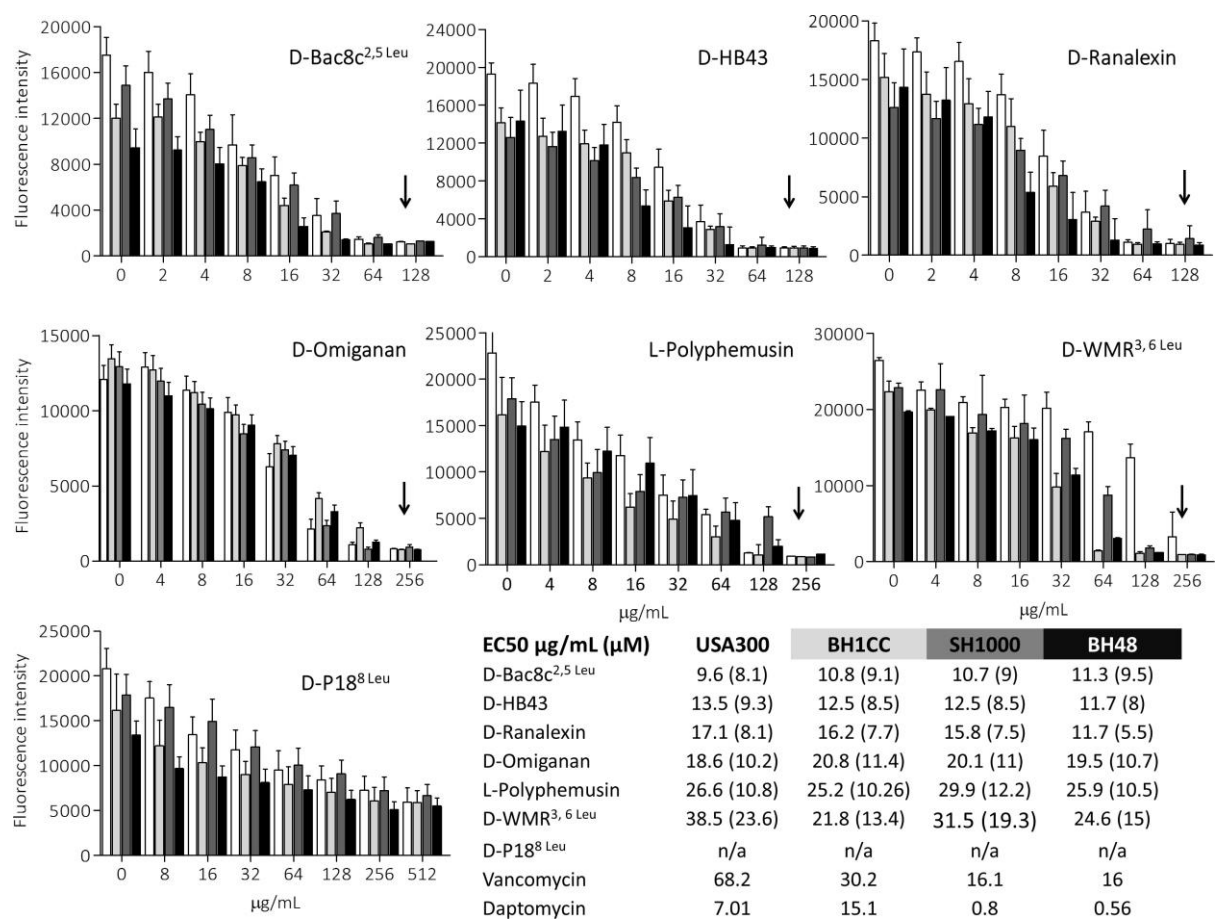
Table 2. Cytotoxicity and haemolysis of AMPs. Cytotoxicity was studied using an MTT assay against human keratinocytes (HaCaT) and umbilical endothelial cells (HUVEC). Haemolysis assays were performed using red blood cells purified from human blood. Half

511 inhibitory concentrations (IC_{50}) at $\mu\text{g/mL}$ & μM were determined as means of three
512 independent experiments.

