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In vivo efficacy of the antimicrobial peptide ranalexin in combination with the endopeptidase lysostaphin against wound and systemic meticillin-resistant *Staphylococcus aureus* (MRSA) infections

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ABSTRACT

New treatments are urgently required for infections caused by meticillin-resistant *Staphylococcus aureus* (MRSA) as these strains are often resistant to multiple conventional antibiotics. Earlier studies showed that ranalexin, an antimicrobial peptide (AMP), in combination with lysostaphin, an antistaphylococcal endopeptidase, synergistically inhibits the growth of MRSA, meaning that it deserved consideration as a new anti-*S. aureus* therapy. Using haemolysis and Vero cell viability assays, ranalexin with lysostaphin is proven to be non-toxic at antibacterial concentrations. In human serum, ranalexin with lysostaphin is significantly more effective against MRSA than treatment with either component alone. In a rabbit model of wound infection, ranalexin with lysostaphin reduced MRSA in the wound by ca. 3.5 $\log_{10}$ colony-forming units (CFU) compared with the untreated control. The combination is significantly more effective than treatment with ranalexin or lysostaphin alone. In a mouse model of systemic infection, ranalexin with lysostaphin reduced MRSA kidney burden by ca. 1 $\log_{10}$ CFU/g compared with untreated controls or treatment with ranalexin or lysostaphin alone. Importantly, the combination is synergistically bactericidal against various *S. aureus* isolates *in vitro*, including those with reduced susceptibility to lysostaphin or vancomycin. Ranalexin and lysostaphin could be incorporated in wound dressings for the prevention and treatment of topical *S. aureus* infections. That AMPs can enhance the antibacterial effectiveness of lysostaphin *in vivo* highlights a new avenue of research in the fight against drug-resistant staphylococci.

1. Introduction

Staphylococcus aureus is a serious opportunistic pathogen that can infect open wounds such as burns, ulcers and sites of surgery and can cause potentially lethal systemic infections [1]. Meticillin-resistant *S. aureus* (MRSA) strains are a particular problem as infections caused by these pathogens are more difficult and expensive to treat [2,3]. Moreover, MRSA strains can be resistant to multiple antibiotics, meaning that effective treatment can be unreliable, especially with the emergence of strains with reduced susceptibility to the 'antibiotic of last resort', vancomycin [4]. Thus, new treatments for topical and systemic MRSA infections are urgently required.

Antimicrobial peptides (AMPs) are considered to have potential as new therapeutic agents for treating drug-resistant bacterial infections [5–7]. Concomitantly, the antistaphylococcal endopeptidase lysostaphin has attracted attention in the treatment of drug-resistant *S. aureus* infections [8,9]. Recently, it has been shown that combinations of AMPs with lysostaphin achieve enhanced or synergistic antibacterial effects against *S. aureus* and MRSA compared with the compounds individually [10,11]. One such synergistic combination consists of lysostaphin with ranalexin, a cationic AMP originally isolated from the North American bullfrog, *Rana catesbeiana* [12]. This bactericidal combination kills bacteria within 5 min, is effective in low pH and high salt conditions and, importantly, is effective on human skin [11]. Moreover, individually ranalexin and lysostaphin are efficacious and non-toxic in animal models of bacterial infection [6,13,14]. These earlier observations suggested that ranalexin in combination with lysostaphin warranted further assessment for its ability to treat MRSA infections *in vivo*.

The present study was aimed to assess whether the combination of ranalexin with lysostaphin was effective in the treatment of wound and systemic MRSA infections in animal models and whether this combination was more effective compared with each component individually.

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2. Materials and methods

2.1. Reagents, culture media and microorganisms

High-performance liquid chromatography (HPLC)-grade reagents (or the highest grade available) and culture media were purchased from Sigma Aldrich Ltd. (Poole, UK). Ranalexin (>95% purity) and recombinant lysostaphin (>93% purity) were sourced as previously described [11]. Stocks and all other solutions were made with ultrapure deionised water unless stated. Solutions and media were autoclaved at 121 °C for 15 min or filter-sterilised (polyethersulfone 0.22 μm; Millipore, Watford, UK). Stocks of the following *S. aureus* isolates were kind gifts: meticillin-resistant strain MRSA252 and meticillin-susceptible *S. aureus* (MSSA) strain MSSA476 (from Prof. Mark Enright, Imperial College London, UK); BB270, Newman and four lysostaphin-resistant mutants (from Dr Angelika Gründling, Imperial College London, UK); six isolates with reduced susceptibility to vancomycin [vancomycin-intermediate *S. aureus* (VISA) isolates] (from Dr Sue Lang, Glasgow Caledonian University, UK); RN4220 and SH1000 (from Prof. Simon Foster, University of Sheffield, UK); and MRSA4 (Huntingdon Life Sciences, Alconbury, UK). *Staphylococcus epidermidis* was an in-house clinical isolate.

