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Aurein-Derived Antimicrobial Peptides Formulated with Pegylated Phospholipid Micelles to Target Methicillin-Resistant *Staphylococcus aureus* Skin Infections

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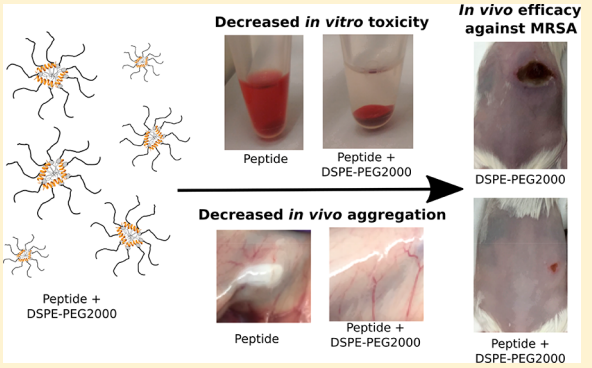
Supporting Information

ABSTRACT: Antimicrobial peptides have been the focus of considerable research; however, issues associated with toxicity and aggregation have the potential to limit clinical applications. Here, a derivative of a truncated version of aurein 2.2 (aurein 2.2Δ3), namely peptide 73, was investigated, along with its D-amino acid counterpart (D-73) and a retro-inverso version (RI-73). A version that incorporated a cysteine residue to the C-terminus (73c) was also generated, as this form is required to covalently attach antimicrobial peptides to polymers (e.g., polyethylene glycol (PEG) or hyperbranched polyglycerol (HPG)). The antimicrobial activity of the 73-derived peptides was enhanced 2- to 8-fold, and all the derivatives eradicated preformed *Staphylococcus aureus* biofilms. Formulation of the peptides with compatible polyethylene glycol (PEG)-modified phospholipid micelles alleviated toxicity toward human cells and reduced aggregation. When evaluated *in vivo*, the unformulated D-enantiomers aggregated when injected under the skin of mice, but micelle encapsulated peptides were well absorbed. Pegylated micelle formulated peptides were investigated for their potential as therapeutic agents for treating high-density infections in a murine cutaneous abscess model. Formulated peptide 73 reduced abscess size by 36% and bacterial loads by 2.2-fold compared to the parent peptide aurein 2.2Δ3. Micelle encapsulated peptides 73c and D-73 exhibited superior activity, further reducing abscess sizes by 85% and 63% and lowering bacterial loads by 510- and 9-fold compared to peptide 73.



KEYWORDS: *biofilm, toxicity, aggregation, MRSA, DSPE-PEG2000, abscess*

A growing concern of modern medicine is the dearth of novel antibiotics.[1] Microorganisms resistant toward multiple classes of antibiotics are steadily increasing.[2−4] Consequently, the treatment of bacterial infections is in substantial jeopardy, and therapeutic treatments are being compromised.[5,6] Alternatives to conventional antibiotics or antibiotic adjuvant strategies are urgently needed. One promising complementary approach uses naturally occurring host defense peptides (HDPs) or synthetic derivatives thereof. HDPs, sometimes referred to as antimicrobial peptides (AMPs) due to their modest activity of killing micro-organisms,[7−11] are produced by most multicellular organisms as a component of the innate immune system.[12] Synthetic peptides based on naturally occurring HDPs offer the potential for the design of novel drug candidates. Key attributes like positive charge, hydrophobicity, and amphipathic structures are linked to the antimicrobial potency of peptide candidates.[13]

Biofilms are ubiquitously found on earth, and bacteria living in a biofilm matrix are often recalcitrant toward antibiotic treatment.[14] Certain peptides have proven effective against biofilms formed by a broad range of multidrug resistant pathogens by inhibiting biofilm formation below their antimicrobial, minimal inhibitory concentration. These can be used to eradicate preformed biofilm structures or used in conjunction with antibiotics to boost antibiofilm activity.[15−17] Other advantages of AMPs include their anti-inflammatory and immunomodulatory activity,[7] neutralization of virulence factors, and slow resistance development.[8,18] Although HDPs possess various activities with the potential to be exploited as therapeutics, none have as-yet been FDA approved due to

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Table 1. Peptide Characteristics and Antimicrobial Activity against *S. aureus* USA300 (All Peptides Amidated at the C-Terminus)

peptide	sequence	molecular weight	net charge	hydrophobicity[a]	MIC (μg/mL)	MIC (μg/mL) in DSPE-PEG2000
aurein 2.2Δ3	GLFDIVKKVVGAL	1614.97	+2	0.617	16	>64
73	RLWDIVRRWVGWL	1755.10	+3	0.815	2	8
73c	RLWDIVRRWVGWLC	1857.24	+3	0.866	8	>64
D-73	rlwdivrrwvGwl	1755.10	+3	n.d.[b]	2	4
RI-73	lwGvwrrvidwlr	1755.10	+3	n.d.	2	8

[a]Calculated from heliquest (http://heliquest.ipmc.cnrs.fr/) with α helix type and full window size. [b]n.d., not determined.

various factors including, likely, their reduced potency under physiological conditions,[19] susceptibility to protease degradation and rapid kidney clearance,[20−22] potential toxicities at biologically relevant concentrations,[23] and tendency to self-associate into aggregates.[24] Many chemical design strategies have been employed to try and mitigate some of these issues including the use of non-natural D-enantiomeric amino acids, incorporation of non-natural amino acids, use of nonpeptidic scaffolds, and head-to-tail cyclization.[25] More recently, the use of various delivery vehicles such as liposomes or micelles has been shown to mitigate peptide toxicity.[26]

Thus, in the design of new synthetic peptides, it is important not only to maintain biological activity but also to address lability, toxicity, and aggregation issues. Recently, we demonstrated that conjugating aurein 2.2 or its derivatives to highly biocompatible branched compact polymers, such as hyperbranched polyglycerol (HPGs), can significantly increase the efficacy of the HDPs while decreasing the toxicity.[8,27,28] Moreover, we have previously used hyaluronic-acid-based nanogels to improve the biosafety of the antibiofilm peptide DJK-5 *in vivo*,[29] as well as hyperbranched polyglycerols to prevent aggregation of the peptide IDR-1018 *in vitro* and *in vivo*.[30] Other nanocarriers such as lipid-based delivery systems[31] or hypromellose ointment[32] have been successfully introduced as new drug vehicles. Pegylated phospholipids, such as 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2000), appear to be promising carriers due to their capability to form stabilized micelles with DSPE as the hydrophobic core and PEG as a hydrophilic assembly around the core.

