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Thrombocidin-1-derived antimicrobial peptide TC19 combats superficial multi-drug resistant bacterial wound infections

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ABSTRACT

Antimicrobial peptides are considered promising candidates for the development of novel antimicrobial agents to combat infections by multi-drug-resistant (MDR) bacteria. Here, we describe the identification and characterization of the synthetic peptide TC19, derived from the human thrombocidin-1-derived peptide L3. Biophysical experiments into the interaction between TC19 and mimics of human and bacterial plasma membranes demonstrated that the peptide is highly selective for bacterial membranes. In agreement, TC19 combined low cytotoxicity towards human fibroblasts with efficient and rapid killing in human plasma of MDR strains of several bacterial species of the ESKAPE panel. In addition, TC19 induced minor resistance *in vitro*, neutralized pro-inflammatory activity of bacterial cell envelope components while displaying slight chemotactic activity for human neutrophils. Importantly, topical application of TC19-containing hypromellose gel significantly reduced numbers of viable methicillin-resistant *Staphylococcus aureus* (MRSA) and MDR *Acinetobacter baumannii* in a superficial wound infection in mice. Together, TC19 is an attractive candidate for further development as a novel agent against (MDR) bacterial skin wound infections.

1. Introduction

The emergence of multi-drug resistant (MDR) bacterial strains belonging to the so-called ESKAPE panel (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter species*) [1], all listed on the recent global priority pathogens list of the World Health Organization (WHO) [2], is accelerated by the selective pressure exerted by extensive use and abuse of antimicrobials. Antimicrobial resistance is a major problem worldwide, at present causing an estimated 50,000 deaths annually in Europe and the US alone, with many hundreds of thousands more dying in other areas of the world [3]. To prevent the dreaded scenario of 10 million deaths worldwide in 2050 [3], novel antimicrobials capable of eliminating antibiotic-resistant strains of pathogenic bacteria, are urgently needed.

Naturally occurring antimicrobial peptides have broad antimicrobial activities and are considered excellent templates for the development of novel antimicrobials [4,5]. Thrombocidin-1 (TC-1), the major microbicidal protein isolated from human blood platelets, has indeed been used as a design template for such peptides [6,7]. Initial screens using 15-mer overlapping peptides covering the TC-1 sequence led to identification of a peptide designated as L3 [7] with moderate antibacterial activity. Peptide L3 has a strongly hydrophobic N-terminus, hydrophilic, non-charged residues in the central loop and a cationic C-terminal region. In order to improve the antimicrobial activity of L3, peptides have been synthesized with subsequent single amino acids replaced by lysine. Lysine replacement of isoleucine 14 (position according to the position in the original TC-1 protein) generated peptide L3-I14K (LRCMCIKTTSGKHPK) which had the highest microbicidal activity against *Bacillus subtilis*, *Escherichia coli*, *S. aureus* and *Cryptococcus neoformans* of the peptides, and decreased hemolytic activity compared to the unmodified L3 peptide [6]. To further improve

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its antimicrobial activity the hydrophobicity of the central domain of peptide L3-I14K was increased by replacing either threonine 11 or 12 with tryptophan, resulting in peptides TC18 (LRCMCIK**W**TSGKHPK) and TC17 (LRCMCIKT**W**SGKHPK), respectively, or by replacing both threonine residues with tryptophans, *i.e.* TC19 (LRCMCIK**WW**SGKHPK). This strategy of tryptophan introduction followed the approach reported earlier for tritrpticin [8] and indolicidin [9]. This central hydrophobic domain supposedly interacts with the interfacial region of the bacterial membrane, resulting in peptide's penetration into the hydrophobic membrane core [10,11].

In this study, we investigated the antibacterial activities of these novel L3-I14K-derived synthetic antimicrobial peptides. We show that the most potent peptide, TC19, displays limited cytotoxicity and has potent *in vitro* bactericidal activity against MDR strains of bacterial species of the ESKAPE panel, rapidly kills these bacteria and effectively eradicates methicillin-resistant *S. aureus* (MRSA) as well as MDR *A. baumannii* from infected, wounded murine skin, underlining the peptide's potential for further translational development.

2. Materials and methods

2.1. Peptides

L3-I14K (previously designated I29K [6] and the L3-I14K-derived peptides shown in Table 1 were synthesized by 9H-fluorenylmethyloxycarbonyl (Fmoc)-chemistry as described previously [12]. All peptides were C-terminally amidated. Purity of the final products was > 95% (RP-HPLC, detection at 214 nm) and their identity were confirmed with MALDI-ToF (Microflex, Bruker).

2.2. Microorganisms

The extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* isolate [13], *Staphylococcus aureus* JAR060131 [14] and the clinical strains of ESKAPE bacteria (and their antibiotic resistance profiles) [15] used in this study are listed in Table 3. Multi-drug resistance (MDR) was defined as non-susceptible to at least one agent in three or more classes of antimicrobials, and pan-drug resistant (PDR) as non-susceptible to any tested agent in all classes of antimicrobials as described [16]. Prior to each experiment, bacteria from frozen stocks were grown overnight at 37 °C on sheep blood agar plates (BioMerieux). For each experiment, fresh subcultures were made in tryptic soy broth (TSB, Oxoid) for *Enterococcus faecium* and *S. aureus*, in brain heart infusion broth (BHI, Oxoid) for *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Enterobacter cloacae* and *Escherichia coli*, and in lysogeny broth (LB, Oxoid) for *Acinetobacter baumannii*.

2.3. In vitro killing assays

Bacteria were cultured to the mid-logarithmic growth phase in the appropriate growth media at 37 °C and 200 rpm, washed once with phosphate-buffered saline (PBS; 140 mM NaCl, pH 7.4), resuspended and diluted in low sodium phosphate buffer supplemented with 1% (*v*/v) TSB (PT; 10 mM NaCl, pH 7.0) or in PBS to 5×10^6 colony forming units (CFU)/ml, based on the optical density of the suspension at 600 nm (OD_{600}). Twenty microliter of this bacterial suspension [final concentration of 1×10^6 CFU/ml] were added to 80 μl of peptide solution [final concentrations ranging 0.9375–60 μM for the peptide screening study and from 0.8–204.8 μM for the bactericidal spectrum study] in PT, PBS or PBS with a final concentration of 50% (*v*/v) pooled human plasma (Sanquin) in polypropylene round bottom microtiter plates (Greiner). As negative controls, bacteria were incubated in PT, PBS or PBS with 50% plasma. After 2 h of incubation at 37 °C and 200 rpm the numbers of viable bacteria were determined by quantitative culture. Bactericidal activity is expressed as the 99.9% lethal concentration (LC99.9), *i.e.* the lowest peptide concentration that killed ≥99.9% of bacteria within 2 h.