2.2. Efficacy in human serum

Briefly, 48 μL of MRSA252 cell suspension [1×10^6 colony-forming units (CFU)/mL, exponential phase] made up in heat-inactivated human serum (Lonza, Wokingham, UK) was added to each well of a 96-well plate. The bacterial inoculum had been cultured in a shaking incubator (37 °C, 220 rpm) from a single colony in tryptone soya broth (TSB). Then, 2 μL of stock solutions were added to experimental wells to give final well concentrations of 25 mg/L ranalexin alone, 0.125 mg/L lysostaphin alone or these compounds together at these concentrations; water only was added to control wells. Each treatment was performed in quadruplicate. Following incubation (ca. 25 °C, 35 min), viable bacteria were determined for each well by serial dilution in phosphate-buffered saline (PBS) [for 1 L: 8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4 (pH 7.3)] and plating on tryptone soya agar (TSA). Plates were incubated (37 °C, 24 h) and colony counts were performed. The number of colonies recovered from replicate wells for each treatment was transformed by $\log_{10}$ and then averaged to give a mean value.

2.3. Haemolysis assay

To each well of a 96-well plate was added 96 μL of 2% defibrinated horse blood (Oxoid, Basingstoke, UK) in PBS (ca. 1.3×10^8 erythrocytes/mL) and 4 μL of various stock solutions to give final well concentrations of 10–100 mg/L ranalexin, 0.1–5 mg/L lysostaphin or these compounds in combination; water only was added to negative control wells. For positive controls, erythrocytes were suspended in 1% sodium dodecyl sulphate in water, which causes 100% haemolysis. The plate was incubated (37 °C, 4 h, shaker speed 2) on a microplate spectrophotometer (PowerWave XS with KC4 software; Bio-Tek Instruments Inc., Winooski, VT) with A_{570} readings collected every 2 min. A reduction in A_{570} indicated haemolysis.

2.4. Mammalian cell toxicity assay

Mammalian cell toxicity was assessed using the neutral red assay modified from Babich and Borenfreund [15]. Vero cells, routinely cultured in 75 cm^2 flasks (5% CO_2, 95% humidity, 37 °C, ca. 72 h) in 5 mL of RPMI 1640 medium supplemented with 10% bovine calf serum (Cambrex Corp., East Rutherford, NJ), penicillin (50,000 U/L) and streptomycin (50 mg/L), were exposed to trypsin and harvested with fresh medium. Then, 40,000 cells (200 μL) were added to each well of a 96-well plate and the plate was incubated for 24 h to allow cell monolayers to develop. The medium was then removed and replaced with 196 μL of fresh medium and 4 μL of various stock solutions to give the same concentrations of ranalexin and lysostaphin as described in Section 2.3. The plate was incubated for 48 h. The medium was removed, the cells were washed with 200 μL of PBS and then 100 μL of fresh medium supplemented with neutral red (100 mg/L) was added to each well. The plate was incubated (90 min), the medium was removed and the cells were washed with PBS before the addition of 200 μL of acidified isopropanol (0.33% HCl) to each well. A_{540} was determined for each well, with lower A_{540} values indicating reduced cell viability.