Cutaneous abscesses represent one of the most common skin conditions that are caused by a bacterial infection and are characterized by the accumulation of pus in the skin that further manifests as a local raised or indurated area on the skin surface.[33] Although both Gram-positive and Gram-negative bacteria can cause abscesses, community-associated methicillin resistant *Staphylococcus aureus* (CA-MRSA) is the most common causative agent.[34] In 2013, approximately 2.7 million people with cellulitis and abscesses were treated in hospital emergency departments in the USA alone.[35] Presently, most abscesses are treated by surgically draining the pus with a single small incision; however, an optimal treatment strategy is still debated. While traditional treatment methods identified that antibiotics do not provide additional benefits, it was recently demonstrated that trimethoprim-sulfamethoxazole administration after drainage resulted in higher cure rates,[36] thereby decreasing recurrent infections. Nevertheless, most antibiotics show poor tissue penetration[37] and even complete drainage together with antibiotic administration might not clear remaining bacteria growing within biofilms.[38] Due to the increasing problem with antibiotic resistant bacteria there is an urgent need for alternatives and antibiotic adjuncts to treat these infections.

Here, we investigated the *in vitro* and *in vivo* activity of aurein 2.2Δ3, a shorter form of the naturally occurring aurein 2.2 peptide, with three residues removed from the C-terminus. The shorter form was previously shown to have the same activity as the natural AMP.[39] Here, we studied the activities of derivative peptide 73, obtained from a screen of 91 peptides,[27] as well as the modified versions 73c (with an additional cysteine at the C-terminus, required for conjugation to HPG, which is not the focus of this study but has been published elsewhere),[27] D-73, and RI-73. Peptide 73 shares an identical sequence to the previously reported peptide 77,[27] but with residues 9 and 10 (Trp and Val) swapped. Overall, our results demonstrate that several factors should be considered when designing synthetic HDPs since enhanced *in vitro* antimicrobial potency alone is likely insufficient to predict potential clinical efficacy of synthetic HDPs.

RESULTS AND DISCUSSION

Aurein 2.2Δ3 Derivates Showed Enhanced Antimicrobial Activity against *S. aureus* USA300. In previous studies, the activity of aurein peptides isolated from Australian southern bell frog *Litoria aurea* skin was examined in terms of their ability to act against planktonic (free swimming) bacteria.[40,41] We identified that a truncated version of aurein 2.2, aurein 2.2Δ3 (last three residues at the C-terminus removed), showed similar antimicrobial potency compared to the parent peptide, and this sequence served as the starting point for further optimization. In a previous peptide array based on aurein 2.2Δ3, several peptides with increased antimicrobial activity against *S. aureus* were identified.[27] One of these improved peptides was peptide 73, which was chosen for further investigation in this study.

Peptide 73 differs from aurein 2.2Δ3 by six residues that were substituted for either Arg or Trp side chains at positions 1 (G → R), 3 (F → W), 7 (K → R), 8 (K → R), 9 (V → W), and 12 (A → W), respectively (Table 1). Although it appears that a significant number of residues were changed, it should be noted that the substitutions are of one hydrophobic residue (e.g., F, V, A) for another (W) and one positively charged residue (K) for another (R). Arginine and tryptophan have been shown to play a critical role in antimicrobial activity for various other HDPs.[42] Overall, these modest substitutions increased the net charge from +2 to +3 and hydrophobicity from 0.6 to 0.8. As net charge and hydrophobicity are also linked to antimicrobial activity,[43] increasing these factors might offer a possible strategy to enhance the activity of peptides. A number of studies have demonstrated that there is a correlation between charge and antimicrobial activity.[44−48] For instance, it has been shown that an increase in the charge of magainin 2 from +3 to +5 improved activity against both Gram-positive

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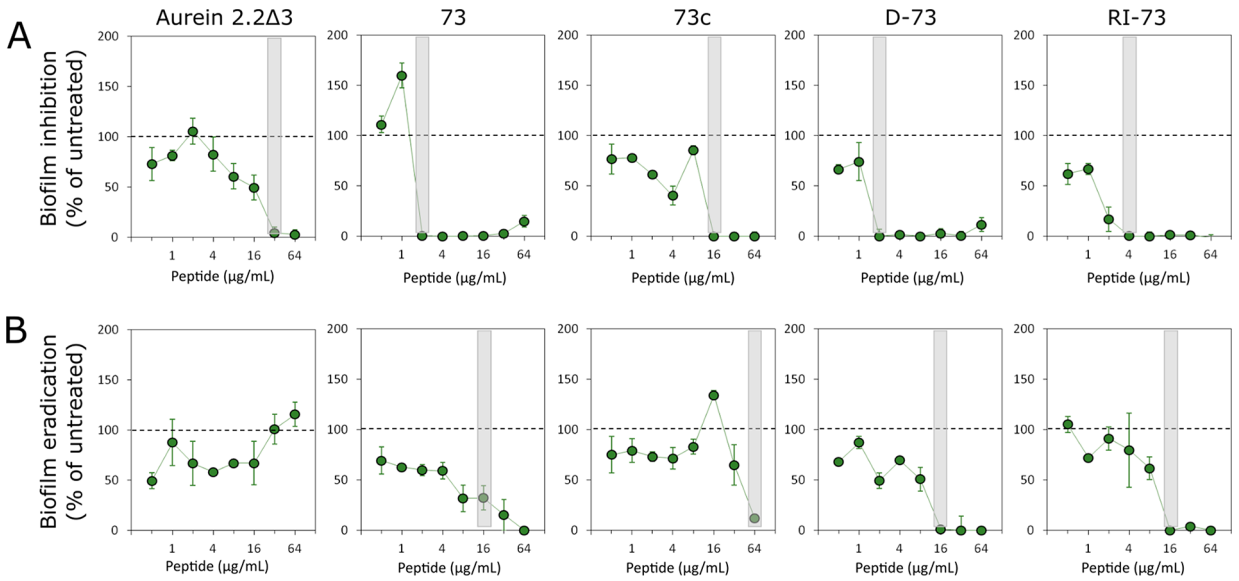

