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ANTIMICROBIALS

The antimicrobial peptide SAAP-148 combats drug-resistant bacteria and biofilms

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Development of novel antimicrobial agents is a top priority in the fight against multidrug-resistant (MDR) and persistent bacteria. We developed a panel of synthetic antimicrobial and antibiofilm peptides (SAAPs) with enhanced antimicrobial activities compared to the parent peptide, human antimicrobial peptide LL-37. Our lead peptide SAAP-148 was more efficient in killing bacteria under physiological conditions in vitro than many known preclinical- and clinical-phase antimicrobial peptides. SAAP-148 killed MDR pathogens without inducing resistance, prevented biofilm formation, and eliminated established biofilms and persister cells. A single 4-hour treatment with hypromellose ointment containing SAAP-148 completely eradicated acute and established, biofilm-associated infections with methicillin-resistant *Staphylococcus aureus* and MDR *Acinetobacter baumannii* from wounded ex vivo human skin and murine skin in vivo. Together, these data demonstrate that SAAP-148 is a promising drug candidate in the battle against antibiotic-resistant bacteria that pose a great threat to human health.

INTRODUCTION

The escalating crisis of multidrug resistance is raising fears of untreatable infections killing substantial numbers of patients (*1*, *2*). Such nosocomial infections are often caused by multidrug-resistant (MDR) strains belonging to the so-called ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) panel (*3*). The ability of these bacteria to form biofilms, aggregates of bacteria embedded within a self-produced matrix of extracellular substances (*4*), further complicates treatment because biofilm-encased bacteria can be 10 to 1000 times more tolerant to conventional antibiotics than their planktonic counterparts (*5*). Moreover, persisters, a minor subpopulation of bacteria in a dormant and drug-tolerant state, are largely responsible for the recalcitrance and recurrence of biofilm-associated infections (*6*).

Synthetic peptides based on the sequences of naturally occurring antimicrobial peptides are considered promising treatment options to combat infections by bacteria not effectively treatable with conventional antibiotics. Antimicrobial peptides (AMPs) are generally small, cationic peptides that exhibit a broad range of antimicrobial and immunological properties. LL-37 is one of the principal human AMPs that play important roles in the defense against local and systemic infections (*7*). This peptide displays direct bactericidal activities against Gram-positive and Gram-negative bacteria by disrupting the bacterial membrane in a phospholipid-dependent fashion (*8*, *9*). A synthetic derivative of LL-37, designated OP-145 or P60.4Ac, which includes the core antimicrobial region of LL-37 (*10*), has improved antimicrobial and similar endotoxin-neutralizing activities as LL-37 (*11*). We recently showed that OP-145, when incorporated in a biodegradable implant coating, can prevent *S. aureus*–induced biomaterial-associated infection in rabbits (*12*). Moreover, this 24–amino acid peptide was successfully used in the treatment of chronic otitis media in a clinical phase 2 trial (*13*).

Apart from broad bactericidal and antibiofilm activities, desired characteristics of a novel systemic and/or topical antimicrobial agent include stability of the antimicrobial activity in situ, for example, in the presence of human plasma, wound fluid, or urine. Because OP-145 has reduced antimicrobial activity in these biological fluids (*12*), we aimed to develop novel synthetic peptides with improved antimicrobial and antibiofilm activities (SAAPs) under physiological conditions. For this purpose, we synthesized a set of LL-37–inspired peptides, assessed their antimicrobial activities under physiological conditions, and compared these to the activities of promising preclinical- and clinical-phase peptides. The lead peptide of this set, SAAP-148, was tested against MDR pathogens, biofilms, and persisters. In addition, its efficacy and safety were determined after topical application on ex vivo and in vivo wound infection models.

RESULTS

Screening of LL-37–inspired peptides reveals lead peptide with improved bactericidal activity

Using amino acid substitutions enhancing cationicity and improving helicity of the C-terminal part of LL-37 that is responsible for its functional activities, we designed 25 peptide analogs, P139 to P163, based on the human AMP LL-37 (Table 1). Amino acid substitutions were made randomly in the 24–amino acid sequence derived from the C-terminal part of LL-37 while keeping the computer-predicted α-helical structure intact and increasing the hydrophobic region. In all peptides, the anionic glutamine (Q) residue present in LL-37 was exchanged by cationic amino acids [arginine (R) or lysine (K)], resulting in an increased net cationic charge. We screened this set of peptides for their activity against *S. aureus* in phosphate-buffered saline (PBS) with and without 50%

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Table 1. Screening of LL-37–derived peptides reveals peptides with improved bactericidal activity against *S. aureus*. Bactericidal activity in phosphate-buffered saline (PBS) and in PBS with 50% human plasma. Results are expressed as the 99.9% lethal concentration ($LC_{99.9}$), that is, the lowest peptide concentration that resulted in ≥99.9% killing of *S. aureus* JAR060131. Results are medians (and ranges) of three to four independent experiments. If no range is indicated, then the $LC_{99.9}$ was identical in all experiments. The most effective synthetic antimicrobial and antibiofilm peptides (SAAPs) are depicted in bold.

Peptide	Sequence	$LC_{99.9}$ (μM)			
		PBS		50% plasma	
LL-37	L L G D F F R K S K E K I G K E F K R I V Q R I K D F L R N L V P R T E S	**1.6**	(1.6–6.4)	**>204.8**	
P139	L K K L W K R V F R I W K R I F R Y L K R P V R	**1.6**	(0.8–1.6)	**51.2**	
P140	L R R L W K R L V R I I K R I Y R Q L K R P V R	**1.6**		**38.4**	(25.6–51.2)
P141	L R R L Y K R V F R L L K R W W R Y L K R P V R	**1.6**	(0.8–1.6)	**38.4**	(25.6–51.2)
P142	L R R L W K R L V K I L K R W F R Y L R R P V R	**1.6**	(0.8–1.6)	**51.2**	(51.2–102.4)
P143	L R R L Y K R V V K L W K R L F R Q L R R P V R	**1.6**	(1.6–3.2)	**51.2**	(51.2–102.4)
P144	L K K L Y K R V A K I W K R W I R Y L K K P V R	**1.6**		**38.4**	(25.6–51.2)
P145	**(SAAP-145) L K R L Y K R L A K L I K R L Y R Y L K K P V R**	**1.6**	**(0.8–1.6)**	**12.8**	**(12.8–25.6)**
P146	L K K L Y K R L F K I L K R I L R Y L R K P V R	**1.2**	(0.8–1.6)	**51.2**	(25.6–51.2)
P147	L K K L W K R L A R L L K R F I R Q L R R P V R	**1.6**		**51.2**	(25.6–51.2)
P148	**(SAAP-148) L K R V W K R V F K L L K R Y W R Q L K K P V R**	**1.6**		**12.8**	**(12.8–25.6)**
P149	L K K V Y K R L A R L L K R Y I R Y L R R P V R	**1.6**		**25.6**	(25.6–51.2)
P150	L K K V W K R V A R L I K R W F R Y L R R P V R	**1.6**		**25.6**	(25.6–51.2)
P151	L K K L Y K R L F K L W K R L Y R Y L K K P V R	**1.6**		**25.6**	(25.6–51.2)
P152	L R R V Y K R L A R L I K R Y L R Q L K K P V R	**1.6**	(1.6–3.2)	**25.6**	
P153	L R K L W K R V V K I W K R Y L R Q L R R P V R	**1.6**		**19.2**	(12.8–25.6)
P154	L R K L W K R L A K I I K R L Y R Y L R R P V R	**1.6**	(0.8–1.6)	**25.6**	(12.8–25.6)
P155	L K K V Y K R V A R L I K R L F R Y L K R P V R	**1.6**		**25.6**	(12.8–25.6)
P156	L R R L W K R L V K L W K R F F R Y L K K P V R	**1.6**		**51.2**	(51.2–102.4)
P157	L K K V W K R V F R I L K R F L R Y L K R P V R	**1.6**	(0.8–1.6)	**51.2**	(25.6–51.2)
P158	L R R V Y K R L F R L W K R I I R Q L R R P V R	**1.6**		**25.6**	(12.8–25.6)
P159	**(SAAP-159) L K R L Y K R V F R L L K R Y Y R Q L R R P V R**	**1.6**	**(1.6–3.2)**	**12.8**	
P160	L K K L W K R L A R L W K R I I R Q L K K P V R	**1.6**	(1.6–3.2)	**51.2**	(25.6–51.2)
P161	L R R V W K R V A R I I K R L Y R Y L K R P V R	**1.6**	(1.6–3.2)	**19.2**	(12.8–25.6)
P162	L K R L W K R L F K I L K R Y Y R Y L R R P V R	**1.6**		**25.6**	(25.6–51.2)
P163	L R R L W K R V F K I I K R L F R Q L K K P V R	**1.6**	(0.8–1.6)	**19.2**	(12.8–25.6)
P276	**(SAAP-276) L K R V W K A V F K L L K R Y W R Q L K K P V R**	**0.8**		**6.4**	**(6.4–12.8)**

pooled human plasma. The antimicrobial activity of these peptides in PBS was similar to that of LL-37, killing ≥99.9% of bacteria [99.9% lethal concentration ($LC_{99.9}$)] at 0.8 to 1.6 μM (Table 1). However, the activity of the peptides in the presence of 50% human plasma was markedly higher than that of LL-37 (Table 1). P145, P148, and P159 showed the highest activity, killing *S. aureus* in plasma at concentrations of 12.8 μM (Table 1). Alanine scanning of these three peptides did not indicate specific amino acids responsible for their improved activity (fig. S1). The peptides with the highest activity, P145, P148, P159, and P276, the latter being derived from the alanine scan of P148, were named SAAP-145, SAAP-148, SAAP-159, and SAAP-276.