For time-kill experiments, 200 μl of *S. aureus* JAR060131 suspension (final concentration of 1×10^6 CFU/ml) were added to 800 μl of peptide solution in PBS (final concentrations of 3.75–15 μM) in polypropylene tubes (Micronics). As untreated control, bacteria were incubated in PBS. After incubation at 37 °C and 200 rpm for various intervals up to 4 h a 100-μl sample was mixed with 100 μl of a 0.05% (*v*/v) sodium polyanethole sulfonate (SPS; Sigma) solution to neutralize peptide activity [17], after which the number of viable bacteria was determined by quantitative culture.

2.4. Interaction with membrane mimics

All phospholipids (purity > 99%) were purchased from Avanti Polar Lipids. For leakage experiments, large unilamellar vesicle (LUVs) composed of 1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPG) or 1-palmitoyl-2-oleyl-phosphatidylcholine (POPC) loaded with 8-aminonaphthalene-1,3,6-trisulfonic acid/p-xylene-bis-pyridinium bromide (ANTS/DPX; Molecular Probes) were prepared according to previously described protocols [18]. Leakage of the aqueous content of ANTS/DPX-loaded LUVs upon incubation with peptide was related to full leakage induced by addition of 1% Triton X-100 [18].

For differential scanning calorimetry (DSC), lipid vesicles composed of 1,2-dipalmitoyl-phospatidylglycerol (DPPG) and 1,2-dipalmitoyl-phospatidylcholine (DPPC) were prepared according to [19]. Thermograms in the presence and absence of TC19 were recorded at a constant rate of 30 °C/h using a lipid concentration of 1 mg/ml. Data were analyzed after baseline correction and normalization to the mass of phospholipid using Microcal's Origin software.

Table 1
Bactericidal activity of L3-I14K-derived peptides against *E. coli* ESBL and *S. aureus* JAR060131.

			LC99.9 in μM				
			E. coli ESBL		*S. aureus* JAR060131		
Peptide	Sequence	ΔG_{woct}	PT	PBS	PT	PBS	50% plasma
L3-I14K	LRCMCIKTTSGKHPK	14.01	7.5	> 60	15	> 60	> 60
TC17	LRCMCIKT**W**SGKHPK	11.67	3.75	≥60	7.5–15	> 60	> 60
TC18	LRCMCIK**W**TSGKHPK	11.67	3.75	> 60	3.75–7.5	> 60	> 60
TC19	LRCMCIK**WW**SGKHPK	9.33	3.75	15	3.75	7.5–15	15–30

Results are expressed as the lethal concentration (LC) 99.9%, *i.e.* the lowest peptide concentration that resulted in ≥99.9% killing of bacteria after 2 h of incubation in PT buffer (10 mM phosphate buffer, pH 7.0, with 1% (v/v) TSB), phosphate buffered saline (PBS, pH 7.4) and in PBS with 50% (*v*/v) human plasma (one experiment, performed in duplicate). Hydrophobicity expressed as transfer free energy of peptides from water to *n*-octanol (ΔG_{WOCT} in kcal/mol) was calculated from the whole residue hydrophobicity scale taking into account the contribution of C-terminal amidation.