Figure 1. Minimal biofilm inhibitory and eradication concentration of peptides against *S. aureus* LAC USA300-GFP. The peptides aurein 2.2Δ3, 73, 73c, D-73, and RI-73 were dissolved in water and used at concentrations between 0.5 and 64 μg/mL. (A) Peptides were added to the growth medium and biofilm inhibition measured after 24 h. (B) Biofilms were grown for 24 h and subsequently treated with peptides for another 24 h. (A, B) *S. aureus* with constitutive GFP expression was grown in 10% TSB supplemented with 0.1% glucose and biofilms measured and quantified at an excitation wavelength of 488 nm and emission of 530 nm. The percent biofilm inhibition or eradication was based on the amount of biofilm growth without peptides (100%). The data represent the mean with standard deviation as error bars. Experiments were performed three times. The gray bars indicate the required peptide concentration that resulted in growth inhibition of the planktonic cells measured before biofilm quantification.

and Gram-negative bacteria.[45] However, a further increase in charge to +6 or +7 led to increased hemolytic activity and loss of antimicrobial activity.[45] In addition, increasing hydrophobicity can lead to peptide aggregation (self-association), increased toxicity, and/or loss of selectivity.[43,49−51] Finding an optimal balance between these two parameters is crucial.

Since peptide 73 showed increased net charge and hydrophobicity, it prompted us to investigate the antimicrobial activity against a community acquired methicillin resistant *S. aureus* strain USA300. The antimicrobial activity of peptide 73 was compared to its parent peptide aurein 2.2Δ3. Intriguingly, peptide 73 (2 μg/mL) was 8-fold more active than aurein 2.2Δ3 (16 μg/mL; Table 1). The addition of a C-terminal cysteine residue to peptide 73 (peptide 73c) resulted in a 4-fold decreased antimicrobial activity (8 μg/mL) compared to peptide 73 against *S. aureus*, demonstrating that enhanced antimicrobial activity is likely sequence dependent (Table 1). To further modulate the activity of peptide 73, a D-enantiomeric (D-73) and a retro-inverso peptide (RI-73) were synthesized. Both peptides showed similar antimicrobial activity as peptide 73 (MICs of 2 μg/mL), suggesting that the use of D-amino acids or reversing the amino acid sequence of the D-peptide did not affect the antimicrobial activity. Similar observations have been made by Zhao et al. where the D-enantiomer of the cationic polybia-MPI peptide was similar to that of the L-peptide.[52] Indeed, the stereochemistry of many D-peptides has been shown not to affect their antimicrobial activity, presumably because their mechanism of action is not based on a specific membrane receptor.[13] However, there are also examples in the literature where the D-version was less active, e.g., the natural all L-RAWVAWR peptide was more active than the synthetic all rawvawr.[53] Overall, increased hydrophobicity and the Arg- and Trp-rich properties of the peptides might allow them to interact better with the bacterial cell membrane,[42] thereby accounting for the higher activities.

Aurein 2.2Δ3 Derivates Eradicated *S. aureus* USA300 Biofilms. Biofilms are a complex and dense assembly of cells that display novel physiology leading to emergent properties, such as adaptive antibiotic resistance relative to planktonic bacteria.[54,55] A biofilm is a community of bacterial cells encased in a polymeric matrix which protects bacteria from host immune defenses.[56] Biofilms are often associated with recurring and chronic infections.[57] Given the serious complications associated with biofilm-related infections and the fact that it has been previously reported that some HDPs can inhibit and/or eradicate biofilms,[58] we further assessed aurein 2.2Δ3 and 73-derivative peptides for their antibiofilm activity against *S. aureus* biofilms.

To evaluate the potential of the peptides as agents to inhibit *S. aureus* biofilm formation, minimal biofilm inhibitory concentrations (MBICs) were determined. The MBIC of aurein 2.2Δ3 was somewhat similar to the growth inhibitory concentration of 32 μg/mL under biofilm growth conditions. A similar trend was observed for peptide 73 and its derivative 73c and D-73 (MBICs of 2, 16, and 4 μg/mL, respectively). Intriguingly, peptide RI-73 showed enhanced biofilm inhibition at a subinhibitory concentration of 2 μg/mL (Figure 1).

Biofilms tremendously impact natural, clinical, and industrial environments and can form on many biological and non-biological surfaces. Biological surfaces include any human tissue from the skin to the bone, and biofilms can impede the healing processes of acute and chronic wounds.[58] Non-biological surfaces include any kind of medical device ranging from pacemakers to contact lenses.[58] The eradication of biofilms with traditional antibiotics has been a challenge for years, and no compounds have been clinically developed that specifically target bacteria growing in biofilms. Having previously shown that synthetic peptides could be designed to target biofilm cells,[59] we investigated the ability of the aurein 2.2Δ3 and 73-derived peptides to eradicate preformed GFP-

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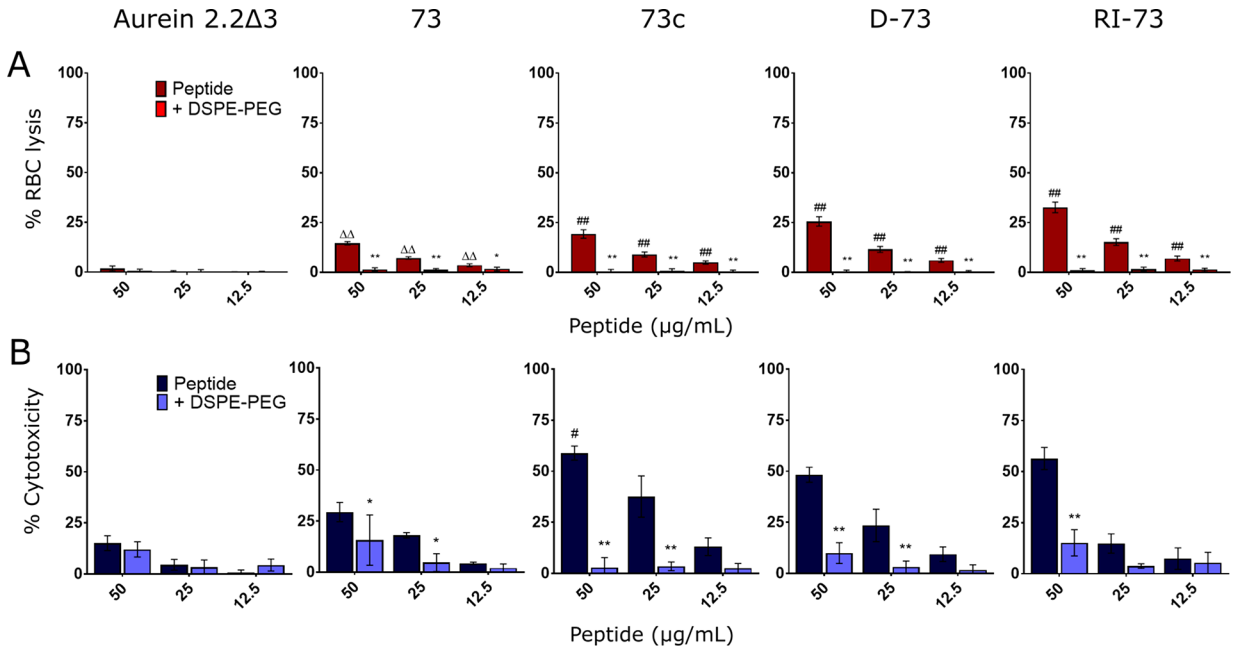