513

514

515 Figure 1



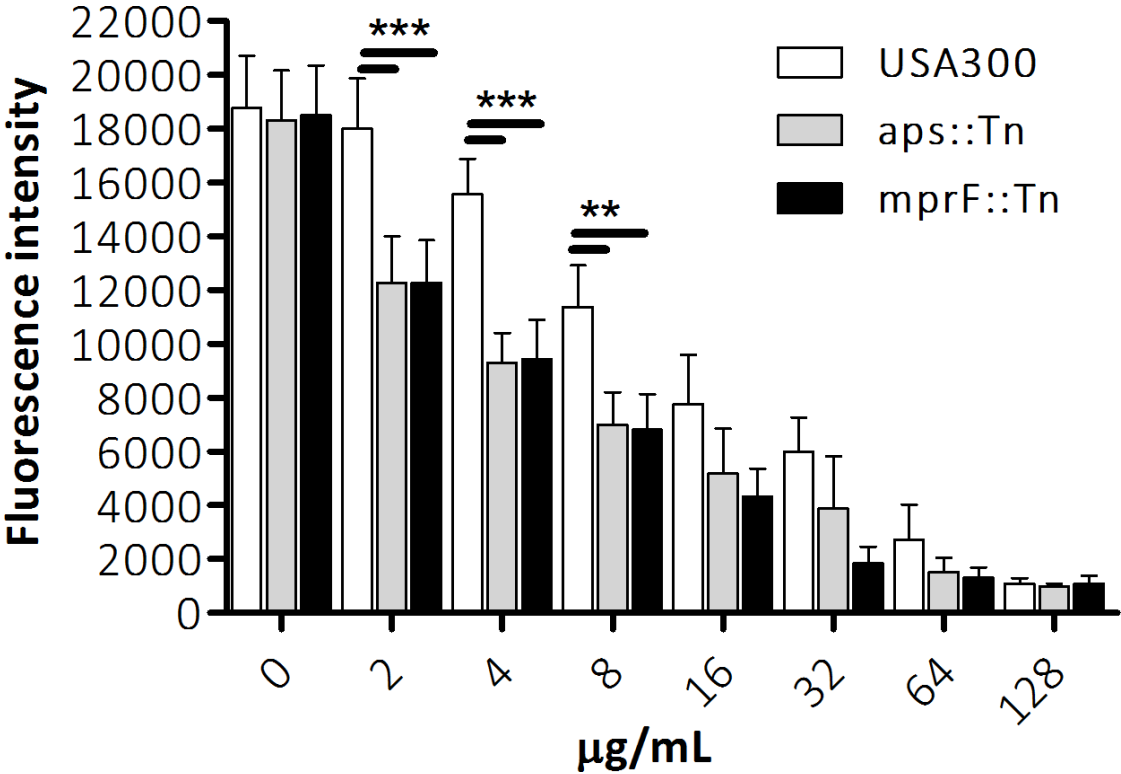
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520 **Figure 2**