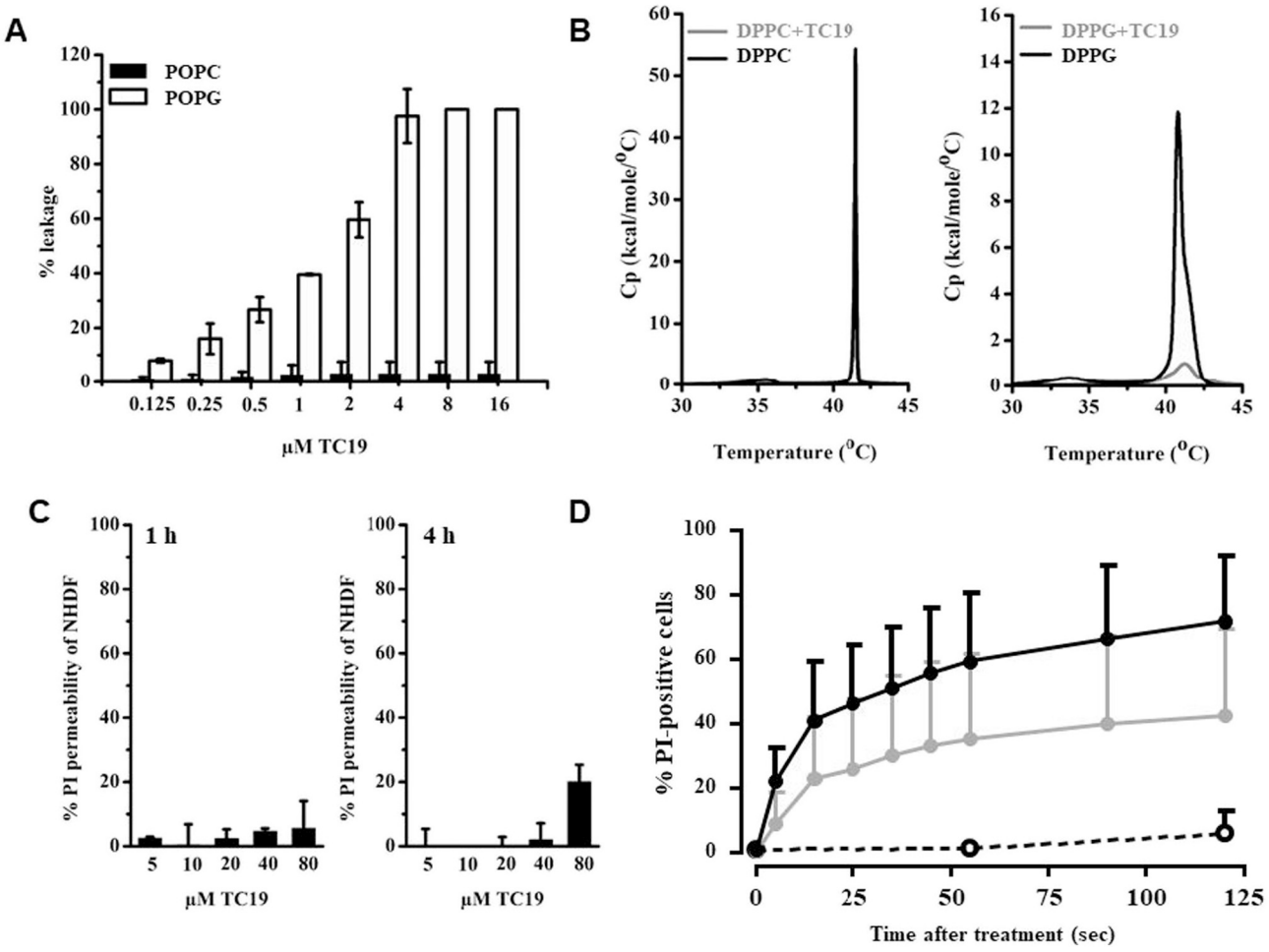

Fig. 1. TC19 is highly selective for bacterial membranes and shows low toxicity towards human cells. **A**, Leakage of LUVs composed of POPG (white bars; mimicking bacterial membranes) and POPC (black bars; mimicking eukaryotic membranes) at indicated TC19 concentrations. **B**, Effect of TC19 on mammalian and bacterial membrane mimics. Representative DSC heating scans of DPPC (left) and DPPG (right) liposomes in the absence (black line) and presence of 2 μM TC19 (grey line) corresponding to a lipid-to-peptide molar ratio of 25:1. Three independent experiments were performed with standard deviations of < 5%. **C**, PI-uptake by normal human dermal fibroblasts (NHDF) after 1 h (left) and 4 h (right) of incubation with TC19 at indicated concentrations. Values are means and standard deviations of three replicates. **D**, Membrane permeabilization in *S. aureus* JAR060131 after addition of 7.5 (grey solid circles) or 15 μM TC19 (black solid circles) or no peptide (open circles), as measured by PI influx during 120 s. Results are means and standard deviations of three independent experiments.

Table 2
Thermodynamic parameters of DPPC and DPPG bilayers in the absence and presence of TC19.

	Pre-transition			Main transition		
	Tpre	ΔHpre	ΔT1/2	Tm	ΔHm	ΔT1/2
	(°C)	(kcal/mol)		(°C)	(kcal/mol)	
DPPC	35.4	1.8	1.9	41.5	9.2	0.1
DPPC + TC19	35.0	1.8	2.7	41.5	10.0	0.2
DPPG	33.8	1.3	3.3	40.8	10.9	0.6
DPPG + TC19	–	–	–	41.3	2.2	1.4

Pretransition and main phase transition temperature (Tpre/Tm) and corresponding enthalpies (ΔHpre/ΔHm). Data are from a representative experiment out of three independent experiments with standard deviations < 5%.

2.5. Cytotoxicity assay

Normal human dermal fibroblasts (NHDF; PromoCell GmbH, Heidelberg, Germany) were cultured in fibroblast growth medium 2 (PromoCell GmbH). All cells were kept in a 5% CO_2 atmosphere at 37 °C. Cells were cultured to ~90% confluency and then detached by accutase (PAA; Pasching, Austria). Enzymatic activity was stopped with PBS containing 10% (*v*/v) fetal bovine serum. Cells were washed with PBS and resuspended in the culture medium. Approximately 1×10^5 cells were incubated with 5–80 μM peptide in PBS for 1 and 4 h at 37 °C in a total volume of 100 μl. Cytotoxicity of TC19 was determined by the propidium iodide (PI)-uptake assay and expressed as the fraction of the percentage of PI-positive cells in the presence of peptide over the percentage of PI-positive cells upon exposure to 2.5% (*v*/v) Triton X-100. The background signal was determined by using cells in medium without peptide and subtracted from all measurements [20].

2.6. Resistance development

The resistance development assay was adapted from others [21]. *S. aureus* JAR060131 and MDR *A. baumannii* RUH875 were cultured overnight in modified RPMI-1640 medium (with 20 mM Hepes and L-glutamine, without sodium bicarbonate; Sigma) at 37 °C and 150 rpm. Modified RMPI medium was used as it supports bacterial growth, without affecting peptide activity [22]. In wells of a 96-wells polypropylene (peptide) or polystyrene (antibiotic) flat bottom plate, 2 μl of the overnight bacterial suspension were added to 100 μl of peptide or antibiotic solution in RPMI, with final concentrations of 3.75–240 μM of peptide, and with final concentrations of 0.031–512 μg/ml of rifampicin or ciprofloxacin (Sigma). All incubations were performed in

Table 3
Antimicrobial activity of TC19 against ESKAPE panel multi-drug resistant and pan-drug resistant bacterial strains.

Species	Strain	Class	Antibiotic resistance[1]	LC99.9 TC19 (μM)	
				PBS	50% plasma
E. faecium	LUH10330	MDR		1.6 (1.6-3.2)	6.4 (3.2-6.4)
S. aureus	LUH14616	MDR (MRSA)		3.2 (3.2-6.4)	12.8 (3.2-12.8)
K. pneumoniae	LUH8995	MDR		1.6 (0.8-1.6)	3.2
A. baumannii	RUH875	MDR		1.6 (1.6-3.2)	6.4 (6.4-12.8)
P. aeruginosa	LUH15100	PDR		4.8 (3.2-6.4)	>204.8
E. cloacae	LUH15114	MDR		25.6	204.8

Antibiotic resistance[1]: aminoglycosides, ansamycins, carbapenems, cephalosporins, fluoroquinolones, fusidanes, glycopeptides, glycylcyclines, lincosamides, lipopeptides, macrolides, monoxycarbolic acid, oxazolidinones, penicillins, polymyxins, sulfonamides, tetracyclines

Results are expressed as the lethal concentration (LC) 99.9%, *i.e.* the lowest peptide concentration that resulted in ≥99.9% killing of multi-drug resistant (MDR) and pan-drug resistant (PDR) bacteria after 2 h of incubation in phosphate buffered saline (PBS, pH 7.4) and PBS with 50% human plasma. Values are medians and ranges of at least 3 independent experiments. If no range is depicted, the LC99.9 values were identical for each experiment.
[1]Bacteria susceptible to all (green boxes) or intermediate/resistant to at least one (red boxes) of the antibiotics per class. Grey boxes are shown if the susceptibility to agents in this class is not assessed.