Figure 2. *In vitro* hemolysis and cytotoxicity upon exposure to peptides. The peptides aurein 2.2Δ3, 73, 73c, D-73, and RI-73 were dissolved in water or with DSPE-PEG2000 (10:3 mass ratio) and used at concentrations of 12.5, 25, and 50 μg/mL. (A) Hemolysis was measured after 1 h of incubation of the peptides with red blood cells at 37 °C. (B) Cytotoxicity measured as release of lactate dehydrogenase from peripheral blood mononuclear cells after overnight incubation with peptide at 37 °C. (A, B) Statistical analysis was performed using the Mann−Whitney test for comparison of peptide 73 to aurein 2.2Δ3 and one-way ANOVA, Kruskal−Wallis test with Dunn's correction for comparison of the peptide derivatives to peptide 73. The delta (Δ) indicates a significant difference between peptide 73 and aurein 2.2Δ3. The hashtag (#) indicates a significant difference between the peptide derivatives and peptide 73 at a specific concentration. The asterisk (*) indicates a significant difference between the peptide in water and the peptide + DSPE-PEG2000 (*, #, $p \leq 0.05$; **, ##, ΔΔ, $p \leq 0.01$). The data represent the mean with standard deviation as error bars. Experiments were performed three times.

expressing *S. aureus* biofilms *in vitro*. Aurein 2.2Δ3 showed poor biofilm eradication efficacy, as no reduction in GFP signal was seen at any concentration of peptide evaluated. Peptide 73c also exhibited weak biofilm eradication activity as concentrations >32 μg/mL were required to eradicate >50% of the preformed GFP-expressing *S. aureus* biofilm (Figure 1B). Interestingly, the D-73 and RI-73 peptides both eradicated *S. aureus* biofilms as well as peptide 73 (Figure 1B). Comparable observations have been made against *P. aeruginosa* biofilms by Dean et al.[60] where a D-enantiomer of LL-37 retained its antibiofilm activity and by de la Fuente-Nunez et al.[15] where they showed that D-enantiomer and retro-inverso variants of 10 peptides often either retained or even enhanced the eradication activity.

Aurein 2.2Δ3 Derivatives Increased Red Blood Cell Lysis and Peripheral Blood Mononuclear Cell Cytotoxicity. Host toxicity is one of the major limitations of synthetic HDPs that has prevented advancement of these drugs into clinical trials.[23] It is crucial that peptides lack cytotoxicity toward human cells in order to have a therapeutic potential. Hence, the toxicity of all the aurein 2.2Δ3 derived peptides was assessed by measuring human red blood cell (RBC) lysis and cytotoxicity toward human peripheral blood mononuclear cells (PBMCs).

Aurein 2.2Δ3 did not induce RBC lysis at 50 μg/mL, the highest tested concentration (Figure 2A). Peptide 73, on the other hand, caused significant dose-dependent hemolysis at concentrations between 12.5 and 50 μg/mL (Figure 2A). Moreover, at a concentration of 50 μg/mL, all peptide 73 derivatives also caused significantly higher RBC lysis with hemolysis values ranging from 33% for RI-73, 26% for D-73, and 19% for 73c, compared to 15% for peptide 73 (Figure 2A). An interesting observation was that peptides D-73 and RI-73, which have the same antimicrobial activity against *S. aureus* as peptide 73 (Table 1), both showed increased hemolytic activity (Figure 2A).

A similar toxicity trend was observed using the LDH release assay with PBMCs (Figure 2B). All peptides showed concentration dependent cytotoxicity with aurein 2.2Δ3 causing about 15% LDH release at the highest peptide concentration evaluated. These were followed by 30% cytotoxicity recorded for peptide 73, 50% for D-73, 55% for RI-73, and 60% 73c. The increase in toxicity between aurein 2.2Δ3 and peptide 73 was not significant, and only peptide 73c showed a significant increase in cytotoxicity over peptide 73, suggesting that 73c might have a preference for eukaryotic membranes over prokaryotic cell membranes. Overall peptide hydrophobicity is known to have varying effects on prokaryotic and eukaryotic cells, which might explain why aurein 2.2Δ3 derivatives showed enhanced antimicrobial activity and increased toxicity toward human RBCs and PBMCs. Jiang et al.[61] demonstrated that increased hydrophobicity had tremendous effects on eukaryotic cells (286-fold increased hemolytic activity) compared to prokaryotic cells (3-fold increased antimicrobial activity).

DSPE-PEG2000 Reduced Peptide Aggregation *in Vitro* and *in Vivo*. Not only were aurein-derivative peptides more toxic toward RBCs and PBMCs, they also aggregated in tissue culture conditions when incubated with PBMCs (Figure 3A upper panel, Supporting Information Figure S1). Several synthetic and natural HDPs have been found to have a tendency to aggregate, which may be related to their toxicity