In contrast to LL-37 (*14*), these SAAPs were not rapidly degraded by plasma proteases: Preincubation of SAAP-145, SAAP-148, SAAP-159, and SAAP-276 in pooled, not heat-inactivated human plasma, for up to 6 hours before their use to treat *S. aureus* did not affect their antimicrobial activity (fig. S2A). Twenty-four hours of preincubation resulted in only a twofold (for SAAP-148 and SAAP-276) or fourfold (for SAAP-145 and SAAP-159) reduced activity against *S. aureus*.

Moreover, purified high-density lipoprotein and albumin, which bind to LL-37 and abolish its activity (*15*), at concentrations that resemble those in 50% plasma, reduced the antimicrobial activity of the SAAPs to a level similar to that in plasma ($LC_{99.9}$ of 12.8 to 25.6 μM; fig. S2B). These results indicate that enhanced stability and availability of the peptides in plasma may explain their improved activity compared to LL-37.

To assess the relative potency of the novel SAAPs, we compared their antimicrobial activity to that of 16 synthetic AMPs that showed promising antimicrobial activities against Gram-positive and/or Gram-negative bacteria in preclinical or clinical trials (Table 2). All peptides, except CZEN-002 and Ghrelin, killed *S. aureus* and/or *P. aeruginosa* at concentrations ranging from 0.8 to 25.6 μM in 10 mM sodium phosphate buffer (table S1), which is routinely used for screening of antimicrobial activities of AMPs. However, the activity of these (pre-)clinical phase peptides, as well as LL-37 and the previously developed LL-37–inspired peptides OP-145 and P10 (*16*), was strongly reduced in the presence of physiological salt concentrations and 50% human plasma (Table 2). The SAAPs had higher activity against *S. aureus* and *P. aeruginosa* than most of the (pre-)clinical phase peptides. Only iseganan, KABT-AMP, and pexiganan approximated the efficacy of the SAAPs against *S. aureus* and *P. aeruginosa* (Table 2). We selected SAAP-148, one of the peptides with the highest activity against both *S. aureus* and *P. aeruginosa*, for further characterization of its potency as a therapeutic agent against MDR bacteria.

SAAP-148 kills ESKAPE pathogens without resistance selection

First, we assessed the activity of SAAP-148 against a panel of MDR pathogens belonging to the ESKAPE panel (table S2). SAAP-148 killed these pathogens at concentrations of 0.4 to 12.8 μM in PBS and in PBS with 50% plasma or in the case of *Escherichia coli* in 50% urine (Fig. 1A). The activity of SAAP-148 was two- to eightfold lower in plasma than in PBS, except against *Enterobacter cloacae*, which was killed at a fourfold lower concentration in plasma than in PBS. Notably, SAAP-148 was

Table 2. LL-37–derived peptides are more effective against *S. aureus* and *P. aeruginosa* than (pre-)clinical phase peptides. Bactericidal activity ($LC_{99.9}$) in PBS and in PBS with 50% human plasma against *S. aureus* JAR060131 and *P. aeruginosa* PAO1. Results are medians (and ranges) of three to four independent experiments. If no range is indicated, then $LC_{99.9}$ values were identical in all experiments.

Peptide	Development phase	$LC_{99.9}$ (μM)							
		S. aureus				*P. aeruginosa*			
		PBS		50% plasma		PBS		50% plasma	
Ci MAM-A24	Preclinical	**6.4**		**51.2**	(25.6–51.2)	**4.8**	(1.6–6.4)	**51.2**	
CZEN-002	IIb	**>102.4**		**>102.4**		**>102.4**		**>102.4**	
DPK060	II	**>102.4**		**>102.4**		**>102.4**	(102.4–>102.4)	**>102.4**	
Ghrelin	II	**>102.4**		**>102.4**		**>102.4**		**>102.4**	
HB1345	Preclinical	**51.2**	(25.6–102.4)	**>102.4**		**51.2**	(25.6–102.4)	**>102.4**	
Hepcidin-25	Preclinical	**>102.4**		**>102.4**		**>102.4**		**>102.4**	
hLF1-11	I/II	**102.4**		**>102.4**		**>102.4**	(51.2–>102.4)	**>102.4**	
Iseganan	III	**3.2**	(3.2–6.4)	**12.8**	(6.4–12.8)	**2.4**	(0.8–6.4)	**51.2**	(51.2–102.4)
KABT-AMP	Preclinical	**1.6**		**25.6**	(12.8–25.6)	**1.6**		**25.6**	(12.8–25.6)
Omiganan	III	**51.2**		**>102.4**		**6.4**	(6.4–12.8)	**>102.4**	
OP-145	I/II	**1.6**	(1.6–3.2)	**>102.4**		**3.2**	(0.8–3.2)	**>102.4**	
Pexiganan	III	**12.8**	(6.4–12.8)	**25.6**		**1.6**	(0.8–1.6)	**6.4**	(6.4–25.6)
Plectasin	Preclinical	**>102.4**		**>102.4**		**>102.4**		**>102.4**	
POL7001	Preclinical	**>102.4**		**>102.4**		**51.2**	(51.2–>102.4)	**>102.4**	
S-Thanatin	Preclinical	**>102.4**	(102.4–>102.4)	**>102.4**		**102.4**		**>102.4**	
XOMA-629	II	**>102.4**		**>102.4**		**>102.4**		**>102.4**	
LL-37		**1.6**	(1.6–6.4)	**>102.4**		**3.2**	(1.6–3.2)	**>102.4**	
SAAP-145		**1.6**	(0.8–1.6)	**12.8**	(12.8–25.6)	**1.6**	(0.8–1.6)	**25.6**	(12.8–25.6)
SAAP-148		**1.6**		**12.8**	(12.8–25.6)	**1.6**		**12.8**	(6.4–12.8)
SAAP-159		**1.6**	(1.6–3.2)	**12.8**		**1.6**	(1.6–3.2)	**12.8**	(6.4–12.8)
SAAP-276		**0.8**		**6.4**	(6.4–12.8)	**0.8**	(0.8–1.6)	**51.2**	

A

Species	Strain	Aminoglycosides	Ansamycins	Carbapenems	Cephalosporins	Fluoroquinolones	Fusidanes	Glycopeptides	Glycylcyclines	Lincosamides	Lipopeptides	Macrolides	Monobactams	Monoxycarbolic acid	Oxazolidinones	Penicillins	Phenicols	Phosphonic acids	Polymyxins	Streptogramins	Tetracyclins	$LC_{99.9}$ SAAP-148 (μM) PBS	$LC_{99.9}$ SAAP-148 (μM) 50% plasma
E. faecium	LUH15122																					1.6	6.4
S. aureus	LUH14616																					1.6 (0.8–1.6)	3.2 (3.2–12.8)
K. pneumoniae	LUH15104																					6.4 (3.2–6.4)	12.8 (12.8–25.6)
A. baumannii	RUH875																					0.8	1.6 (1.6–3.2)
P. aeruginosa	LUH15103																					6.4 (3.2–6.4)	12.8 (12.8–25.6)
E. cloacae	LUH15114																					12.8 (6.4–12.8)	3.2 (3.2–12.8)
E. coli	LUH15117																					0.8	6.4

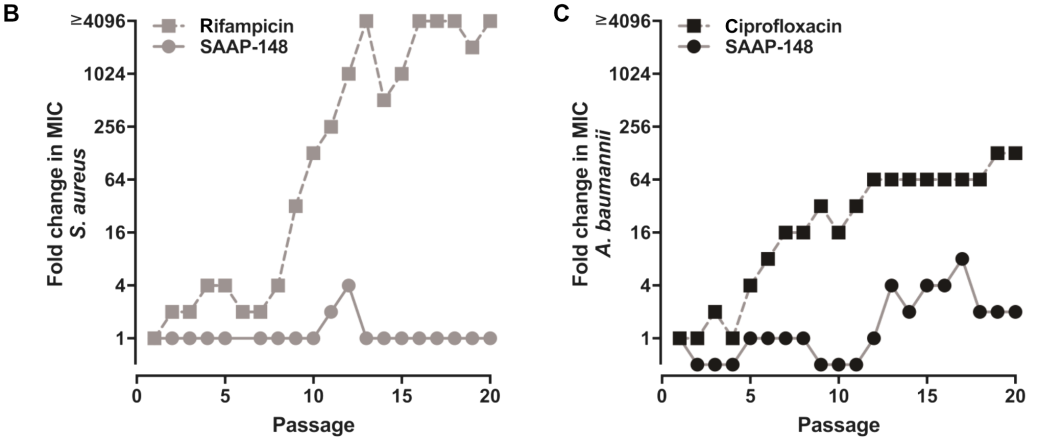

Fig. 1. SAAP-148 kills MDR ESKAPE pathogens and colistin-resistant *E. coli* without resistance selection. (**A**) Susceptibility of multidrug-resistant (MDR) ESKAPE (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter* species) pathogens and colistin-resistant *E. coli* to antibiotics and SAAP-148. Bacteria susceptible to all (green boxes) or intermediate/resistant to at least one (red boxes) of the antibiotics per class. Gray boxes are shown if the susceptibility to agents in that class is not assessed. Bactericidal activity of SAAP-148 in PBS and in PBS with 50% human plasma (or 50% urine in case of *E. coli*). Results are expressed as the $LC_{99.9}$, the lowest peptide concentration in micromolar that resulted in ≥99.9% killing. Results are medians (and ranges) of three independent experiments. If no range is indicated, then the $LC_{99.9}$ was identical in all experiments. (**B** and **C**) Resistance development of *S. aureus* JAR060131 (B) and *A. baumannii* RUH875 (C) to SAAP-148 and the antibiotics rifampicin and ciprofloxacin, respectively. Values are fold changes (in log_2) in minimal inhibitory concentration (MIC) relative to the MIC of the first passage.

also highly effective against an *E. coli* isolate resistant to colistin, which is considered a last resort antibiotic. Of note, for each species, between 2 and 17 isolates were tested, which all showed similar susceptibility to SAAP-148 ($LC_{99.9}$ of 0.8 to 6.4 μM in PBS and 6.4 to 25.6 μM in plasma/urine).