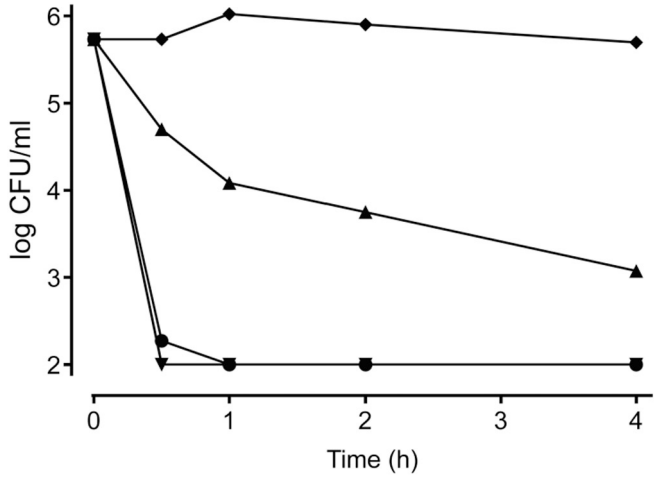

Fig. 2. TC19 rapidly kills *S. aureus*. Killing of *S. aureus* JAR060131 after 0.5–4 h exposure to 0- (diamond; no peptide), 0.5- (upward triangle; 3.75 μM), 1- (circle; 7.5 μM) or 2-fold (downwards triangle; 15 μM) LC99.9 concentration of TC19. Results are expressed as number of viable bacteria in log CFU per ml. Values are means of two replicates.

quadruplicate. The plates were sealed with breathseals (Greiner) and incubated for 20 h at 37 °C and 150 rpm. After incubation, plates were visually inspected for growth and the minimal inhibitory concentration (MIC), *i.e.* the lowest concentration without visible growth, was determined. For each individual incubation series, 2 μl of the culture with 0.5-fold MIC were added to a new concentration series, ranging from 0.25- to 8-fold MIC and incubated as described above. This was repeated for 22 passages.

2.7. Human blood

Blood was obtained from human volunteers using heparin-containing tubes. Written informed consent was obtained from all blood donors.

2.8. Lipopolysaccharide and S. aureus inflammatory activity neutralization assay

To assess the lipopolysaccharide (LPS) neutralizing capacity of TC19, 250 ng/ml of LPS (*E. coli* J5; Sigma) was pre-incubated with increasing doses (0.002–156.25 μM) of TC19 in PBS for 30 min at 37 °C. To assess the *S. aureus* inflammatory activity neutralizing capacity of TC19, 5×10^8 CFU/ml of UV-killed *S. aureus* JAR060131 were pre-

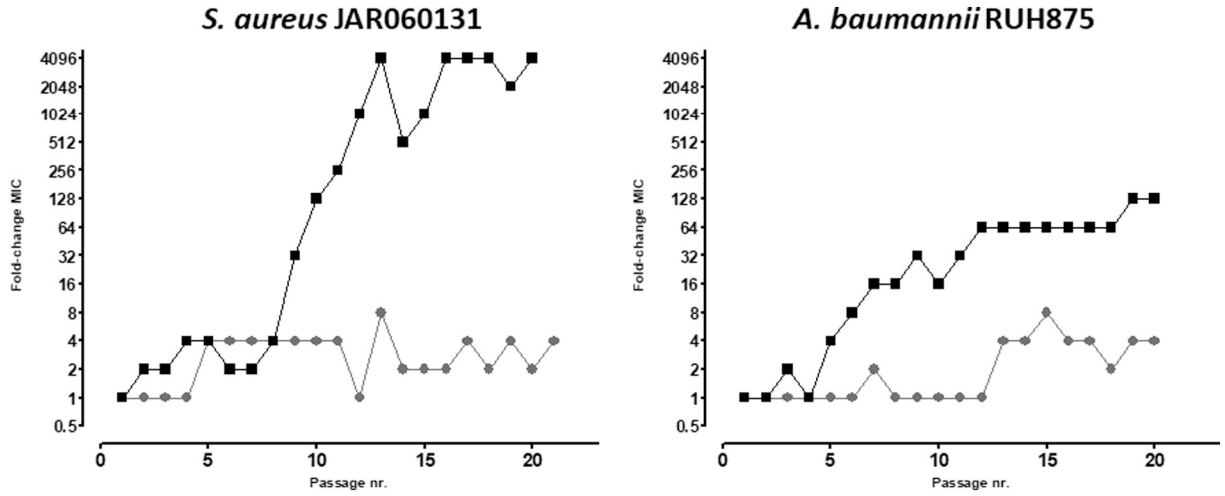

Fig. 3. Development of resistance of *S. aureus* JAR 060131 and MDR *A. baumannii* RUH875 to TC19 and rifampicin (*S. aureus*) or ciprofloxacin (*A. baumannii*). The fold increase in minimal inhibitory concentration (MIC) of TC19 (grey circles) and rifampicin or ciprofloxacin (black squares) for the different bacterial strains serially passaged (20 h per passage) in RPMI is shown. Values are medians of four replicates.