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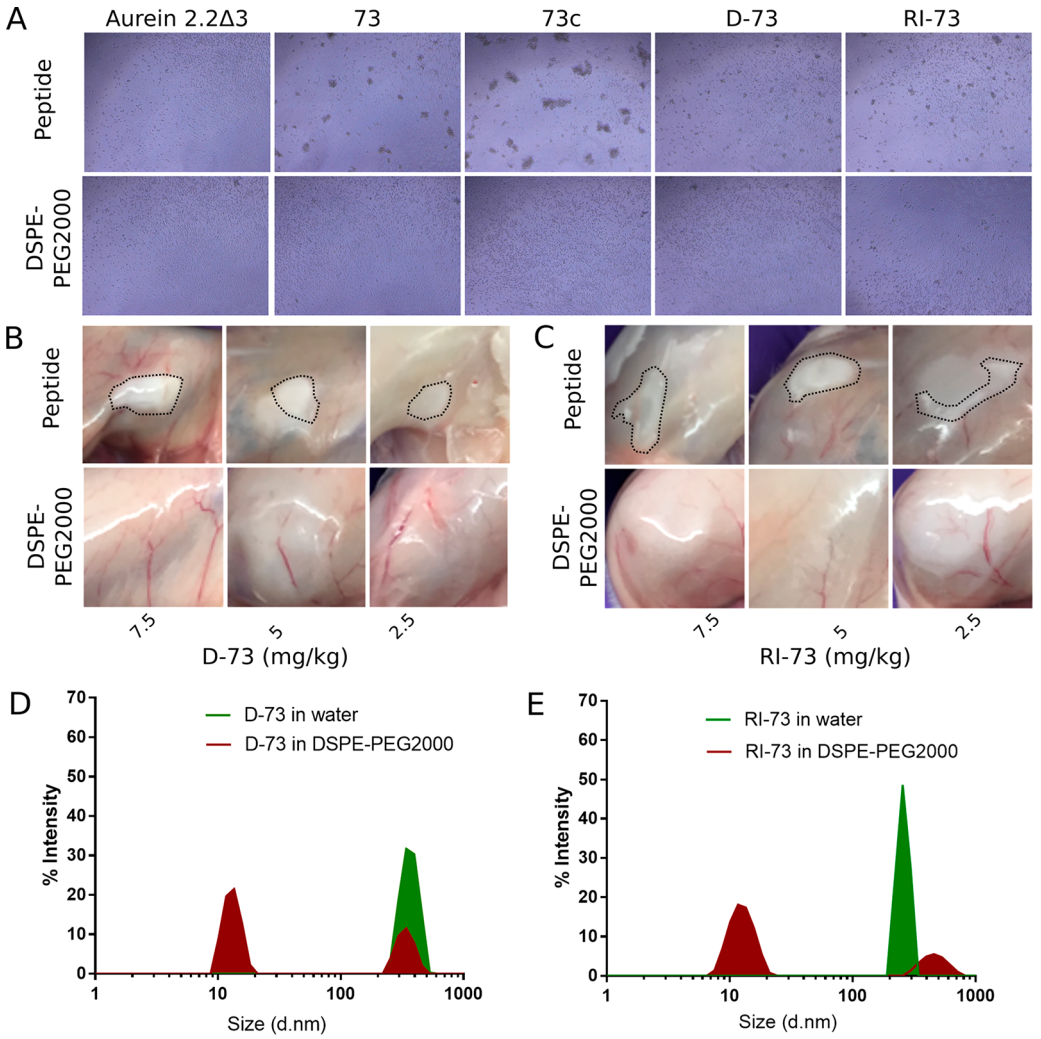

Figure 3. Peptide aggregation in tissue culture medium in the presence of peripheral blood mononuclear cells (PBMCs), precipitation under the skin of 7-week-old CD-1 female mice, and size distribution from dynamic light scattering (DLS). Peptides aurein 2.2Δ3, 73, 73c, D-73, and RI-73 were dissolved either in water or with DSPE-PEG2000 (10:3 mass ratio). (A) PBMCs from healthy donors were exposed to 50 μg/mL peptide overnight and then visualized with a Nikon Eclipse TS100 microscope. Experiments were performed on three individual donors, and representative results from one donor are shown. (B) Peptide D-73 injected under skin of mice. (C) Peptide RI-73 injected under the skin of mice. (D) DLS data of 1 mg/mL peptide D-73. (E) DLS data of 1 mg/mL peptide RI-73. (B, C) Peptide dissolved in water (upper panel) and with added DSPE-PEG2000 (lower panel). 50 μL of peptides at concentrations of 2.5, 5, and 7.5 mg/kg was injected into the right and left sides of the dorsum. The dotted line indicates peptide precipitation. Experiments were performed twice on individual mice. Mice were euthanized 3 days post infection, and the skin flap visually inspected for precipitation (white discoloration). (D, E) Peptide dissolved in water (green) and with added DSPE-PEG2000 (red). Experiments were performed twice.

and other biological functions of these molecules.[24] One strategy to alleviate the toxicity and aggregation tendencies of HDPs is the use of various drug delivery systems, particularly liposomal and micelle-based systems,[62−64] that prevent these negative effects while preserving the biological functions of HDPs. For instance, the encapsulation of the cationic peptide LL-37 within liposomes composed of DSPC/DSPE-PEG/cholesterol ensured enhanced bioactivity and reduced toxicity in cell cultures.[65] Other carriers including polyethylene glycol (PEG)-modified lipids such as DSPE-PEG2000 micelles showed improved survival rates in sepsis mouse models for a cationic antimicrobial decapeptide self-associated with the micelles.[66] Hence, to address peptide toxicity issues, a similar approach was used with a 10:3 mass ratio of DSPE-PEG2000/peptide, and these formulated peptides were further evaluated for toxicity and aggregation tendencies *in vitro* and *in vivo*.

First, we determined the hydrodynamic sizes of all peptides in water as well as with DSPE-PEG2000. All water-dissolved peptides showed a single peak per peptide with varying hydrodynamic sizes ranging from approximately 180 to 480 nm, indicating the formation of large aggregates. Interestingly, the addition of dithiothreitol (DTT) to 73c in water resulted in aggregates that were slightly smaller in size (compared to 73c in water), suggesting that disulfide bond formation may play a role in the peptide−peptide interactions present in the aggregates. All encapsulated peptides displayed a hydrodynamic size of 12−14 nm with an intensity of 55−73%, suggesting that this formulation partner prevented the formation of large peptide aggregates under these conditions. The dynamic light scattering (DLS) data also revealed a second peak around 270−430 nm with an intensity of 27−45%, indicating that some peptide aggregates persisted (Supporting Information Table S1). In a study by Yang et