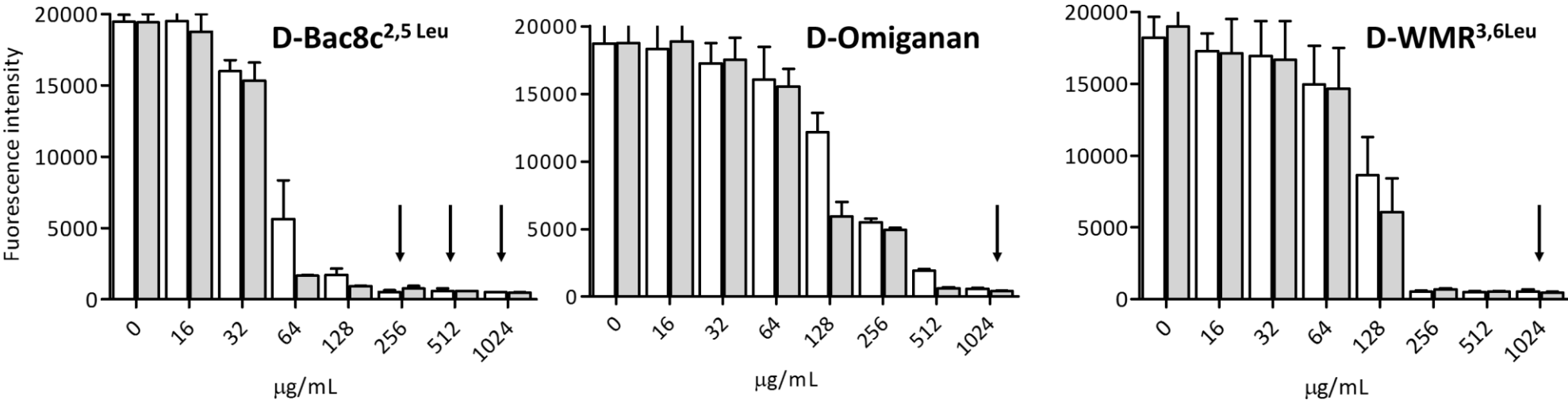


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523 **Figure 3**

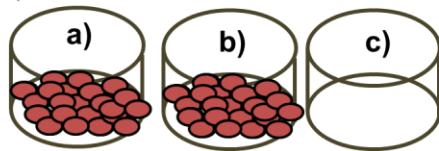
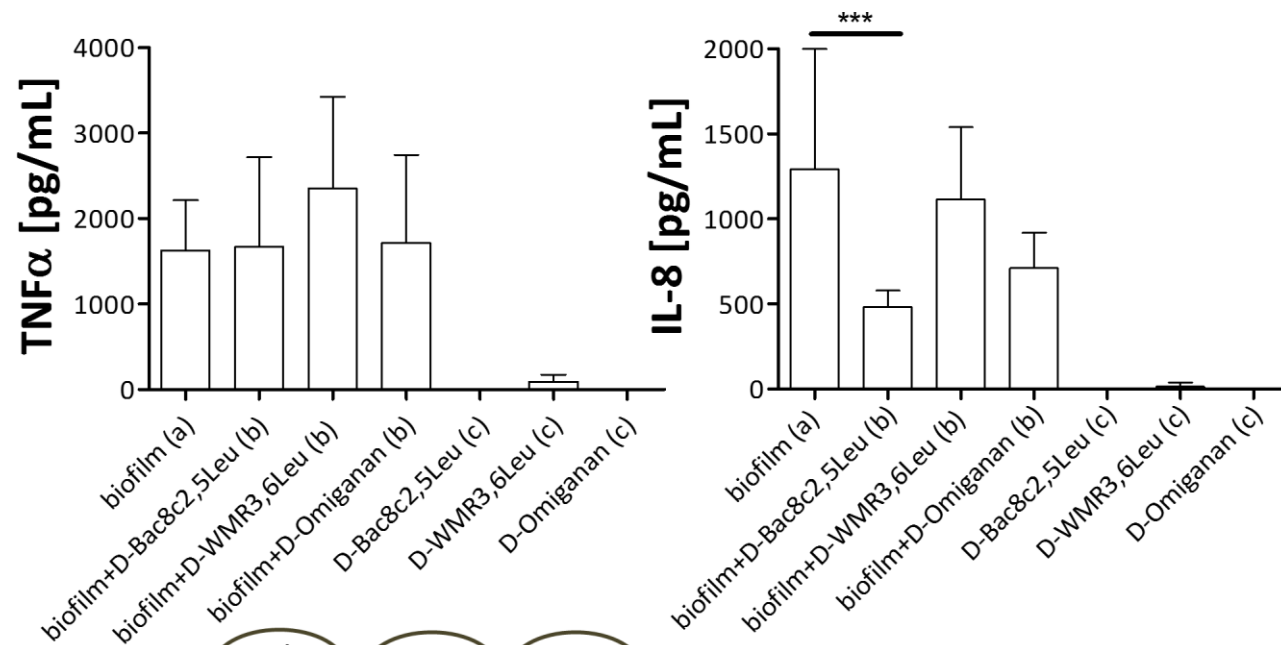
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526 **Figure 4**

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biofilm (24h old)	+	+	-
Bac8c^{2,5Leu} or D-Omiganan or D-WMR (sub-MIC, 2h)	-	+	+
blood , (donors n=1, 2 or 3)	+	+	+