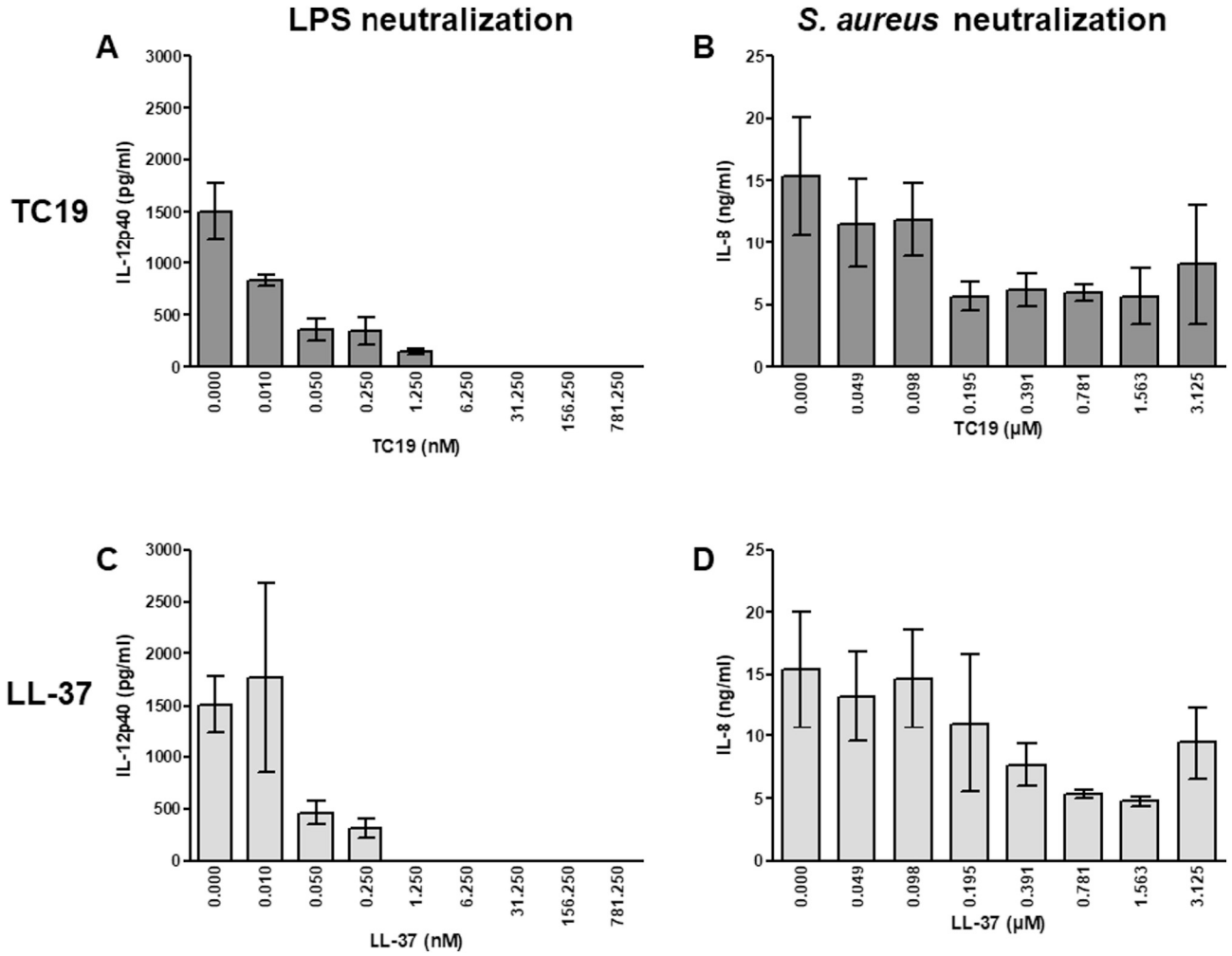

Fig. 4. LPS- and *S. aureus*-neutralizing activity of TC19. Samples containing 250 ng/ml of LPS (*E. coli*) or 5 × 10^8 CFU/ml of UV-inactivated *S. aureus* JAR060131 were pre-incubated with 0.002–156.25 μM or 9.8–625 μM peptide, respectively, in PBS for 30 min. Next, the LPS/*S. aureus*-peptide mixture was added 1:200 to 4-times diluted blood from a healthy volunteer. After 18–20 h incubation, the level either of LPS-induced IL-12p40 (**A** and **C**) or *S. aureus*-induced IL-8 (**B** and **D**) in the supernatants was determined by ELISA. Values are medians and ranges of two donors.

incubated with 9.8–625 μM of TC19 in PBS for 30 min at 37 °C. For comparison, the known immunomodulatory antimicrobial protein LL-37 was taking along in the experiments at same concentrations as TC19 [23]. Next, the LPS- or *S. aureus*-peptide mixture was added 1:200 to freshly drawn heparinized blood from a healthy volunteer, resulting in final concentrations of 1.25 ng/ml LPS with 0.01–781.3 nM peptide, and 2.5 × 10^6 CFU/ml *S. aureus* with 0.05–3.125 μM peptide, respectively. After 18–20 h of incubation at 37 °C in a 5% CO_2 atmosphere, the levels of LPS-induced IL-12p40 and *S. aureus*-induced IL-8 in the supernatants were determined by ELISA (Invitrogen). The ELISAs were performed according to manufacturer's instructions.

2.9. Chemotaxis assay

Human neutrophils were isolated from freshly drawn heparinized human blood by Ficoll-amidotrizoate (1.077 g/ml, Pharmacy of LUMC) density centrifugation. The erythrocytes in the neutrophil-containing pellet were lysed by ammonium chloride-EDTA lysis buffer (1 mM EDTA, 1.8 M NH_4Cl, 100 mM $KHCO_3$). After erythrocyte lysis the pellet was washed four times in PBS and the neutrophils were resuspended in RPMI 1640 (Gibco) to a concentration of 2 × 10^6 neutrophils/ml. Next, the permeable filters Corning® HTS Transwell 12 well permeable support pore size 3.0 μm (Sigma) were coated with 500 μl of 0.1% BSA (wt/v) in PBS for 10 min at room temperature. After removal of the BSA solution 100 μl of 2 × 10^6 cells/ml plus 300 μl of RPMI 1640 were applied to the upper chamber of the well, while 1 ml of various doses of TC19 (range 0–12.8 μM) in RPMI 1640 or as positive control 1 ml of 10 nM N-formylmethionyl-leucyl-phenylalanine (fMLP; Sigma-Aldrich) plus 10% (*v*/v) heat-inactivated fetal calf serum was applied to the lower compartment. After 90 mins at 37 °C and 5% CO_2 the cells in the upper compartment were removed and 50 μl of 50 mM EDTA were added to harvest emigrating neutrophils from the filter to the lower compartment. After careful removal of the 12 well filter rack the cell suspension in the lower compartment was transferred to polypropylene tubes (Falcon) and the cell numbers enumerated by flow cytometry on an Accuri (Becton Dickinson). Results are expressed as the percentage of cells migrated in response to the peptide compared to that migrated upon fMLP.