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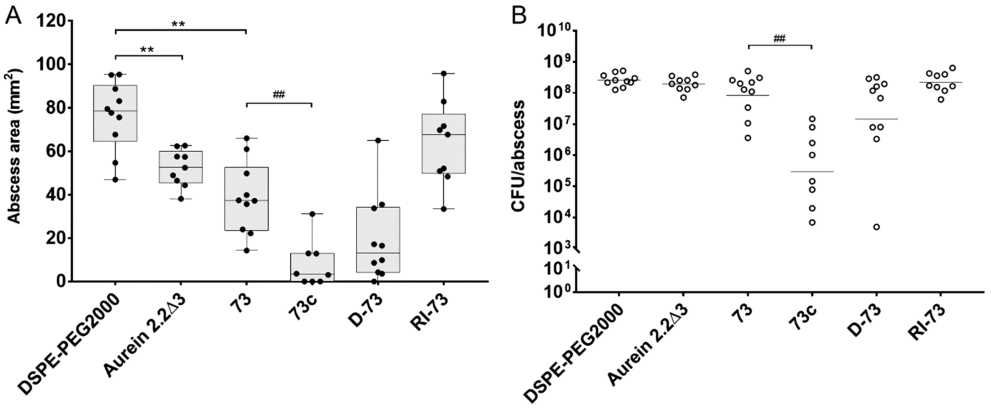

Figure 4. Therapeutic treatment of *S. aureus* mouse cutaneous abscesses with various peptides. CD-1 female mice were subcutaneously infected with 5×10^7 CFU *S. aureus* LAC-USA300 and treated intra-abscess 1 h post infection with 50 μL of either DSPE-PEG2000 (10 mg/mL; control) or peptides (5 mg/kg) dissolved with DSPE-PEG2000 (10 mg/mL; 10:3 mass ratio). Lesion sizes and CFU counts were determined 3 days post infection. (A) Box and whiskers plot of dermonecrosis measurements. (B) CFU counts/abscess with geometric mean. (A, B) All experiments were done at least two times with 3−4 mice/group, $n = 56$ biologically independent animals. Statistical analysis was performed using the Mann−Whitney test to compare aurein 2.2Δ3 or peptide 73 to the DSPE-PEG2000 control or one-way ANOVA, Kruskal−Wallis test with Dunn's correction (two-sided) comparing each peptide derivate to peptide 73. The asterisk (*) indicates significant differences between aurein 2.2Δ3 or peptide 73 to the control. The hashtag (#) indicates a significant difference between peptide derivate and peptide 73 (*, #, $p < 0.05$; **, ##, $p < 0.01$).

al.,[67] it was shown that DSPE-PEG2000 micelles encapsulated cabozantinib, an anticancer drug, with similar hydrodynamic sizes of approximately 11 nm and with approximately 75% of the drug encapsulated.

Promisingly, the DSPE-PEG2000 encapsulated peptides not only decreased aggregation when incubated with human PBMCs (Figure 3A lower panel, Supporting Information Figure S1) but were also less toxic toward RBCs and PBMCs (Figure 2). Compared to the parent peptide aurein 2.2Δ3, which showed no visual aggregation and only minor toxicity when incubated with RBCs or PBMCs, none of the encapsulated peptides caused hemolysis, and all derivatives exhibited significantly decreased cytotoxicity toward PBMCs. These results clearly demonstrated the utility of DSPE-PEG2000 micelles as a delivery vehicle for aurein-derived peptides to prevent toxicity and increase peptide solubility. However, this decreased aggregation and toxicity profile was also coupled with a reduction in the antimicrobial activity for DSPE-PEG2000 formulated peptides (Table 1). The parent peptide aurein 2.2Δ3 and the C-terminus cysteine version 73c completely lost their *in vitro* antimicrobial activity (MIC > 64 μg/mL), while peptide 73 and RI-73 showed a 4-fold decrease. Intriguingly, the D-enantiomer D-73 showed only a 2-fold decrease. This was a promising observation indicating that the DSPE-PEG2000 micelles could be used as a potential delivery option for peptides.[68]

To further investigate the aggregation tendency and therapeutic potential of the peptides under physiological conditions, we tested them for skin toxicity in mice. Although we observed aggregation in tissue culture medium with PBMCs with all peptides (Figure 3A), only the D-73 and RI-73 peptides precipitated following injection under the skin of mice at concentrations from 2.5 to 7.5 mg/kg (Figure 3B and C, upper panel). Intriguingly, when the same peptides were prepared in the presence of DSPE-PEG2000 and administered to mice, no obvious precipitation was visible (Figure 3B and C, lower panel). None of the L-peptides showed precipitation when dissolved either in water or DSPE-PEG2000 when injected under the skin at a concentration of 5 mg/kg (data not shown).

Peptides 73c and D-73 Showed Enhanced Efficacy in a Cutaneous Mouse Infection Model. We have previously shown that *in vitro* biofilms and *in vivo* abscesses caused by MRSA could be targeted by the antibiofilm peptide DJK-5.[69] Hence, to further investigate the therapeutic potential of the peptides in this study, we used a *S. aureus* skin abscess mouse model to treat a high bacterial density infection with a single subcutaneous drug administration. The efficacy of each peptide was evaluated based on tissue dermonecrosis and bacterial burden in the abscess after 3 days.

Initially, water/saline-dissolved peptide 73 was assessed for its *in vivo* activity in comparison to a saline control. Treatment with peptide 73 significantly reduced the abscess sizes by ∼68% and the bacterial load by 8.9-fold (Supporting Information Figure S2). However, since we encountered solubility issues with peptides 73c, D-73, and RI-73 when dissolved in water/saline, all subsequent experiments were performed with peptides in DSPE-PEG2000 (10:3 mass ratio). Peptide 73, when dissolved with DSPE-PEG2000, still decreased the abscess sizes by approximately 49% and reduced the bacterial load by 2.9-fold, indicating that the DSPE-PEG2000 delivery was suitable with this *in vivo* model.

S. aureus infected mice treated with the control DSPE-PEG2000 showed an average of ∼76 mm^2 dermonecrosis, while the infections treated with aurein 2.2Δ3 significantly reduced the abscess sizes to ∼52 mm^2. Peptide 73 showed a further reduction over aurein 2.2Δ3 to ∼39 mm^2. Intriguingly, in comparison to peptide 73, peptides 73c and D-73 reduced nearly all tissue necrosis, while RI-73 was only as good as aurein 2.2Δ3 (Figure 4A). When comparing to DSPE-PEG2000, peptide 73 reduced abscess sizes by more than 50%, and D-73 as well as 73c showed over 80% reduction. In terms of bacterial burden, mice treated with either aurein 2.2Δ3 or RI-73 had no impact on reducing bacteria from the infection site, while peptide 73 showed a 2.2-fold reduction over aurein 2.2Δ3. Promisingly, treatment with peptide D-73 reduced the bacterial load by 9-fold, and more strikingly, peptide 73c significantly reduced bacterial loads by 510-fold when compared to peptide 73 (Figure 4B). When comparing

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to DSPE-PEG2000, peptide 73 reduced bacterial loads by 2.9-fold, D-73 by 11.9-fold, and 73c by 680-fold.