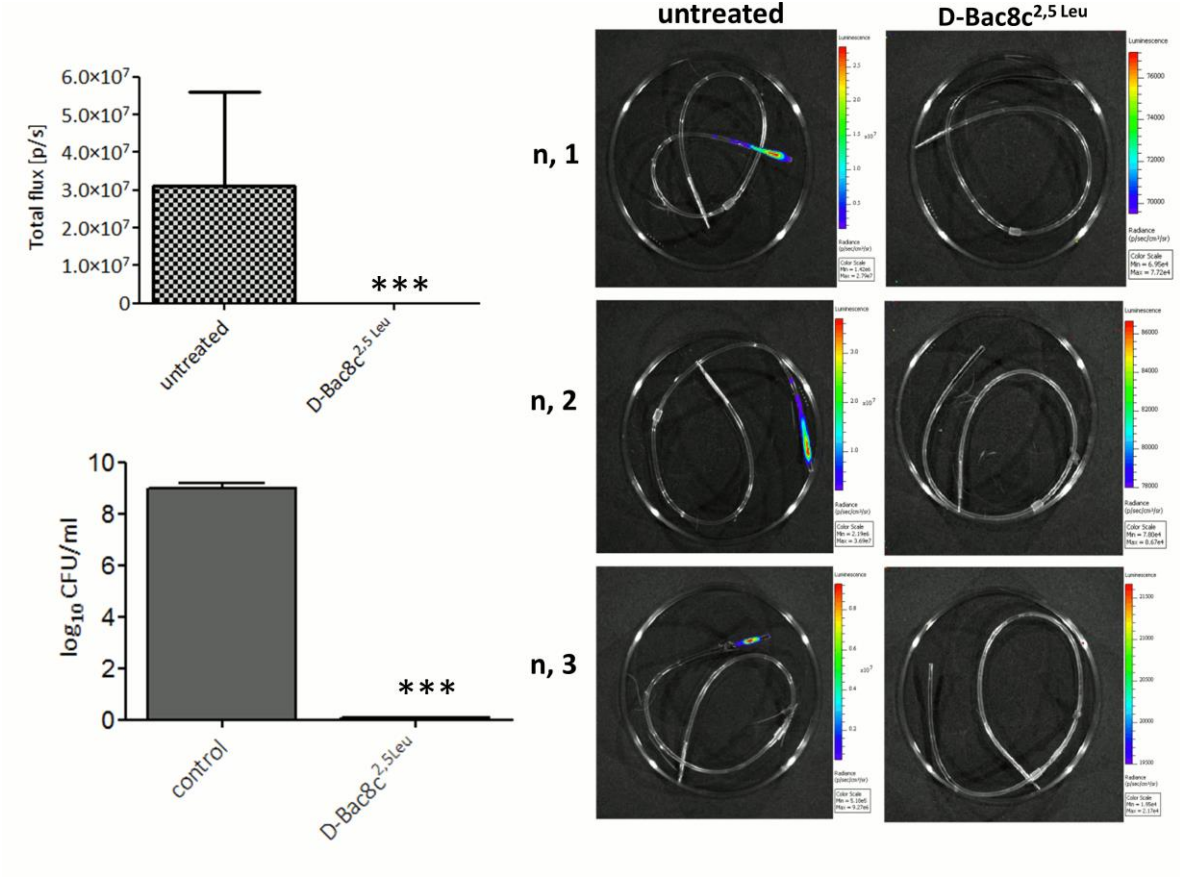
plasma collection

detection (hIL2, hIL4, hIL6, hIL8, hIL10, INFγ, TNFα and human GM-CSF)

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531 **Figure 5**



534 **Table 1**

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AMP	aa sequence (peptide amides)	Average mass (g/mol)	Disulphide bridge	Net charge	MIC µg/mL (µM)
D-Bac8c ^{2,5Leu} [24]	rlwvlwrr	1183.47	-	+4	8 (6.75)
D-HB43 [25]	fakllaklakkll	1455.94	-	+5	4 (2.75)
D-P18 ^{8Leu} [26, 27]	kwklfkklpkflhlakkf	2299.97	-	+8.5	64 (27.8)
D-WMR ^{3,6Leu} [28]	wglrrllkygkrs	1631.99	-	+6	8 (4.9)
D-Omiganan [29]	ilrwpwwpwrk	1821.21	-	+4	8 (4.4)
D-Ranalexin [30]	flgglikivpamicavtkkc	2105.73	14-20	+4	8 (3.8)
L-Polyphemusin [31]	RRWCFRVCYRGFCYRKCR	2457.981	4-17, 8-13	+7	16 (6.5)

536

537 **Table 2**

	IC₅₀ µg/mL (µM)	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.)	n/a	0.4 %
538	haemolysis	414 (350)	77 (4.8)	195 (92.6)	1885 (766.8)	> 4000 (2450)	873 (380.5)	250 (137)	n/a