Because resistance development is a major concern, we assessed the ability of a Gram-positive and Gram-negative bacterium from the ESKAPE panel to develop resistance to SAAP-148. Serial passaging of *S. aureus* in the presence of subinhibitory concentrations of this peptide did not select for isolates resistant to SAAP-148 [minimum inhibitory concentration (MIC) of 1.875 μM at the first and 20th passages], whereas exposure to the antibiotic rifampicin resulted in a rapid increase in MIC already after eight passages, resulting in a ≥4096-fold increased MIC after 15 passages (from 0.125 to >512 μg/ml; Fig. 1B). When cultured in the presence of SAAP-148 for 20 passages, no significant change in MIC was observed for *A. baumannii* (MIC of 1.875 μM at the first passage and 3.75 μM at the last passage) when cultured in the presence of SAAP-148. The MIC for ciprofloxacin started to increase after five passages and had increased ≥128-fold after 19 passages (from 4 to >512 μg/ml; Fig. 1C). No resistance to SAAP-148 was observed for various other bacteria, including (MDR) *S. aureus* and *E. coli* strains (table S3).

SAAP-148 eliminates biofilms and kills persister cells

SAAP-148 dose-dependently inhibited the formation of biofilms on uncoated and plasma-coated polypropylene microtiter plates by *S. aureus* (Fig. 2, A and B) and *A. baumannii* (Fig. 2B). The biofilm mass as measured using crystal violet staining after 24 hours was maximally reduced after exposure to 12.8 μM of SAAP-148 (by 81 and 72% for *S. aureus* in plasma-coated and uncoated wells, respectively, and by 85% for *A. baumannii* in plasma-coated wells). Notably, in the biofilm-adjusted medium used for these assays, SAAP-148 did not have bactericidal activity against *S. aureus* and *A. baumannii* at concentrations of ≤12.8 and ≤6.4 μM, respectively, indicating that the inhibition of biofilm formation at these concentrations was not due to killing of the bacteria.

Established biofilms of *S. aureus* and *A. baumannii* formed on a plasma-coated surface were also eradicated by SAAP-148 in a dose-dependent fashion (Fig. 2C). Within 2 hours, 51.2 μM of SAAP-148 killed 99.9% of biofilm-encased *S. aureus* (Fig. 2C). Biofilms of *A. baumannii* were eradicated at lower concentrations: 12.8 μM of SAAP-148 killed 99.9% of biofilm-encased *A. baumannii*, and complete eradication was observed at ≥25.6 μM.

The biofilm matrix of staphylococci may be composed mainly of polymers of β-1-6-linked *N*-acetylglucosamine (PNAG), the so-called *ica* (intercellular adhesin)–dependent biofilm, or of proteins, the *ica*-independent biofilm (*17*). The *S. aureus* JAR060131 used was positive in polymerase chain reactions for the *icaA* and *icaD* genes, showing presence of the *ica* operon responsible for PNAG formation in this strain. To demonstrate the effect of SAAP-148 on *ica*-independent biofilms, we used the well-described *S. epidermidis* O-47 strain and its *ica*-deletion mutant (*18*). As expected, the *ica* mutant formed a smaller biofilm mass than the wild type (WT) but with the same number of viable bacteria as in biofilms of the WT. SAAP-148 significantly ($P < 0.0001$) inhibited the formation of biofilms by both the WT and its *ica*-deletion mutant (50% inhibition at 6.4 and 12.8 μM, respectively;

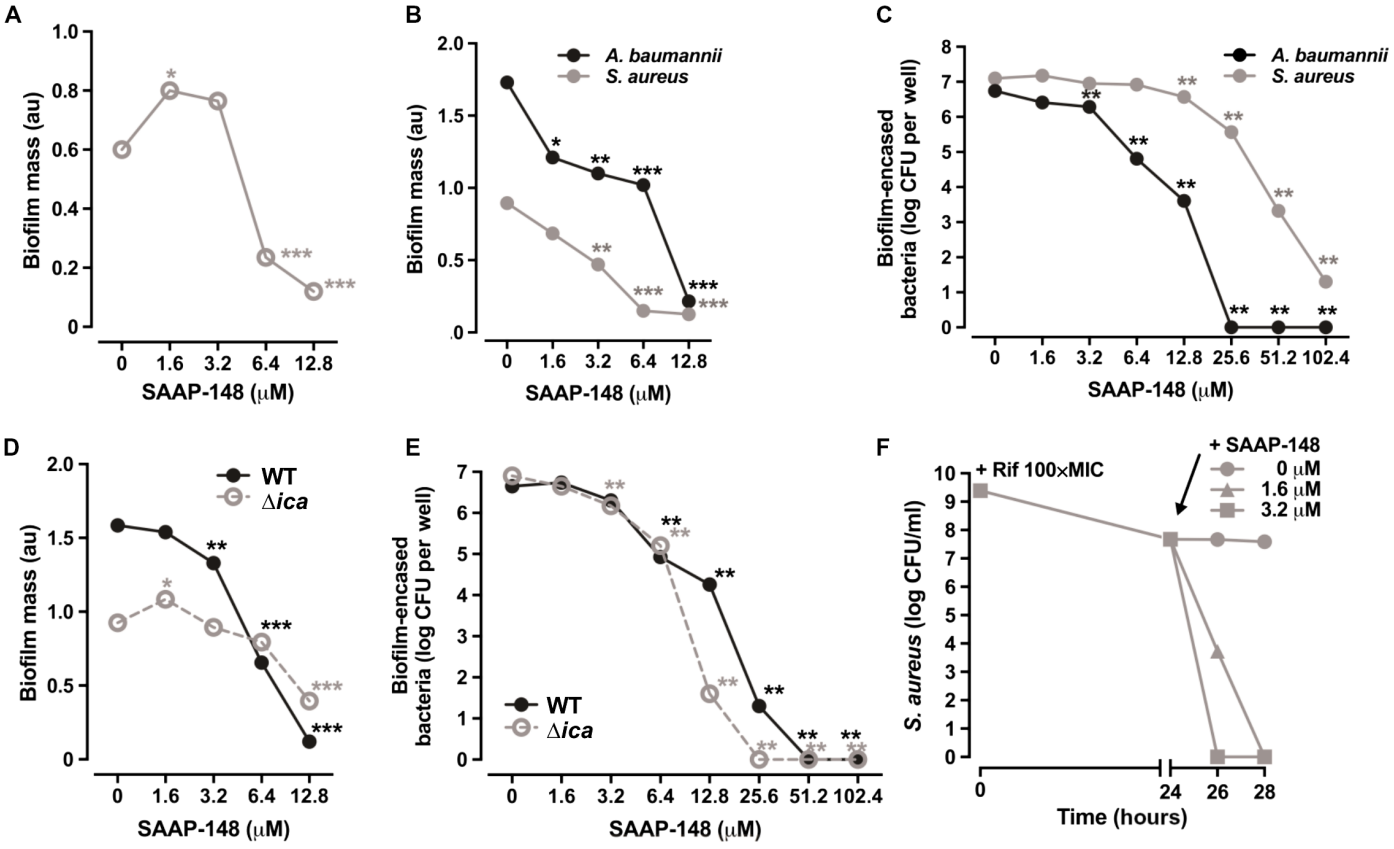

Fig. 2. SAAP-148 prevents biofilm formation, eliminates established biofilms, and kills persisters. (**A** and **B**) Prevention of biofilm formation by SAAP-148. Biofilm formation by *S. aureus* JAR060131 (gray lines) and *A. baumannii* RUH875 (black lines) after 24 hours incubation in uncoated wells (for *S. aureus*; A) and plasma-coated wells (B) in biofilm medium (BM) 2 medium containing 0 to 12.8 μM of SAAP-148. Results are expressed as the biofilm mass, measured using crystal violet staining, in arbitrary units (au). Values are medians of 14 to 18 replicates from three independent experiments. (**C**) Bactericidal activity of SAAP-148 against established biofilms of *S. aureus* JAR060131 (gray lines) and *A. baumannii* RUH875 (black lines). Results are expressed as the number of viable bacteria [in $\log_{10}$ colony-forming units (CFU)] after 2-hour exposure of 24-hour-old biofilms to SAAP-148. Values are medians of four to six replicates from three independent experiments. (**D**) Prevention of biofilm formation by SAAP-148. Biofilm formation by *S. epidermidis* O47 wild type (WT) (black lines) and its *ica*-deletion mutant Δ*ica* (gray dashed lines) after 24 hours in BM2 medium containing 0 to12.8 μM SAAP-148. Results are expressed as the biofilm mass, measured using crystal violet staining, in arbitrary units. Values are medians of 18 replicates from three independent experiments. (**E**) Bactericidal activity of SAAP-148 against established biofilms of *S. epidermidis* O47 WT (black lines) and its *ica*-deletion mutant Δ*ica* (gray dashed lines). Results are expressed as the number of viable bacteria (in $\log_{10}$ CFU) after 2-hour exposure of 24-hour-old biofilms to SAAP-148. Values are medians of five to six replicates from three independent experiments. (**F**) Bactericidal activity of SAAP-148 against persister cells derived from biofilms. Biofilms of *S. aureus* JAR060131 were treated for 24 hours with 100× MIC of rifampicin. Antibiotic was removed (arrow), and persisters were exposed to PBS containing 1.6 or 3.2 μM SAAP-148 or no peptide for an additional 2 or 4 hours. Results are expressed as the number of viable bacteria in $\log_{10}$ CFU per milliliter. Values are medians of six replicates from three independent experiments. *, significantly different (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$) as compared to control (0 μM), as calculated using the Mann-Whitney rank sum test.