Comparing the novel findings from this *in vivo* study with our previous published data on the D-peptide DJK-5 against high-density MRSA infections,[69] peptide 73, which is comprised of L-amino acids and thus not protected from host proteases, showed a similar trend by reducing abscesses' sizes by approximately 50% and bacterial loads by more than 10-fold. Intriguingly, the activity of peptide 73 was further enhanced by the D-amino acid version or with the simple addition of a C-terminal cysteine residue. It will be interesting to see in the future whether the addition of a cysteine to a D-enantiomer would even further enhance the peptide activity in an *in vivo* model. Moreover, the broad-spectrum activity of these peptides against other pathogens also remains to be tested.

Peptide Design Paradigms. Determining the MIC is one of the most prominent parameters used in the screening of novel antibiotics. Although it offers a good starting point, the drug with the best MIC does not always guarantee that it will be effective under physiological conditions. Furthermore, *in vivo* differences in activity between peptides might be indirectly correlated with their *in vitro* toxicity as well as antimicrobial activity. As such, peptide 73c when encapsulated in DSPE-PEG2000 lost its antimicrobial activity as well as toxicity *in vitro* but showed superior activity *in vivo*, indicating that *in vitro* experiments are not necessarily predicting *in vivo* outcomes, as previously discussed.[70] This is an important consideration since many drugs are not even considered for *in vivo* investigations if they fail to show *in vitro* activity under lab conditions. Therefore, it is important to develop *in vitro* screening methods that better resemble physiologically relevant conditions. In addition, as previously suggested,[71] it is also crucial to screen *in vitro* toxicity as this property of HDPs appears to be linked to activity *in vivo*.

Further evidence of this discrepancy between *in vitro* and *in vivo* assays was observed in our study since peptide 73 and RI-73 showed the same MIC *in vitro* when encapsulated in DSPE-PEG2000, and similar cytotoxicity toward human PBMCs, but *in vivo*, the retro-inverso peptide RI-73 proved to be inactive. This was interesting since peptide 73 would presumably be susceptible to degradation by host proteases, although this might be mitigated by formulation. As such, encapsulated peptides 73, D-73, and RI-73 showed less than 16% toxicity toward PBMCs (Figure 2B at 50 μg/mL), while there was almost no cytotoxicity with encapsulated peptide 73c, which might correlate with the even better activity of peptide 73c *in vivo*.

Overall, our data indicated that a fine balance is needed in terms of the *in vitro* toxicity, chosen formulation, and antimicrobial activity for optimum *in vivo* activity. In this context, the choice of an appropriate delivery vehicle could significantly influence the activity of novel drugs and should therefore be considered in the design of novel peptides.

METHODS

Chemicals Used in This Study. All chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON) and used without further purification. The dialysis membrane was obtained from Spectra/Por Biotech (Rancho Dominguez, CA). DSPE-PEG2000 (1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-methoxy-poly[ethylene glycol 2000]) was purchased from Avanti Polar Lipids (Alabaster, AL).

Peptide Synthesis, Purification, and Stock Solution Preparation. Solid phase peptide synthesis (Fmoc) was carried out using a solid phase peptide synthesizer from CS Bio Co. (Menlo Park, CA, US) to make aurein 2.2Δ3, as well as arginine (R) and tryptophan (W) substitution peptides 73 and 73c (Table 1), using a previously described protocol.[27,72] Briefly, the first residue at the C-terminus was double-coupled for 24 h to the Rink resin to produce C-terminally amidated peptides; the last four residues were double coupled for 4 h. The crude peptides were purified by preparative reverse phase HPLC on a Waters 600 system (Mississauga, Canada) monitored using a UV detector (229 nm) and a Phenomenex (Torrance, USA) C4 preparative column (20.0 μm, 2.1 cm × 25.0 cm). The mobile phase was composed of two buffers (I and II) with gradient flow. Buffer I consisted of 90% ddH_2O, 10% acetonitrile, and 0.1% TFA, and buffer II was made up of 10% ddH_2O, 90% acetonitrile, and 0.1% TFA. The peptides were purified to >95% purity, and the identities of the peptides were confirmed by MALDI-time-of-flight mass spectrometry. Peptide D-73 and RI-73 were purchased from CanPeptide (Montréal, Canada).

Peptides were solubilized at a stock concentration of 10 mg/mL either in water or with DSPE-PEG2000 (10:3 mass ratio).

Bacterial Strains and Growth Conditions. Bacterial strains used in this study were the *S. aureus* LAC (USA300) strain[73] and its GFP variant expressing the USA300 LAC-GFP derivative.[74] Organisms were cultured in either LB, double Yeast Tryptone (dYT), or tryptic soy broth (TSB) supplemented with 0.1% glucose (w/v), with shaking at 250 rpm, at 37 °C. Bacterial growth was monitored using a spectrophotometer at the optical density of 600 nm (OD_{600}).

Antimicrobial Activity Assay. The minimum inhibitory concentration (MIC) of the peptides against *S. aureus* LAC-USA300 was measured based on a previously described methodology.[75]

Biofilm Susceptibility Assay. Both the biofilm inhibitory capacity as well as the ability to eradicate preformed *S. aureus* biofilms were evaluated in a static microtiter plate assay as previously described.[76] For all experiments, 96-well Costar polypropylene plates (Corning) were used. The antibiofilm activity was evaluated for peptides aurein 2.2Δ3, 73, 73c, D-73, and RI-73 (each dissolved in water) at concentrations of 64, 32, 16, 8, 4, 2, 1, and 0.5 μg/mL. An overnight culture of USA300 LAC-GFP was diluted to an OD = 0.01 in tryptic soy broth (TSB) supplemented with 0.1% glucose.

For inhibitory experiments, 90 μL of the diluted overnight culture was added to each well in the plate containing 10 μL of either peptide or water control. After overnight growth under static conditions, planktonic cells were removed, adhered biomass was rinsed three times with distilled water, and subsequently the remaining adhered biomass was resuspended in 150 μL of 10% LB/90% PBS (v/v). Total biofilm mass was quantified by measuring the fluorescence (GFP, λ_{ex} = 488 nm, λ_{em} = 530 nm) using the Synergy H1 microtiter plate reader (BioTek). The percent biofilm inhibition was calculated in relation to the amount of biofilm grown in the absence of peptide (defined as 100%) and the media sterility control (defined as 0% growth). Results from three separate biological replicates were averaged.