2.10. Mouse superficial skin wound infection model

The mouse study was approved (DMB103170) by the Animal Ethical Committee of the Amsterdam University Medical Center, Amsterdam, the Netherlands and carried according to the guidelines of the EU Directive 2010/63/EU for animal experiments. Specific pathogen-free C57Bl/6J OlaHsd immune-competent female mice (Harlan), weighing 18 to 20 g, were used. Prior to the experiments, mice were housed in individually ventilated cages (IVCs) and were provided with sterile food

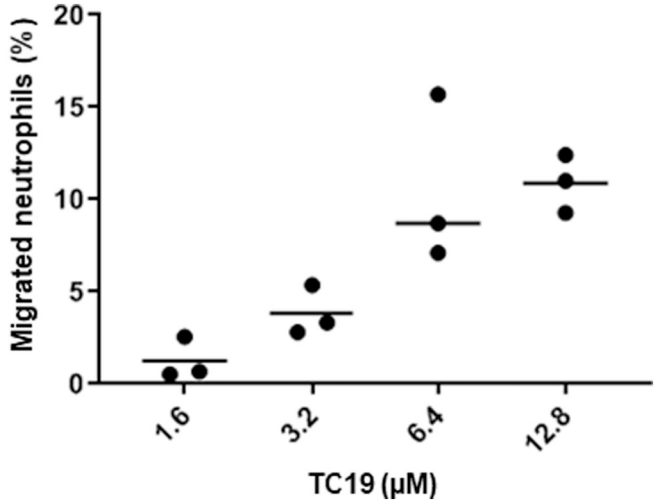

Fig. 5. Chemotactic activity of TC19. Human neutrophils were applied to the upper compartment of a transwell system equipped with a 3.0 μm filter and the peptide was introduced in the lower compartment. After 90 min at 37 °C the number of neutrophils that have migrated to the lower compartment was enumerated by flow cytometry. Results are expressed as the percentage of cells migrated in response to TC19 compared to that upon exposure to fMLP, which induced 8.2% (range 8.3–6.8) of the neutrophils to migrate from the upper compartment to the lower compartment. Values are means of three donors.

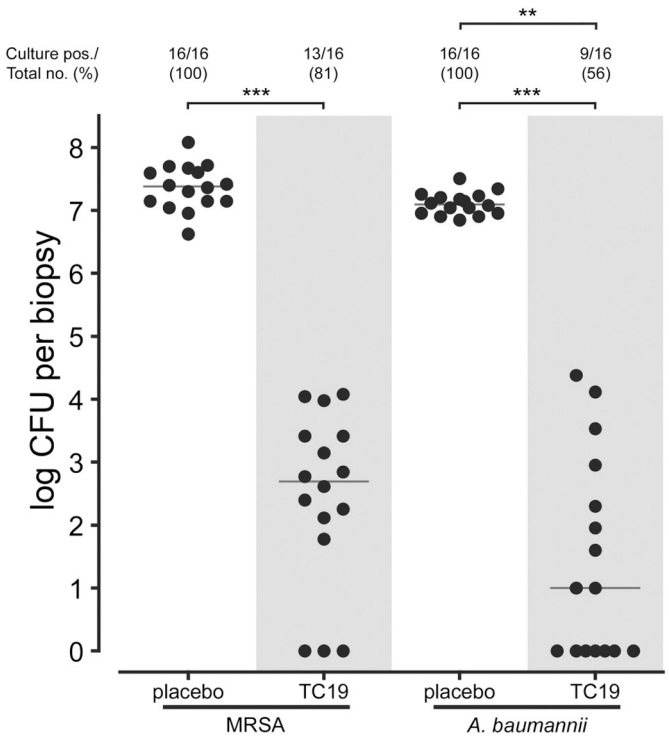

Fig. 6. Efficacy of 2% wt/wt TC19 hypromellose ointment against drug-resistant bacteria in a murine wounded skin infection model. The skin of the mouse was abraded, infected with 1×10^7 CFU MRSA LUH14616 (left) or MDR *A. baumannii* RUH875 (right) and treated with ointments containing no peptide (placebo) or 2% TC19 following infection. After 4 h, the number of viable bacteria (log CFU) in the skin and the frequency (expressed as percentage) of culture-positive skin samples was determined. The horizontal line represents the median value. Frequencies of culture-positive samples and log numbers of CFUs were analyzed by Fisher's exact and Mann-Whitney tests, respectively. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

and water *ad libitum*. Per bacterial strain, mice were randomized over the experimental groups. The mice were randomized over the different groups using the online random sequence generation at www.random.org. The investigators were blinded for the group allocation during the experiment and processing of the outcome.

The mouse superficial skin wound infection study was adapted from [24]. Briefly, the back of the mice was shaved using an electric razor one day before the experiment. The mice were anaesthetized with 2% (*v*/v) isoflurane (Pharmachemie) in oxygen in a laminar flow cabinet, followed by a subcutaneous injection of buprenorphine (Temgesic, RB Pharmaceuticals Limited; 0.05 mg/kg) 15 min prior to the start of the experiment for pain control. Mice were kept under anaesthesia and at an optimal body temperature with a temperature-controlled heat mat during the entire experiment. The backs of the mice were disinfected with 70% ethanol. At the middle of the back, the skin in an area of approximately 2 cm^2 was stripped with Tensoplast (Smith & Nephew Medical), an elastic adhesive bandage. By tape stripping 7 times in succession, with replacing the tape after the first time, the skin became visibly damaged, characterized by reddening and glistening but no bleeding. After stripping of the skin, 5 μl of the bacterial inoculum containing 1×10^7 CFU were applied to the stripped skin. Hypromellose-based ointments containing 2% (wt/wt) of peptide or no peptide (placebo) were prepared as described previously [25]. Ten min post-infection, approximately 30 mg of ointment was applied to the infected stripped skin, and covered with a circular piece of parafilm (Ø 8 mm). As control, placebo ointment was applied on the skin. After 4 h mice were sacrificed by cervical dislocation. The skin was separated from the underlying fascia and muscle tissue and approximately 2 cm^2 of skin from the infected area was excised. The skin was homogenized in 0.5 ml saline using 5 zirconia beads (Ø 2 mm, BioSpec Products) and the MagnaLyser System (Roche), with 3 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. Groups of 8 mice with 2 infected wounds each ($n = 16$ samples per group) were used in this study.