Fig. 2D). The peptide also significantly ($P < 0.01$) reduced the numbers of viable bacteria in established biofilms of *S. epidermidis* WT and its *ica*-deletion mutant to undetectable levels at 51.2 and 25.6 μM, respectively (Fig. 2E).

To assess the activity of SAAP-148 against persisters, we exposed biofilms of *S. aureus* to a high dose of rifampicin (100× MIC) for 24 hours. About 2% of biofilm-encased bacteria, that is, a median of 4.8×10^7 colony-forming units (CFU) per milliliter, survived the rifampicin treatment. These remaining viable bacteria, that is, the persisters, were efficiently killed by SAAP-148: 1.6 μM of peptide killed ≥99.9% of persisters within 2 hours (Fig. 2F). After treatment of these bacteria for 4 hours with 1.6 μM or ≥2 hours with 3.2 μM SAAP-148, no viable bacteria could be detected—even after prolonged incubation of the agar plates—indicating that the peptide eliminated persisters to levels below detection.

SAAP-148 permeabilizes the bacterial membrane, resulting in killing within minutes

Bacterial killing by LL-37 is known to involve binding of the peptide to the bacterial membrane, followed by permeabilization and ultimately lysis of the bacterium (*9*, *19*). To assess whether SAAP-148 interacts with the membrane in a similar fashion, we first performed differential scanning calorimetry (DSC) and fluorochrome leakage analyses of bacterial cytoplasmic membrane mimics before and after exposure to the peptide (*20*). DSC showed a loss of the pretransition of the membrane lipid 1,2-dipamitoyldipalmitoyl-*sn*-glycero-3-phospho-rac-glycerol (DPPG), a decrease of the main transition enthalpy by about 10% and a marked decrease of the main transition temperature upon cooling (Fig. 3A), indicating that SAAP-148 strongly perturbed the hydrophobic core of the lipid bilayer (*21*). This, in turn, may lead to membrane thinning in the physiological relevant fluid phase, as described previously for LL-37 (*22*) and OP-145 (*20*).

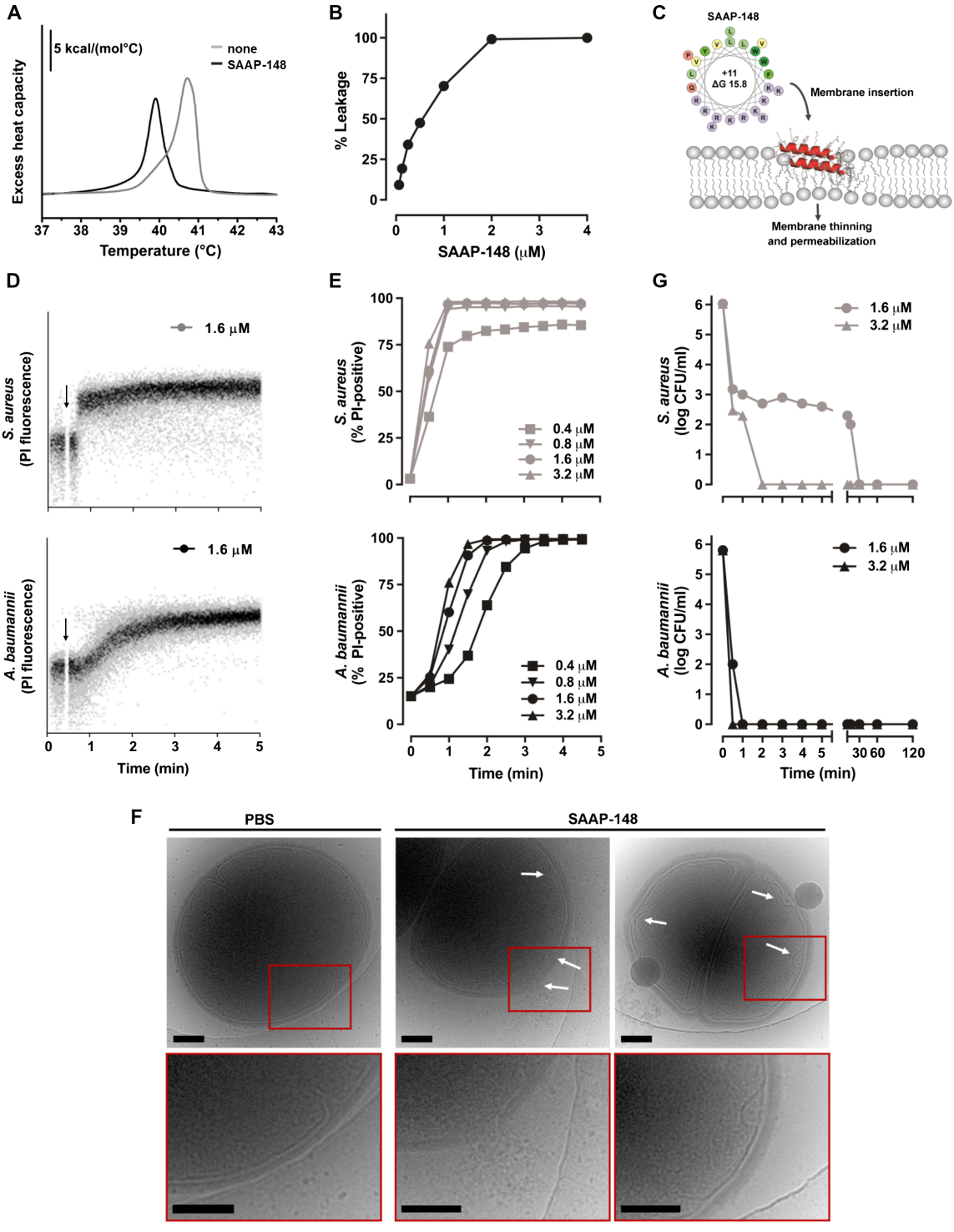

Fig. 3. SAAP-148 inserts into the bacterial membrane, causing rapid permeabilization and killing of *S. aureus* and *A. baumannii*. (**A**) Effect of SAAP-148 on phase transition of DPPG liposomes upon cooling. Results of one representative experiment out of three experiments. (**B**) Leakage of POPG liposomes in the presence of SAAP-148. Results are expressed as percentage leakage of the fluorescent dye relative to the total amount of the fluorescent dye. Values are medians of four independent experiments. (**C**) Schematic model of the interaction of SAAP-148 with the bacterial membrane. Peptide structure prediction based on PEP-FOLD. Helical wheel projection indicating the hydrophilic and hydrophobic region along the helical axes. Charged amino acids are in purple, apolar amino acids are in yellow, and C, P, and Q are in red. Net charge of the peptide at neutral pH and hydrophobicity expressed as transfer free energy of peptides from water to *n*-octanol (ΔG_{WOCT} in kilocalories per mole) calculated from the whole-residue hydrophobicity scale, taking into account the contribution of the C-terminal amidation and N-terminal acetylation, is shown in the middle of the wheel. (**D**) Membrane permeabilization in *S. aureus* JAR060131 and *A. baumannii* RUH875 by SAAP-148, as measured by propidium iodide (PI) influx. After 20 s, SAAP-148(1.6 μM) was added to bacteria (indicated by arrow), and PI fluorescence was measured for 5 min. (**E**) The percentage of PI-positive *S. aureus* and *A. baumannii* cells was calculated at different time intervals after addition of 0.4 to 3.2 μM SAAP-148. Results are medians of three independent experiments. (**F**) Cryo-transmission electron micrographs of *S. aureus* LUH14616 exposed to PBS or subinhibitory concentrations of SAAP-148. Arrows indicate membrane disruption and perturbation. Lower figures are magnifications of the area depicted in red in the upper figures. Scale bars, 200 nm. (**G**) Killing of *S. aureus* JAR060131 and *A. baumannii* RUH875 after 0.5- to 120-min exposure to 1.6 to 3.2 μM SAAP-148. Results are expressed as the number of viable bacteria in $\log_{10}$ CFU per milliliter. Values are medians of three independent experiments.

Ultimately, this resulted in permeabilization of the membrane as demonstrated by the leakage of the fluorescent marker 8-aminonaphthalene-1,3,6-trisulfonic acid/*p*-xylene-bis-pyridinium bromide (ANTS/DPX) from the membrane mimics in a dose-dependent manner (Fig. 3B).

We verified the proposed permeabilization of the plasma membrane by SAAP-148 (Fig. 3C) in live bacteria using flow cytometric analysis of propidium iodide (PI) influx and cryo-transmission electron microscopy (cryo-TEM). SAAP-148 dose-dependently permeabilized

the membranes of *S. aureus* and *A. baumannii* in a time-dependent manner (Fig. 3D). Within 60 s after exposure to ≥0.8 μM of SAAP-148, more than 90% of the *S. aureus* cells were permeabilized. This concentration of SAAP-148 also caused permeabilization of *A. baumannii* cells, albeit at a slightly slower rate: 0.8 μM peptide induced permeabilization in more than 90% of cells after 120 s (Fig. 3E). In agreement, whereas cryo-TEM evaluation of *S. aureus* before treatment showed intact cytoplasmic membranes in all cells (Fig. 3F), exposure to subinhibitory concentrations of SAAP-148 resulted in disrupted membranes and pores traversing the cell wall in most of the cells (Fig. 3F).