For eradication experiments, 100 μL of the diluted overnight culture was added to each well. After another 24 h of growth under static conditions, planktonic cells were removed, adhered biomass was washed 3 times with sterile TSB, and

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180 μL of TSB followed by 20 μL of peptide or water control was added to each well. The plates were again incubated overnight under static conditions, and subsequently biofilm mass was quantified as described above.

Treatment of RBCs and Hemolysis Assay. Blood was donated by unmedicated healthy individuals and collected into a 3.8% sodium citrated tube with a 9:1 (blood/coagulant) ratio or serum tube at the Centre for Blood Research, University of British Columbia. Red blood cells (RBC) for lysis studies were prepared by washing packed RBC with phosphate-buffered saline (PBS; three times) to yield 80% hematocrit. The red blood cell lysis profile of the peptides was measured based on a previously described methodology.[28] Ten microliters of each of the stock peptide concentrations either in PBS or DSPE-PEG2000 (10:3 DSPE-PEG2000/peptide mass ratio) was mixed with 90 μL of 10% hematocrit and incubated for 1 h at 37 °C. One-hundred percent lysis of red blood cells was defined by the lysis caused by dH_2O, and PBS acted as the normal control (minimal lysis). The Drabkin method was used to measure the percent of red blood cell lysis. Five microliters of the incubated blood cells/peptide solution was added to 1 mL of Drabkin's solution. After centrifugation of the incubated samples, 50 μL of the supernatant was also subjected to 1 mL of Drabkin's solution. The absorbance was measured at 540 nm. The percent of red blood cell lysis in the sample was the adjusted absorbance of the supernatant (hemoglobin released) divided by the adjusted absorbance of the blood/peptide solution (total hemoglobin).[77]

PBMC Isolation and LDH-Release Assay. Donated human blood was collected from healthy, consenting volunteers in accordance with the University of British Columbia ethics guidelines. Written consent was obtained from all blood donors, and the samples were subsequently anonymized. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy donors as described previously.[78] Cells were seeded to a final cell density of 1×10^6 cells/mL in a 96-well flat bottom tissue culture treated plate (Corning). Peptides were added to PBMCs at concentrations of 50, 25, and 12.5 μg/mL (total volume per well was 100 μL). Treated PBMCs were incubated overnight at 37 °C in a 5% CO_2 humidified atmosphere, and the supernatants were collected in fresh 96-well plates following centrifugation at 1150 rpm for 5 min. Sample supernatants were used immediately in the lactate dehydrogenase (LDH) assay to determine peptide toxicity. The cytotoxicity detection kit (Roche) was used according to the manufacturer's instructions. Aggregation was visually inspected under the microscope at 10× magnification for the 50 μg/mL peptide treatment conditions using a Nikon Eclipse TS100 microscope (Tokyo, Japan).

Dynamic Light Scattering (DLS) Size Measurement of the Peptide Aggregates. The average hydrodynamic radius was measured using the Zetasizer Nano ZS (Malvern Panalytical, Montreal, CA). Samples for DLS measurement were filtered using a 0.22 μm membrane and repeated three times, with 15 subruns at 25 °C. Peptides (1 mg/mL) were either dissolved in water or with DSPE-PEG2000 (10:3 DSPE-PEG2000/peptide mass ratio).

Animal Ethics Statement. Animal experiments were performed in accordance with The Canadian Council on Animal Care (CCAC) guidelines and were approved by the University of British Columbia Animal Care Committee (certificate number A14−0363). Mice used in this study were female outbred CD-1. All animals were purchased from Charles River Laboratories (Wilmington, MA), were 7 weeks of age, and weighed about 25 ± 3 g at the time of the experiments. One to three percent isoflurane was used to anesthetize the mice. Mice were euthanized with carbon dioxide.

Peptide Skin Toxicity and Cutaneous Mouse Infection Model. The peptides aurein 2.2Δ3, 73, 73c, D-73, and RI-73 were tested for skin toxicity at a concentration of 2.5, 5, and 7.5 mg/kg prior to efficacy testing. Therefore, 50 μL of the peptide solution (either dissolved in water/saline or with DSPE-PEG2000 [10:3 mass ratio]) was injected underneath the skin layer of skeletal muscle on the right or left side of the dorsum on the back of CD-1 female mice. Superficial skin integrity was visually inspected every day. Three days post injection, the region around the injection was cut and flipped over for further inspection. For the subsequent infection model, we only used peptide concentrations that did not show any visual precipitation or inflammation.

The cutaneous mouse abscess infection model was performed as described earlier.[69,79] Briefly, *S. aureus* LAC was grown to an OD_{600} of 1.0 in dYT broth and subsequently washed twice with sterile PBS and adjusted to 5×10^7 CFU/mL. The fur on the backs of the mice was removed by shaving and application of chemical depilatories. A 50 μL bacterial suspension was injected into the right side of the dorsum. Peptides or saline (50 μL) were directly injected subcutaneously into the infected area (intra-abscess injection) at 1 h post infection. The progression of the disease/infection was monitored daily and abscess lesion sizes (visible dermonecrosis) on day three measured using a caliper. Swelling/inflammation was not considered in the measurements. Skin abscesses were excised (including all accumulated pus), homogenized in sterile PBS using a Mini-Beadbeater-96 (Biospec products) for 5 min, and bacterial counts determined by serial dilution. Experiments were performed at least two times independently with three to four animals per group.

Statistical Analysis. Statistical evaluations were performed using GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA). *P*-values were calculated using the Whitney-Mann test or one-way ANOVA, Kruskal−Wallis multiple-comparison test followed by the Dunn procedure. Data were considered significant when *p*-values were below 0.05 or 0.01 as indicated.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsinfecdis.8b00319.

Table listing the hydrodynamic sizes found for the various peptides used in this study, as well as formulated versions (Table S1); Figure S1 showing peptide aggregation in tissue culture medium with peripheral blood mononuclear cells (PBMCs) from two donors; Figure S2 illustrating the results obtained upon infection and therapeutic treatment of mouse cutaneous abscesses with peptide 73 (in saline) (PDF)

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Notes

The authors declare no competing financial interest.
The raw and processed data required to reproduce the findings presented herein are available upon request by emailing the corresponding author.

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