2.11. Statistics

All statistical analyses were performed with Graphpad Prism. Two-sample comparisons were made using a two-tailed 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*-values of ≤ 0.05 were considered significant.

3. Results

3.1. Peptide TC19 has improved bactericidal activity

The hydrophobicity of the central domain of peptide L3-I14K was enhanced by replacement of one, *i.e.* in TC17 and TC18, or both threonine residues, *i.e.* in TC19, by tryptophan residues (Table 1). As these replacements may affect the functional activities of the peptide, we first compared the bactericidal activity of TC17, TC18 and TC19 against the ESBL-producing *E. coli* and *S. aureus* JAR060131 in various conditions, including 50% (*v*/v) human plasma as a stringent model for complex biomatrices. TC17 and TC18 showed a 2-fold enhanced activity against *E. coli* in PT buffer compared to L3-I14K but lacked bactericidal activity in PBS (Table 1). Of note, the activity against *E. coli* was not assessed in plasma, as this strain proved to be plasma sensitive. TC17 and TC18 showed 2- to 4-fold enhanced activity against *S. aureus* in PT, but lacked activity in PBS and PBS supplemented with plasma. In contrast, TC19 was effective in killing both *E. coli* and *S. aureus* in PBS and *S. aureus* in PBS with 50% plasma (Table 1). Therefore, we selected peptide TC19 for further characterization.

3.2. TC19 is selective for bacterial membrane mimics

To investigate the selectivity of TC19, the interactions between the peptide and vesicles composed of POPC and POPG as mimics of the mammalian and bacterial cytoplasmic membranes, respectively [26–28], were determined. Addition of TC19 to large unilamelar vesicles (LUVs) of the bacterial membrane mimic POPG resulted in a concentration dependent permeabilization reaching full leakage at 4 μM TC19 (Fig. 1A). In contrast, no permeabilization was observed using LUVs of the mammalian membrane mimic POPC at concentrations up to 16 μM, the highest concentration tested. In accordance, TC19 had no effect on the thermotropic phase behavior of DPPC (mammalian mimic), but markedly affected the thermotropic behavior of DPPG (bacterial mimic) (Fig. 1B and Table 2): at 2 μM TC19 abolished the pre-transition (at 33.8 °C for pure DPPG), broadened the phase transition and more importantly dramatically decreased the main transition enthalpy by about 80%. These biophysical experiments clearly demonstrate that TC19 selectively interacts with bacterial membrane mimics. To further substantiate these findings, we assessed the cytotoxic activity of TC19 against human fibroblasts. Up to 40 μM TC19 did not permeabilize human fibroblasts, whereas at 80 μM an increase in PI-positivity was recorded after 4 h of exposure (Fig. 1C).

We verified the proposed permeabilization of the plasma membrane by TC19 in live bacteria using flow cytometric analysis of propidium iodide (PI) influx. TC19 dose-dependently permeabilized the membranes of *S. aureus* in a time-dependent manner (Fig. 1D). Within 120 s after exposure to 7.5 or 15 μM of TC19 > 40 or 75% of the *S. aureus* cells were permeabilized, respectively.

3.3. TC19 kills multi-drug resistant bacteria

Next, we determined the bactericidal activity of TC19 against different MDR and PDR strains of species of the ESKAPE panel. All strains except *E. cloacae* were killed at low concentrations in PBS; *E. faecium*, *S. aureus*, *K. pneumonia* and *A. baumannii* were also killed at low micromolar concentrations (3.2–12.8 μM) in PBS with 50% (v/v) human plasma (Table 3). However, PDR *P. aeruginosa* and MDR *E. cloacae* were not killed in PBS with 50% plasma (Table 3).

These results were obtained after 2 h of exposure to TC19. To assess the kinetics of killing, we tested the survival of bacteria upon exposure to TC19 over time. The peptide killed *S. aureus* in a time-dependent manner with a steady decrease in CFU over time when exposed to 3.75 μM TC19, *i.e.* 0.5-fold of the LC99.9 concentration in PBS, resulting in an approximate 2.5-log reduction in CFU after 4 h of incubation (Fig. 2). When exposed to 7.5 and 15 μM of TC19 ≥99.9% of the *S. aureus* were killed within 30 min.

3.4. TC19 induces minor resistance development

The possibility that *S. aureus* and MDR *A. baumannii* develop resistance to TC19 was assessed *in vitro* by serial passaging. The MIC values of TC19 for 4 independent replicates of *S. aureus* all showed variations in MIC over the course of the experiment with a maximal 8-fold increased MIC at 20 passages (Fig. 3). At the end of the experiment one replicate had the same MIC as at the start of the experiment, *i.e.* 15 μM, and the other replicates had a 2- to 4-fold increased MIC. For comparison, all independent replicates of *S. aureus* had at least a 4096-fold increased MIC (from 0.125 to ≥512 μg/ml) for the antibiotic rifampicin within only 6 passages. Resistance development in MDR *A. baumannii* to TC19 and – for comparison – the antibiotic ciprofloxacin showed similar results, with resistance developing only against the antibiotic (Fig. 3). Thus, *S. aureus* and MDR *A. baumannii* quickly developed resistance to antibiotics, but only a minor reduction of susceptibility to TC19.