This fast permeabilization of the membrane was associated with rapid killing of *S. aureus* and *A. baumannii* after exposure to the peptide: ≥1.6 μM SAAP-148 killed more than 90% of these bacteria within 30 s (Fig. 3G). Complete killing of *A. baumannii* and *S. aureus* was observed 120 and 30 s after exposure to 3.2 μM SAAP-148, respectively. These data indicate that interaction of SAAP-148 with the bacterial membrane leads to permeabilization of the membrane, which contributes to the death of the bacteria within minutes.

SAAP-148 formulated in an ointment is safe in an animal model

To enable topical application of the peptide, we formulated SAAP-148 in a 3.75% (w/w) hypromellose gel base, which we previously selected for the related peptide OP-145 because the peptide's stability and bactericidal activity were unaffected by this ointment (*23*).

Potential adverse effects of the SAAP-148–containing ointment were assessed in rabbits. Results revealed that treatment of rabbits with 500 mg of 1% (w/w) SAAP-148 ointment on shaved intact and abraded skin did not evoke primary irritation within 72 hours after treatment. There were no notable changes in behavior and body weight and no signs of systemic toxicity. Histological examination revealed a minimal degree of focal lymphohistiocytic infiltration in the dermis of most of the wounded animals, which is in line with the physiological restoration of the wound. No difference in severity of these histopathological findings was observed between untreated, vehicle-treated, and SAAP-148–treated skin (Table 3). An additional study using a single dose of 1% (w/w) SAAP-148 ointment (2000 mg/kg) showed no signs of skin irritation or morbidity. Moreover, a 14-day dermal repeated-dose toxicity study showed no signs of skin irritation or pathology using 0.1, 0.3, and 1% (w/w) SAAP-148 ointments.

SAAP-148 ointments are highly effective against (biofilm)-associated skin infections

The efficacy of SAAP-148 ointment was first assessed using an ex vivo wounded human skin infection model. Skin was inoculated with methicillin-resistant *S. aureus* (MRSA) and *A. baumannii* 1 hour before treatment with SAAP-148 ointment for 4 hours, a period during which >90% of SAAP-148 is released from the ointment, as assessed by release experiments in vitro (fig. S3). A single treatment with 0.125 to 2% (w/w) SAAP-148 ointment completely eradicated MRSA, whereas treatment with the vehicle resulted in 100% culture-positive skin models with a median of 1.9×10^3 CFU per skin model (Fig. 4A). SAAP-148 was also highly effective against *A. baumannii*: 0.125% (w/w) SAAP-148 ointment significantly ($P < 0.01$) reduced the number of viable bacteria on the skin (from a median of 2.7×10^5 CFU per skin model to a median of 5.5×10^2 CFU per skin model), whereas ≥0.5% (w/w) SAAP-148 ointment completely eradicated *A. baumannii* from the skin (Fig. 4A). The vehicle did not affect the numbers of bacteria on the skin compared to no treatment.

To assess the efficacy against established wound infections, we applied the treatment 24 hours after inoculation of the wounded human skin, when the bacteria have formed a biofilm (Fig. 4B) (*23*, *24*). Skin models treated with the vehicle had a median of 1.5×10^7 CFU of MRSA per skin model. Treatment with 0.125 and 0.5% (w/w) SAAP-148 ointment caused a significant ($P < 0.01$) 1- and 4-log lower number of CFU of MRSA per skin model, respectively, whereas 2% (w/w) SAAP-148 ointment completely eradicated MRSA in 67% of samples (Fig. 4C). Treatment of established *A. baumannii* infections with 0.125 and 0.5% (w/w) SAAP-148 ointment significantly ($P < 0.05$ and $P < 0.0001$) reduced the number of bacteria (Fig. 4C). A single treatment with 2% (w/w) SAAP-148 ointment completely eradicated *A. baumannii* in 50% of the samples, with a maximum of 1.9×10^3 CFU remaining in the culture-positive samples (Fig. 4C). For comparison, 2% (w/w) mupirocin and 1% chlorohexidine ointment, which are standard treatments

Table 3. Safety of topical application of SAAP-148 ointment in rabbits. Single-dose dermal tolerance test in rabbits. Animals with intact or abraded skin were treated for 4 hours with 500 mg of vehicle or 1% (w/w) SAAP-148 ointment. As a control, untreated rabbits were included. Results are expressed as the number of animals out of the total number of animals within the group ($n = 3$) that showed signs of skin irritation or pathology within 72 hours after treatment.

	Number of rabbits					
	Intact			Abraded		
	Untreated	Vehicle	SAAP-148	Untreated	Vehicle	SAAP-148
Skin irritation						
Erythema formation	0/3	0/3	0/3	0/3	0/3	0/3
Edema formation	0/3	0/3	0/3	0/3	0/3	0/3
Gross pathology findings	0/3	0/3	0/3	0/3	0/3	0/3
Histopathological findings						
Lymphohistiocytic infiltration in the dermis	0/3	0/3	0/3	3/3*	0/3	2/3*
Fibrosis in the dermis	0/3	0/3	0/3	0/3	2/3*	0/3

*Minimal change (score 1).

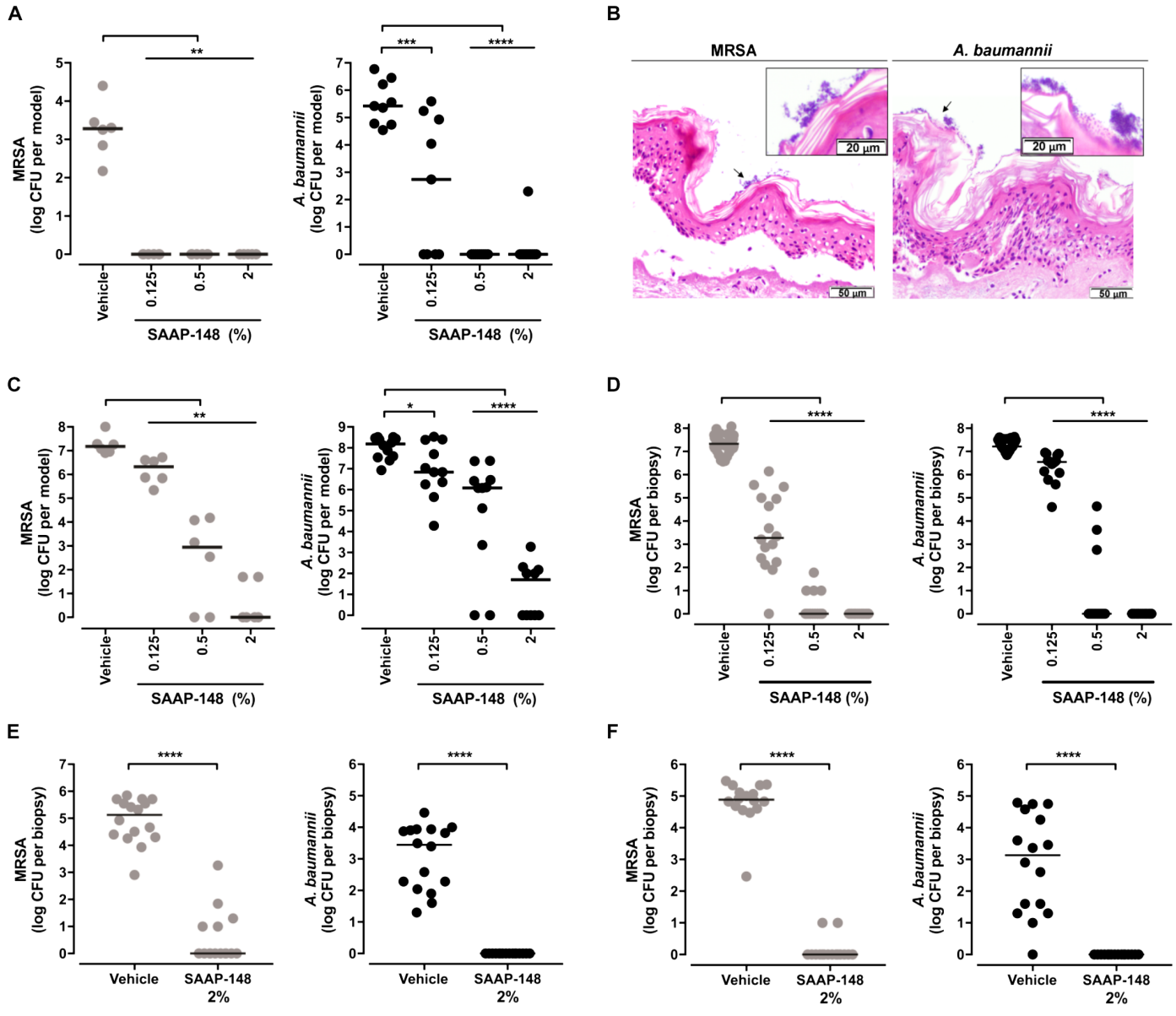

Fig. 4. Topical application of SAAP-148 ointment eradicates acute and established infections of MRSA and *A. baumannii* from the skin. (**A** to **F**) Ex vivo wounded human skin (A to C) and in vivo abraded murine skin (D to F) were inoculated with MRSA LUH14616 (gray circles) or *A. baumannii* RUH875 (black circles). Ten minutes after inoculation (D) or 1 hour (A), 24 hours (C and E), or 48 hours (F) after inoculation, the skin was treated with ointments containing no peptide (vehicle) or 0.125, 0.5, or 2% (w/w) SAAP-148 ointment. Results are expressed as the numbers of viable bacteria (in $\log_{10}$ CFU) per skin model of three to six donors and of 16 skin samples for each group of mice (eight mice per group and two skin samples per mouse). Each circle represents one skin sample, and bars indicate medians. *, significantly different (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$) as compared to the vehicle, as calculated using the Mann-Whitney rank sum test. (B) Light micrographs of hematoxylin and eosin–stained skin biopsies 24 hours after inoculation with MRSA and *A. baumannii* and subsequent vehicle treatment. Arrows indicate biofilm formation, which is shown at a higher magnification in the inset.

used for skin infections caused by *S. aureus* and Gram-negative bacteria, respectively, only reduced the numbers of viable bacteria in the acute ex vivo skin infection model. Their effect was limited and significantly ($P < 0.01$) less than SAAP-148 in the case of established ex vivo skin infections (fig. S4).