2.5. Spectrum of activity

To determine the spectrum of activity, 96 μL of bacterial suspensions in TSB (1×10^6 CFU/mL, exponential phase) and 4 μL of stock solutions were added to wells on a 96-well plate to give various final well concentrations of ranalexin alone, lysostaphin alone or ranalexin with lysostaphin; to control wells was added water only. Each treatment was performed in quadruplicate. Bacterial inocula were prepared as described in Section 2.2 in either TSB, TSB supplemented with 2 mg/L vancomycin for the VISA isolates or TSB supplemented with 10 mg/L erythromycin for the lysostaphin-resistant mutants (as erythromycin is the selectable marker for these transposon mutants from the Phoenix library [16]). The 96-well plate was incubated (37 °C, 24 h, 1000 rpm) on a microplate thermoshaker (PHMP; Grant Instruments Ltd., Shepreth, UK). Following incubation, viable bacteria were determined for each well by serial dilution in PBS and plating on TSA as described in Section 2.2. Bactericidal synergy is defined as a >2 $\log_{10}$ CFU/mL reduction by the combination treatment compared with its most active constituent, providing that the number of viable bacteria recovered from the combination treatment is >2 $\log_{10}$ CFU/mL lower than the starting inoculum [17]. Furthermore, at least one of the components in the combination must not affect the viability of the test organism when used alone.

2.6. Rabbit model of MRSA wound infection

Animal experimentation was performed by Huntingdon Life Sciences and adhered to their in-house ethics policies. The following method is modified from Morton and Malone [18]. New Zealand White rabbits (1.71–3.31 kg, 10–15 weeks old, outbred) were housed in polypropylene or metal cages with grid floors operating a 12 h:12 h light:dark cycle (18 °C, 55% relative humidity) and given access to food and water ad libitum. Each rabbit was anaesthetised with isoflurane and a 3 cm × 3 cm area of the dorsal thoracic region was shaved and disinfected with 5% chlorhexidine digluconate. The area was swabbed and plated on mannitol salt agar (MSA) but no contaminants were ever recovered. A circular wound (ca. 1.5 cm diameter) was made by excision (including the panniculus carnosus and adherent tissues) and inoculated with ca. 1×10^9 CFU of MRSA4 in 0.2 mL of PBS (prepared from surface growth on TSA). The wound was covered with a sterile Hilltop chamber (gauze moistened with saline), which was secured with a dressing and further covered to prevent exposure to the air.

To recover from the anaesthetic, each animal received subcutaneous injections of 0.05 mg/kg buprenorphine immediately after wounding and at 8, 16 and 24 h thereafter. Then animals were

assigned to five treatment groups (two male and two female rabbits for each experimental group and one male and one female rabbit for the two control groups): (i) PBS (untreated control); (ii) ranalexin 0.225 mg; (iii) lysostaphin 0.0045 mg; (iv) ranalexin 0.225 mg + lysostaphin 0.0045 mg and (v) vancomycin 6 mg (positive control). These doses were administered at 24, 48 and 72 h post-inoculation in 0.3 mL of PBS by moistening the gauze of fresh Hilltop chambers. Every 24 h, gauzes from the Hilltop chambers were removed from the wounds and were assessed for MRSA4 burden by vortexing the whole gauze in PBS, serially diluting the liquid and plating on MSA supplemented with enrofloxacin (MSA-E) to select for MRSA4. At 96 h post-inoculation, the rabbits were humanely sacrificed with pentobarbital and the wound area was carefully excised, homogenised in PBS, diluted and plated on MSA-E to assess MRSA4 burden. Group sizes for the untreated control and the vancomycin positive control were smaller than the experimental groups owing to the high cost of this experimentation. Furthermore, the key aim in this experiment was to determine whether there was a beneficial effect of combining ranalexin with lysostaphin compared with the individual components alone.

2.7. Murine model of systemic MRSA infection

Female ICR CD-1 mice (22–26 g, 4–6 weeks old) were housed in vented polycarbonate cages operating a 12 h:12 h light:dark cycle (21 °C, 55% relative humidity) and given access to food and water ad libitum. Each mouse was inoculated intravenously via the tail vein with ca. 1.7×10^7 CFU of MRSA4 (prepared in 0.2 mL of 2% mucin in beef broth from surface growth on TSA). Animals were randomly assigned to five treatment groups (eight mice per group): (i) PBS; (ii) ranalexin 12 mg/kg; (iii) lysostaphin 0.1 mg/kg; (iv) ranalexin 12 mg/kg + lysostaphin 0.1 mg/kg and (v) vancomycin 50 mg/kg. Doses (all in PBS) were administered intravenously at 2, 26 and 50 h post-inoculation (dose volume 10 mL/kg) with the exception of vancomycin, which was administered by subcutaneous injection into the skin around the neck. Mice were inspected for adverse clinical signs at inoculation, every 2 h for the first 6 h and then at 6 h intervals. Body weights were recorded prior to inoculation and then at 20, 44 and 68 h. At 74 h post-inoculation, animals were sacrificed and MRSA4 kidney burden was assessed by excising the kidneys. Kidneys were weighed, homogenised in PBS (in pairs), serially diluted and plated on MSA. Plates were incubated (37 °C, 24 h) and colony counts were performed and converted to CFU/g of tissue.