3.5. TC19 has immunomodulatory properties

To determine whether TC19 has immunomodulatory properties, we assessed its ability to neutralize bacterial cell envelope inflammatory compounds to induce cytokine production by human blood cells. TC19 inhibited > 50% of LPS-induced IL-12p40 production at concentrations as low as 0.01–0.05 nM (Fig. 4A). In addition, TC19 at a final concentration of 0.195 μM or higher inhibited > 50% of IL-8 production upon exposure to UV-inactivated *S. aureus* JAR060131 (Fig. 4B). For comparison, the known immunomodulatory antimicrobial protein LL-37 inhibited LPS-induced IL-12p40 production at similar concentrations as TC19 (> 50% inhibition at 0.01–0.05 μM; Fig. 4C), whereas higher concentrations were required to neutralize *S. aureus* (0.391–1.563 μM LL-37 resulted in > 50% inhibition of IL-8 production in response to *S. aureus*, Fig. 4D).

In addition, we assessed the chemotactic activity of TC19. Results revealed that TC19 dose-dependently enhanced the migration of human neutrophils from the upper compartment of the transwell system through the filter to the lower compartment. Maximal chemotactic activity of the peptide was seen at 12.8 μM (Fig. 5), corresponding to 10% of the activity of 10 nM fMLP.

3.6. TC19 formulated in hypromellose ointment is effective against MRSA as well as MDR A. baumannii superficial skin wound infections in vivo

Finally, we assessed the effect of a TC19-containing hypromellose ointment against MRSA and MDR *A. baumannii* in a murine superficial skin wound infection model. Approximately 10 mins post inoculation of abraded skin, a 2% (wt/wt) TC19 ointment was applied for 4 h on the infected wound. This single exposure resulted in a significant 4.7-log ($p < 0.001$) and 6.1-log ($p < 0.001$) reduction in MRSA LUH14616 and MDR *A. baumannii* RUH875 counts, respectively (Fig. 6). Moreover, the numbers of positive MDR *A. baumannii* skin cultures was significantly reduced from 100% after placebo treatment to 56% after TC19 ointment treatment ($p = 0.0068$).

4. Discussion

Development of novel antimicrobial agents with a mode of action different from current antibiotics is top priority in the fight against multidrug-resistant (MDR) bacteria. In this study we show that TC19 could be a promising candidate for further development of such an agent. This conclusion is based on the following findings. First, TC19 was highly effective against MDR strains of ESKAPE bacteria *in vitro* and in a model for superficial skin wound infections in mice, while the cytotoxicity to human dermal fibroblasts was limited. This preferential interaction with bacterial cells over mammalian cells was confirmed by the results from experiments involving mimics of bacterial and mammalian cell membranes. Secondly, *in vitro* induction of resistance to TC19 in bacteria was minor. Finally, TC19 ameliorated LPS-induced and *S. aureus*-induced pro-inflammatory cytokine production by blood leukocytes while being chemotactic for human neutrophils, indicating that the peptide may balance the inflammatory responses in the infected host.

Biophysical experiments involving membrane mimics suggest that TC19 inserts into and subsequently disorders lipid bilayers *in vitro*. In agreement, PI uptake by bacteria upon TC19 exposure indicates that membrane permeabilization is the dominant mechanism of the bactericidal action of TC19. However, it was noted that maximally about 70% of the bacteria were permeabilized upon TC19 exposure, indicating that mechanisms other than plasma membrane permeabilization may also contribute to the killing of bacteria by TC19. In this respect, transcriptome analysis of *Bacillus subtilis* exposed to sub-lethal concentrations of TC19 showed that this peptide induced a cell envelope stress response [29]. Using *B. subtilis* mutants it was established that the cell wall synthesis stress response was associated with

delocalization of essential membrane bound proteins involved in cell wall synthesis. Moreover, TC19 caused membrane leaks at the site of membrane insertion by altering the organization and fluidity of the membrane [30] similar to the cyclic peptide cWFW [31]. *S. aureus* cells also showed fluid membrane domains already at 5 min after treatment with TC19, likely contributing to the killing of this species as well [30].

TC19 neutralized the ability of LPS and inflammatory cell envelope components of *S. aureus* to stimulate pro-inflammatory cytokine production by blood cells, while exerting moderate chemotactic activity for human neutrophils. TC19 is derived from TC-1, which is identical to the CXC chemokine CXCL7 except for a 2 residue C-terminal deletion [32]. Chemotaxis by CXC chemokines such as CXCL7 and IL-8 is mediated by the ELR sequence in the N terminus of these proteins [33]. Although in TC19 the ELR sequence is only partially present, as “LR”, the peptide does have moderate chemotactic activity. This suggests that either the LR sequence is sufficient in this peptide, or that other structural elements are responsible for the activity. To summarize, by enhancing recruitment of immune cells on the one hand and decreasing pro-inflammatory cytokine production on the other, TC19 may contribute to a balanced host response, which is particularly important for wound repair [34].

In our previous study, we used PBS and PBS with 50% plasma to test a set of novel antimicrobial peptides that showed promising antimicrobial activities in preclinical or clinical trials [15]. The antimicrobial activity of most of these peptides was lower in PBS with 50% plasma than expected based on the activities described in literature, where cation-adjusted Mueller Hinton broth or phosphate buffer were often used to test the antimicrobial activities. This indicates that testing in PBS with 50% plasma is not only a clinically more relevant environment, but also a more stringent condition.

TC19 showed promising results in the mouse superficial skin wound infection model with MRSA and MDR *A. baumannii*. A limitation of this study is that the activity of the peptide was assessed in a superficial wound infection model only. Therefore, in order to assess the full potential of TC19 for wound treatment, further experiments into the effects of formulated TC19 on bacteria in deeper wounds, abscesses, and/or chronic wounds are required. If successful, the rapid clearance of bacteria from the wound by TC19 will prepare the wound for the next phase of the healing process. In this connection, several antimicrobial peptides have been shown to possess direct wound healing capacity, such as histatin [35], esculentin [36] *in vitro*, and LL-37 in patients with hard-to-heal venous leg ulcers [37]. In addition, we have found that TC19 does not negatively affect the viability of fibroblasts, important cells in the tissue repair phase of skin injury. Together, TC19 may combine antibacterial activities with a balanced inflammatory response and possibly direct wound healing properties.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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