Together, these results indicate that a single 4-hour treatment with SAAP-148 ointment is highly effective against both acute and established (biofilm-associated) wound infections caused by MDR bacteria. Notably, topical application of 0.125 to 2% (w/w) SAAP-148 ointment on intact or wounded ex vivo human skin samples did not cause any morphological changes, as assessed by histological examination (fig. S5).

Finally, we assessed the efficacy of SAAP-148 ointment in vivo using murine models of abraded skin infection. In the acute infection model, a single treatment of 4 hours with 0.125% (w/w) SAAP-148 ointment significantly ($P < 0.0001$) reduced the number of viable MRSA from 2.1×10^7 to 1.9×10^3 CFU per biopsy (Fig. 4D). This ointment reduced the number of viable *A. baumannii* per biopsy by 0.7-log ($P < 0.0001$). Treatment with 0.5% (w/w) SAAP-148 ointment completely

eradicated MRSA in 75% and *A. baumannii* in 81% of the biopsies, with a maximum of 1×10^2 and 4×10^4 CFU remaining in the culture-positive biopsies. Treatment with 2% (w/w) SAAP-148 ointment completely eradicated MRSA and *A. baumannii* from the skin of all mice (Fig. 4D).

SAAP-148 ointment was also highly effective in an in vivo model of established infection: Application of 2% (w/w) SAAP-148 ointment 24 and 48 hours after inoculation completely eradicated MRSA from 67 and 87% of mice, respectively (Fig. 4, E and F), compared to 0% after vehicle treatment. Established infections of *A. baumannii* were completely cleared from all mice after a single treatment with 2% (w/w) SAAP-148 ointment (Fig. 4, E and F).

DISCUSSION

Antibiotics are highly successful drugs saving millions of lives annually. However, the emergence of multidrug resistance worldwide, especially among Gram-negative members of the ESKAPE panel raises fears of untreatable infections (*1*, *2*). Here, we report that SAAP-148 is highly effective against MDR Gram-positive and Gram-negative ESKAPE pathogens, as well as isolates of *E. cloacae*, *E. coli*, and *K. pneumoniae* resistant to the last-resort antibiotic colistin. Moreover, preliminary experiments showed that SAAP-148 was able to kill *Clostridium difficile* under anaerobic conditions, demonstrating the broad-spectrum bactericidal activity of SAAP-148. This peptide was more effective under physiological conditions [that is, 50% human plasma, a relevant mimic of wound fluid (*25*)] than most AMPs tested in clinical trials. Compared to conventional antibiotics, SAAP-148 is, in most cases, as active against strains susceptible to the antibiotic and up to 400-fold more active against strains resistant to the antibiotic. Our recent report that the related SAAPs from our screen, that is, SAAP-145 and SAAP-276, were highly effective against biomaterial-associated infections in vivo (*26*) further supports our notion that SAAPs may be good candidates for further development as a novel antibiotic.

Biofilms, as well as persister cells, are commonly overlooked in antibiotic research but are an important cause of chronic and recurrent infections. We have found that SAAP-148 is able to prevent the formation of predominantly polysaccharide, as well as proteinaceous biofilms, and to promote their breakdown and eradicate established *S. aureus* and *A. baumannii* biofilms. Furthermore, persister cells, which are a minor subpopulation of bacterial cells that are in a dormant state and highly tolerant to antibiotics (*6*), are known to pose significant challenges to the treatment of chronic and recurrent infections. One of the few antimicrobials displaying bactericidal activity against persister cells is acyldepsipeptide 4 (ADEP4) (*27*). However, resistance to ADEP4 is rapidly developing in *S. aureus*, requiring an additional antibiotic to completely eradicate the persisters (*27*). Here, we showed that *S. aureus* persisters that survived an extremely high dose of rifampicin were completely eradicated within 2 hours by SAAP-148 at low micromolar concentrations.

Investigations into the mode(s) of action of SAAP-148 involving liposomes mimicking bacterial membranes and live bacteria revealed that this peptide rapidly interacts with and subsequently permeabilizes the cytoplasmic membrane of bacteria, leading to bacterial death. This mode of action, which is similar to that used by LL-37 (*9*) and LL-37 derivatives (*28*), is in line with the potent activity of SAAP-148 against dividing and nondividing, metabolically inactive bacteria residing in a biofilm and against persister cells. It has long been thought that because of this rapid, membrane-based mechanism of action, resistance development to AMPs is very unlikely (*29*). Nonetheless, several recent studies have shown that resistance to such peptides can evolve at least in vitro (*30*). Using a similar assay, we demonstrated the lack of resistance development to SAAP-148 in several bacterial species when cultured for 20 passages in the presence of subinhibitory peptide concentrations. The lack of resistance development further underscores the potential of SAAP-148 for further clinical development.

The rise in antibiotic resistance may lead to a scenario where simple wound infections, which account for more than 4% of all emergency department visits in the United States (*31*), can become life-threatening. In addition, chronic wounds, which affect around 6.5 million patients in the United States (*32*), are also highly prone to bacterial infections, which, in many cases, are biofilm-associated (*33*). Obviously, an ointment that will eradicate these antibiotic-resistant and/or antibiotic-tolerant pathogens has great potential to cure patients with persistent infections and to reduce the emergence of antibiotic resistance and transmission. As a first step toward development as a topical treatment for wound infections, we incorporated SAAP-148 in a hypromellose gel and determined its activities on wounded ex vivo human skin and in vivo abraded murine skin infection models. Previous studies have shown that this ointment base was optimal for the LL-37–inspired peptide OP-145 (*23*) with respect to the peptide's stability, release profile, and antibacterial activity. We demonstrated that SAAP-148–containing ointments are highly efficacious in completely eradicating MRSA and *A. baumannii* in acute and established (biofilm-associated) wound infections of ex vivo human skin models and in vivo murine skin. The efficacy of the SAAP-148 ointments is considerably higher than of the previously developed OP-145– and P10-containing ointments, which reduced the numbers of viable MRSA LUH14616 on human epidermal skin models only by about 2-log in both an acute and an established infection model (*23*). Several other AMPs, including AmyA (*34*), Epi-1 (*35*), TP-3 (*36*), RR (*37*), RRIKA (*37*), GE33 (*38*), and the LL-37–derived 17BIPHE2 (*28*), have recently been tested for their antimicrobial activity against *S. aureus* skin infections. None of these peptides completely eradicated *S. aureus* from the skin in acute wound infection models, except for TP-3 after a prolonged treatment period of 19 days and 17BIPHE2, which prevented biofilm formation on subcutaneous catheters and reduced the numbers of bacteria in the peri-implant tissues in the majority of mice (*28*). The effect on established wound infections was only assessed for RR and RRIKA, which showed a 2-log reduction in the numbers of CFU of *S. aureus* after 3 days of treatment (*37*). For comparison, a single 4-hour treatment with SAAP-148 completely eradicated *S. aureus* from the skin of all mice with an acute skin infection and of 87% of mice with a 48-hour established infection. Mupirocin and chlorohexidine ointments, which are widely used to treat skin infections caused by MRSA and Gram-negative bacteria, respectively, did reduce the numbers of viable bacteria in the acute ex vivo human skin wound infection models. However, their effect was limited and significantly less than the effect of SAAP-148 against established skin infections in this model.

Topical application of the peptide in hypromellose gel at doses up to 300 mg/day per animal proved to be safe. On the basis of these results, it can be concluded that application of SAAP-148 ointments up to 1% on the (wounded) skin is safe and without any adverse effects. In agreement, no signs of skin pathology were observed in ex vivo wounded human skin treated with the ointments. These results, together with the peptide's efficacy profile, imply that topical application of SAAP-148 ointment holds promise as a treatment to remove (MDR) bacteria from wounds, including burn wounds, diabetic foot ulcers, and atopic dermatitis lesions. The main limitation of this study is that the efficacy studies were restricted to superficial wounds. In deeper wounds, therapeutics,

including AMPs, may be hampered by poor penetration to deeper layers, hindrance by wound debris, and/or breakdown by proteolytic enzymes. These challenges can be overcome through the use of tailored drug delivery systems such as nanoparticles for encapsulation and controlled release (*39*–*41*). Our recent report that SAAP-145 and SAAP-276 incorporated in a controlled-release coating were highly effective against biomaterial-associated infections in vivo (*26*) demonstrates the potency of SAAPs in tissues.

Together, we showed that SAAP-148 is an excellent drug candidate to combat hard-to-treat infections because of its broad antimicrobial activity against MDR bacteria, biofilms, and persisters; its lack of resistance development; its safety profile; and its efficacy against ex vivo and in vivo wound infections by MDR Gram-positive and Gram-negative bacteria. The first clinical trial with the peptide formulated in hypromellose gel is scheduled for the first quarter of 2018.

MATERIALS AND METHODS

Study design

A set of LL-37–inspired peptides was synthesized, and their antimicrobial activities under physiological conditions were compared to those of promising preclinical and clinical phase peptides. The lead peptide of this set, SAAP-148, was tested against MDR pathogens, biofilms, and persisters in vitro. Its interaction with bacterial membranes was investigated using membrane model systems and several bacterial species. After formulation into an ointment, the efficacy of SAAP-148 against wound infections caused by MDR bacteria was assessed using wounded ex vivo human skin models following the Declaration of Helsinki principles.