2.8. Statistical analyses

All statistical tests were performed using SPSS v15.0 for Windows (SPSS Inc., Chicago, IL). For the human serum and *in vivo* data, $\log_{10}$ CFU reductions (per mL, per g or in total) were compared between groups by Mann–Whitney *U*-test (one-sided) employing Holm's correction to *P*-values for multiple comparisons [19]. For the haemolysis and Vero cell experiments, Kruskal–Wallis tests were performed on the raw data to compare the combination groups and the 50 mg/L ranalexin treatment and where significant (i.e. heterogeneity between groups detected) this was followed by subsequent Mann–Whitney *U*-tests. In all cases, $P \leq 0.05$ was considered statistically significant.

3. Results

3.1. Efficacy in human serum

To assess the usefulness of the combination for subsequent testing in animal models, an evaluation of efficacy was performed in human serum. Untreated controls showed no change in viable MRSA252 during 35 min of incubation [0.00 ± 0.03 $\log_{10}$ CFU/mL (mean ± 1 standard error)]. Ranalexin at 25 mg/L had little effect on bacterial abundance (increase of 0.10 ± 0.03 $\log_{10}$ CFU/mL), whilst lysostaphin at 0.125 mg/L reduced viable bacteria by 1.25 ± 0.37 $\log_{10}$ CFU/mL. In combination, ranalexin with lysostaphin reduced viable MRSA252 by 3.65 ± 0.14 $\log_{10}$ CFU/mL, which was a significantly greater reduction compared with treatment with ranalexin alone ($P < 0.05$) or lysostaphin alone ($P < 0.05$). Thus, even in human serum, which contains components that can inhibit the action of antibiotics [20], the combination is antibacterial and has enhanced efficacy compared with either ranalexin or lysostaphin alone.

3.2. In vitro toxicity

Ranalexin alone caused haemolysis and showed signs of toxicity to Vero cells at ≥50 mg/L, but lysostaphin alone caused no haemolysis and had no effect on Vero cell viability. In both assays, ranalexin in combination with lysostaphin was no more toxic than the ranalexin component alone (Vero toxicity, $H_{4,30} = 3.864$; $P > 0.05$; haemolysis, $H_{4,30} = 10.591$; $P < 0.05$, but Mann–Whitney *U*-tests confirmed that each combination caused no greater haemolysis compared with ranalexin alone) (Table 1). Reassuringly, the concentrations of the combination that were toxic to erythrocytes and Vero cells were in excess of those required for effective antibacterial activity in human serum.

3.3. Spectrum of activity

The synergistic antibacterial efficacy of ranalexin with lysostaphin was assessed against various strains of *S. aureus*, including lysostaphin-resistant mutants, clinically relevant VISA isolates and MRSA4 (used in animal infection models) as well as a strain of *S. epidermidis*. In combination, ranalexin with lysostaphin synergistically reduced the viability of each *S. aureus* isolate and the *S. epidermidis* strain (Table 2). The concentrations of ranalexin and lysostaphin selected for these experiments were dependent on the isolate under investigation, as the susceptibility of each isolate differed with respect to each component. In addition, while some of these concentrations are high enough to be toxic *in vitro*, they were selected to best highlight the synergistic interaction (lower, non-toxic concentrations are bactericidal but do not show the synergy as distinctly).