Safety of formulated SAAP-148 was assessed in an acute dermal toxicity study, followed by a single-dose and 14-day repeated-dose dermal toxicity study in New Zealand White rabbits according to the *Note for Guidance on Non-clinical Local Tolerance Testing of Medicinal Products* (CPMP/SWP/2145/00, London, 1 March 2001), Council Regulation (EC no. 440/2008, 20 May 2008), Regulation (EC No. 1272/2008), and the Organisation for Economic Co-operation and Development (OECD) guidelines for testing of chemicals 404 (28 July 2015). Finally, mouse models were used to confirm the potential antimicrobial and antibiofilm activities revealed in vitro and ex vivo models. The mouse study was approved by the local Animal Ethics Committee. The investigators were blinded for the group allocation during the experiment and processing the outcomes, and the mice were randomized over the different groups. On the basis of data of a previous study (*42*), the sample size was calculated using a power of 0.9. Figure legends include details of replicates used to generate the different data sets.

Peptides and ointments

Peptides in Tables 1 and 2 were prepared by 9*H*-fluorenylmethyloxycarbonyl chemistry, as described previously (*12*, *43*). Peptides were high-performance liquid chromatography (HPLC)–purified, and their identity was confirmed with mass spectrometry. Peptides in Table 2 have been selected based on (i) their antimicrobial activity against Gram-positive and/or Gram-negative bacteria in clinical trials or in preclinical studies, (ii) the availability of the sequence (table S4), and (iii) the ability to synthesize the peptide in our facility.

Hypromellose-based ointments containing 0.125 to 2% (w/w) peptide or no peptide (vehicle) were prepared as described previously (*23*). Ointments were stored at 4°C. Release of SAAP-148 from hypromellose ointments was assessed using the ERWEKA light dissolution apparatus with a rotating paddle. Briefly, 1 g of 0.5% (w/w) SAAP-148 ointment was incubated in the apparatus for 5 hours at 37°C with a paddle rotating speed of 100 rpm. After 0, 15, 30, 60, 120, 180, 240, and 300 min, the amount of peptide released in 1 ml of the surrounding water was measured using HPLC.

Microorganisms and culture

Clinical isolates of *E. faecium* (LUH15122), *S. aureus* (JAR060131, LUH14616, and LUH15101), *K. pneumoniae* (LUH15104), *A. baumannii* (RUH875), *P. aeruginosa* (LUH15103), *E. cloacae* (LUH15114), and *E. coli* (LUH15117) and the reference strain *P. aeruginosa* PAO1 were used. *S. epidermidis* O-47 WT and its *ica*-deletion mutant (*18*) were included. The bacteria were stored in nutrient broth supplemented with 20% (v/v) glycerol at −80°C. Before each experiment, inocula from the frozen stocks were grown overnight at 37°C on sheep blood agar plates (BioMerieux). For experiments, fresh cultures were prepared in tryptic soy broth (for *E. faecium*, *S. aureus*, and *S. epidermidis*; Oxoid), brain heart infusion broth (for *K. pneumoniae*, *P. aeruginosa*, *E. cloacae*, and *E. coli*; Oxoid), or lysogeny broth (for *A. baumannii*; Oxoid). The bacteria were cultured for 3 or 20 hours at 37°C at 200 rpm to mid-logarithmic and stationary growth phase, respectively, washed once with PBS, and diluted to the desired inoculum concentration, based on the optical density at 600 nm.

Susceptibility to antimicrobial agents was determined using VITEK, E-tests, or broth dilution tests. Multidrug resistance was defined as nonsusceptible to at least one agent in three or more antimicrobial classes, as described by Magiorakos *et al.* (*44*).

Bactericidal activity

Bactericidal activity was assessed as described previously (*12*). Briefly, a mid-logarithmic growth-phase culture of bacteria (1×10^6 CFU/ml) was exposed to peptide solutions (with final concentrations ranging from 0.1 to 204.8 μM) in 10 mM sodium phosphate buffer (NaPB; pH 7.4), PBS (140 mM NaCl; pH 7.4), and PBS supplemented with 50% (v/v) pooled human plasma (further referred to as human plasma), frozen after collection and pooling (Sanquin), or pooled urine (obtained from healthy volunteers after informed consent) in polypropylene microtiter plates (Greiner). As untreated controls, bacteria were exposed to NaPB, PBS, or 50% human plasma or urine without peptides. After 2 hours of incubation at 37°C at 200 rpm, the numbers of viable bacteria were determined microbiologically. Bactericidal activity is expressed as the $LC_{99.9}$, that is, the lowest peptide concentration that killed ≥99.9% of bacteria within 2 hours of incubation.

For time-kill experiments, bacteria (1×10^6 CFU/ml) were incubated with 1.6 and 3.2 μM SAAP-148 in PBS in polypropylene tubes (Micronics) in a total volume of 750 μl. As an untreated control, bacteria were incubated in PBS. After incubation at 37°C and 200 rpm for 0.5 to 120 min, a sample (50 μl) was taken from the suspension and added to 50 μl of a 0.05% (v/v) sodium polyanethol sulfonate (Sigma-Aldrich) solution, which neutralizes remaining peptide activity, and the number of viable bacteria was determined.

Resistance development

Development of resistance to the peptides was assessed as described by Habets and Brockhurst (*45*). For comparison, development of resistance to the clinically relevant antibiotics rifampicin and ciprofloxacin (both from Sigma-Aldrich) was determined. Briefly, bacteria were cultured overnight at 37°C at 200 rpm in modified RPMI 1640 (with 20 mM Hepes and L-glutamine, without sodium bicarbonate; Sigma-Aldrich). In wells of a 96-well polypropylene flat-bottom plate, 5 μl of the

overnight bacterial culture was added to 100 µl of SAAP-148/antibiotic solutions (with final concentrations of 0.2 to 240 µM peptide or 0.06 to 256 µg/ml of rifampicin or ciprofloxacin) in modified RPMI 1640. Plates were sealed with breathseals (Greiner) and incubated for 18 to 20 hours (for *S. aureus*) or 48 hours (for *A. baumannii*) at 37°C and 200 rpm. The MIC, the lowest concentration of peptide/antibiotic that caused lack of visible bacterial growth, was determined for each bacterial species. Thereafter, 5 µl of the growth at the 0.5-fold MIC suspension was added to a fresh medium containing peptides/antibiotics, and these mixtures were incubated as described above. This was repeated for 20 passages.

Prevention of biofilm formation

Prevention of biofilm formation was assessed as described previously (*12*). In short, 96-well polypropylene plates were coated with plasma by overnight incubation with 20% (v/v) pooled human plasma (Sanquin) in PBS at 4°C, as described by Walker *et al.* (*46*). In the plasma-coated wells or in uncoated wells, bacteria (1×10^8 CFU/ml) were exposed to SAAP-148 solutions (with final concentrations ranging from 1.6 to 12.8 µM) in biofilm medium (BM) 2 (*47*). As an untreated control, bacteria were exposed to BM2 medium without peptide. After 24 hours incubation at 37°C in a humidified atmosphere, planktonic bacteria were removed, and biofilms were stained with 1% crystal violet (Sigma-Aldrich) for 15 min, washed, and solubilized with 96% ethanol. The optical density at 595 nm was determined as a measure of biofilm mass.

Treatment of established biofilms

A mid-logarithmic growth-phase culture was diluted in BM2 medium to 1×10^8 CFU/ml. Fifty microliters of this bacterial suspension was added to plasma-coated wells of a flat-bottom polypropylene microtiter plate. After 24 hours of incubation at 37°C in a humidified atmosphere, planktonic bacteria were removed by two washes with PBS. Next, biofilms were exposed to 100 µl of SAAP-148 in PBS (with final concentrations ranging from 1.6 to 102.4 µM). As a control, bacteria were exposed to PBS without peptide. After 2 hours of incubation at 37°C in a humidified atmosphere, plates were sonicated for 5 min to dislodge the adherent bacteria, and the number of viable bacteria was determined microbiologically. Sonication did not affect the viability of the bacteria. The lower limit of detection was 50 CFU. To visualize the data on a logarithmic scale, a value of 1 CFU was assigned when no growth occurred.

Bactericidal activity against persisters

To obtain persisters from a biofilm culture, a mid-logarithmic growth-phase culture of *S. aureus* JAR060131 was diluted in brain heart infusion broth to 1×10^8 CFU/ml. One hundred microliters of this suspension was transferred to wells of a 96-well polystyrene flat-bottom plate (Greiner). Plates were sealed and incubated at 37°C in a humidified atmosphere for 24 hours. Planktonic bacteria were removed by two washes with PBS, and biofilms were exposed to 100 µl of brain heart infusion broth containing rifampicin (6.25 µg/ml; that is, 100× MIC). After 24 hours of incubation at 37°C in a humidified atmosphere, planktonic bacteria were removed by two washes with PBS, and adherent bacteria were dislodged in 100 µl of PBS by 5 min sonication. Bacteria were subsequently exposed to 200 µl of PBS containing 1.6 and 3.2 µM of SAAP-148. As a control, bacteria were exposed to PBS without peptide. After 2 and 4 hours of incubation at 37°C, the number of viable bacteria was determined microbiologically. The lower limit of detection was 50 CFU. To visualize the data on a logarithmic scale, a value of 1 CFU was assigned when no growth occurred.

Interaction with membrane mimics

DSC measurements were performed on lipid vesicles composed of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-rac-glycerol (POPG; Avanti Polar Lipids) and DPPG (Avanti Polar Lipids) in the absence and presence of SAAP-148 in PBS (in a lipid-to-peptide molar ratio of 25:1).

Leakage of the aqueous content of the ANTS/DPX (Molecular Probes)–loaded POPG vesicles upon incubation with SAAP-148 (with final concentrations of 0.125 to 4 µM, corresponding to lipid-to-peptide molar ratios from 400:1 to 12.5:1) was determined as described (*48*). Fluorescence emission was recorded as a function of time using the SPEX FluoroMax-3 spectrofluorimeter combined with Datamax software. The percentage of leakage was calculated relative to the positive control (1% Triton-X-100).

Membrane permeabilization using bacteria

A mid-logarithmic growth-phase culture was diluted in PBS to 1×10^6 CFU/ml. One hundred eighty microliters of this bacterial suspension was added to 20 µl of PI (with a final concentration of 1 µg/ml; Sigma-Aldrich) in polypropylene tubes. After 10 min of incubation on ice in the dark, PI fluorescence was measured over time using the BD Accuri C6 flow cytometer. After about 20 s, 20 µl of SAAP-148 (with final concentrations of 0.4 to 3.2 µM) was added, and samples were measured during 5 min. As a control, bacteria were exposed to PBS without peptide.

Cryo-transmission electron microscopy

A mid-logarithmic growth-phase culture was diluted in PBS to 3×10^8 CFU/ml and exposed to 12.8 µM SAAP-148 (which was a sub-inhibitory peptide concentration for this high inoculum) in PBS for 1 hour at 37°C. As a control, bacteria were exposed to PBS without peptide. Bacteria were fixed and transferred to electron microscopy grids, which were placed into a cooled cryo-holder and visualized using a Tecnai F20 electron microscope [Thermo Fisher Scientific (formerly FEI)].

Safety

The safety of the SAAP-148 ointments was assessed in an acute dermal toxicity study, followed by a single-dose and 14-day repeated-dose dermal toxicity study in New Zealand White rabbits. The study was approved by the Animal Care and Use Committee of Toxi-Coop Zrt (Budapest, Hungary). In total, 40 adult rabbits (5 males and 5 females for the limit test, 6 males for the single-dose study, and 12 males and 12 females for the 14-day repeated-dose study) were used.

For the acute dermal toxicity study, 1% (w/w) SAAP-148 ointment was applied uniformly over the shaved skin of the back of the rabbits in a single dose of 2000 mg/kg and covered with sterile gauze pads. After 24 hours, pads were removed, and any remaining ointment was washed away with water. Clinical observations were made 1 and 5 hours after the treatment and once daily during the 14 days thereafter. Irritation effects were evaluated, and body weight was recorded shortly before the treatment, 7 and 14 days after treatment.

For the single- and repeated-dose study, the intact or scarified shaved skin of the rabbits was treated once with 500 mg of 1% (w/w) SAAP-148 ointment or vehicle (single-dose study) or daily with 300 mg of 0.1, 0.3, and 1% (w/w) SAAP-148 ointment or vehicle for 14 days. The treated skin was covered with gauze pads for 4 hours, after which any remaining ointment was washed away with water. Irritation effects and signs of erythema and edema were scored at 1, 24, 48, and 72 hours after removal of the pads. Animals were weighed at the beginning and end of the experiment. After 72 hours in the single-dose study or after

14 days in the limit test and repeated-dose study, rabbits were humanely sacrificed by an intravenous injection of Release (WDT). Macroscopic and histopathological examination of the control and treated parts of the skin was performed.

Infection and treatment of ex vivo human skin

All primary human skin cells were isolated from surplus tissue collected according to article 467 of the *Dutch Law on Medical Treatment Agreement* and the *Code for Proper Use of Human Tissue* of the Federation of Dutch Medical Scientific Societies. None of the authors were involved in the tissue sampling. Split-thickness human skin grafts (0.3 mm thick) were obtained from healthy donors undergoing abdominoplasty in the Red Cross Hospital in Beverwijk (Netherlands) and from deceased donors via the Euro Tissue Bank in Beverwijk after informed consent was given. Models were thermally wounded for 10 s using a soldering iron set at 95°C, as described (*49*), and then placed onto metal grids in 12-well plates containing about 1 ml of culture medium (*50*). To assess the effect of SAAP ointment on the skin, about 20 mg of SAAP-148 ointments ranging from 0.125 to 2% (w/w) was applied on the wounded skin or on the intact skin. As a control, models were exposed to 20 mg of the vehicle. After 4 hours of incubation at 37°C and 5% CO_2, models were fixed in 4% formaldehyde, sectioned, and stained with hematoxylin and eosin for histopathological examination.

For skin infection experiments, skin models were thermally wounded as described previously. Ten microliters of a mid-logarithmic phase culture, containing 1×10^4 CFU, was applied on the wounded skin models. After 1 or 24 hours of incubation at 37°C and 5% CO_2, about 20 mg of peptide ointment, containing 0.125 to 2% (w/w) SAAP-148, 2% (w/w) mupirocin ointment (Bactroban), 1% (w/w) chlorohexidine ointment (Frago), or no ointment, was applied topically on the inoculated skin. As a control, the vehicle was applied. After an additional 4 hours of incubation, models for histology were fixed in 4% formaldehyde, sectioned, and stained with hematoxylin and eosin for histological examination. Skin models for vital count were transferred to polypropylene tubes containing glass beads (Ø 1.7 mm) and 1 ml of PBS and homogenized using the Precellys bead beater (3 × 20 s). Homogenization did not affect bacterial viability. The number of viable bacteria was determined. The lower limit of detection was 50 CFU. To visualize the data on a logarithmic scale, a value of 1 CFU was assigned when no growth occurred.

Infection and treatment of murine skin

Specific pathogen–free C57BL/6J OlaHsd immune-competent female mice (Harlan), aged 8 to 10 weeks and weighing at least 18 g, were used. For each experiment, mice were randomized over the experimental groups. The investigators were blinded for the group allocation during the experiment and processing the outcome. The mice were randomized over the different groups using the online random sequence generation at www.random.org.

The backs of mice were shaved using an electric razor 1 day before the experiment. Fifteen minutes before the experiment, mice were anesthetized with 2% isoflurane in oxygen and subcutaneously injected with the analgesic Temgesic [buprenorphine (0.05 mg/kg)]. Mice were kept under anesthesia and at an optimal body temperature with a thermostatted heat map during the whole experiment. At the middle of the back, an area of about 2 cm^2 was tape-stripped with Tensoplast seven times in succession, with the tape being replaced after the first time, resulting in a visibly damaged skin.

Five microliters of a mid-logarithmic growth-phase culture, containing 1×10^7 CFU, was applied on the wounded skin. Ten minutes, 24 hours, or 48 hours after inoculation, about 30 mg of the peptide ointment, containing 0.125 to 2% (w/w) SAAP-148, was applied on the skin and covered with a circular piece of parafilm. As a control, the vehicle was applied on the skin. After a treatment period of 4 hours, the skin was separated from the underlying fascia and muscle tissue, and about 2 cm^2 of skin from the infected area was excised. The skin was homogenized in 0.5 ml of saline using five zirconia beads (Ø 2 mm; BioSpec Products) and the MagNA Lyser System (Roche), with three cycles of 30 s at 7000 rpm, with 30-s cooling on ice between cycles. The number of viable bacteria was determined. The lower limit of detection was 10 CFU. To visualize the data on a logarithmic scale, a value of 1 CFU was assigned when no growth occurred. The different dosages of SAAP-148 treatment were tested in separate experiments (first, 2% was compared to the vehicle; second, 0.125 and 0.5% were compared to the vehicle). No significant difference was observed between the placebo-treated samples within the two experiments. To visualize the data, vehicle-treated samples of both experiments were pooled. For statistical analyses, the two vehicle-groups were separated and compared to the treatment dose that was tested within the same experiment.

Statistical analysis

Two-sample comparisons were made using a Kruskal-Wallis, followed by a Mann-Whitney rank sum test. The significance of differences between the frequencies of categorical variables was determined using Fisher's exact test. For all tests, $P < 0.05$ was considered significant.

SUPPLEMENTARY MATERIALS

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Materials and Methods
Fig. S1. Antimicrobial activity of SAAP-145/SAAP-148/SAAP-159 alanine-scanning peptides against *S. aureus* JAR060131 in PBS and in PBS with 50% human plasma.
Fig. S2. Effect of plasma proteins on antimicrobial activity of SAAPs against *S. aureus* JAR060131.
Fig. S3. Release of SAAP-148 from hypromellose gel.
Fig. S4. Effect of topical application of mupirocin and chlorohexidine ointment on acute and established human ex vivo skin infections of MRSA and *A. baumannii*.
Fig. S5. Effect of topical application of SAAP-148 ointment on ex vivo human skin.
Table S1. Bactericidal activity of peptides in preclinical and clinical development against *S. aureus* and *P. aeruginosa* in low-salt buffer.
Table S2. Antibiogram of ESKAPE pathogens used in this study.
Table S3. Resistance development of *S. aureus* and *E. coli*.
Table S4. Sequences of peptides in preclinical and clinical development.
Table S5. Primary data.
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The antimicrobial peptide SAAP-148 combats drug-resistant bacteria and biofilms

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New way to keep bacteria at bay

Antibiotic resistance is a major threat to public health. To develop a new type of weapon in the arms race against bacteria, de Breij *et al.* generated a panel of synthetic peptides based on the human antimicrobial peptide LL-37. The lead candidate from this panel, SAAP-148, can kill dangerous antibiotic-resistant pathogens in many contexts, including on ex vivo human skin and in biofilms. Long-term exposure to SAAP-148 did not induce bacterial resistance. Topical application of SAAP-148 could one day be used in hospitals to help patients combat bacteria resistant to traditional antibiotics.