3.4. Rabbit model of MRSA wound infection

Wound dressings that had been treated with PBS, ranalexin alone, lysostaphin alone, ranalexin with lysostaphin, or vancomycin were applied to infected wounds for 24 h and then replaced with freshly treated dressings. Upon removal from the wound, the abundance of MRSA4 was assessed for each dressing to provide an indication of infection progression and bacterial survival on the dressing. During the experiment, mean viable MRSA4 recovered from the dressings reduced from ca. 7.5 $\log_{10}$ CFU at 24 h post-inoculation to ca. 3 $\log_{10}$ CFU at 96 h for those dressings containing the combination treatment (Fig. 1). At 96 h post-inoculation, the dressings containing the combination contained fewer viable MRSA4 than those containing lysostaphin alone by ca. 2 $\log_{10}$ CFU ($U = 3$; $n_1 = n_2 = 4$; $P > 0.05$) and ranalexin alone by ca. 3.5 $\log_{10}$ CFU ($U = 1$; $n_1 = n_2 = 4$; $P < 0.05$) (Fig. 1). These data confirm that the combination of ranalexin with lysostaphin is effective at reducing the viability of MRSA4 when soaked into a gauze dressing.

The combination treatment of ranalexin with lysostaphin reduced median viable MRSA4 recovered from infected wounds

Table 1
Toxicity of ranalexin and lysostaphin alone and in combination compared with untreated controls as assessed by (i) haemolysis of equine erythrocytes during 4 h incubation and (ii) reduction in viability of Vero cells during 48 h incubation.

Concentration of lysostaphin (mg/L)	Concentration of ranalexin (mg/L)	Haemolysis (%)	Reduction in cell viability (%)[a]
Ranalexin alone			
0	10	−3.54 ± 0.90[a]	3.97 ± 2.79
0	20	−0.07 ± 0.58	−3.15 ± 1.60
0	30	−0.18 ± 0.48	1.51 ± 1.38
0	40	0.62 ± 0.55	8.87 ± 2.28
0	50	20.0 ± 10.9	28.9 ± 9.56
0	100	44.5 ± 5.09	75.6 ± 4.23
Lysostaphin alone			
0.1	0	−3.35 ± 0.77	3.04 ± 1.94
0.5	0	−2.89 ± 0.78	−1.08 ± 1.66
1	0	−1.56 ± 0.40	2.71 ± 1.44
5	0	−3.32 ± 0.53	−3.13 ± 1.24
In combination			
0.1	50	26.0 ± 11.7	43.7 ± 2.83
0.5	50	4.04 ± 0.56	41.0 ± 5.66
1	50	−0.28 ± 0.70	36.9 ± 1.53
5	50	6.86 ± 2.44	43.6 ± 8.19

[a] Note that a negative value in the table indicates that haemolysis was less than the control and that Vero cell viability was greater than the control. In both assays, $n = 6$; ±1 standard error.

by ca. 3.5 $\log_{10}$ CFU compared with the untreated control and ranalexin alone ($U = 0$; $n_1 = n_2 = 4$; $P < 0.05$) groups and by ca. 2 $\log_{10}$ CFU compared with treatment with lysostaphin alone ($U = 1$; $n_1 = n_2 = 4$; $P < 0.05$) (Fig. 2). The combination was more efficacious than vancomycin treatment as the combination reduced viable MRSA4 by a further ca. 2.5 $\log_{10}$ CFU (Fig. 2). The combination treatment significantly reduced viable MRSA4 compared with lysostaphin alone and ranalexin alone, confirming that when soaked into a dressing this combination acts against MRSA in an infected wound with enhanced efficacy compared with treatment with each component of the combination alone.

3.5. Murine model of systemic MRSA infection

The combination treatment reduced median viable MRSA4 recovered from the animals' kidneys by ca. 1 $\log_{10}$ CFU/g compared with the untreated control or treatment with ranalexin alone or lysostaphin alone (Fig. 3). However, the combination treatment was no more effective statistically than treatment with lysostaphin alone ($U = 22$; $n_1 = n_2 = 8$; $P > 0.05$). In the positive control group (vancomycin 50 mg/kg), median viable MRSA4 recovered from the animals' kidneys reduced by ca. 4.5 $\log_{10}$ CFU/g, whilst no MRSA4 was recovered from four of eight animals, indicating complete clearance of infection in these cases.

Table 2
Effect on the viability of *Staphylococcus aureus* isolates and an isolate of *Staphylococcus epidermidis* during 24 h exposure to ranalexin, lysostaphin or ranalexin with lysostaphin.

Strain	Concentration (mg/L)		Mean ($\log_{10}$ CFU/mL)[a]				
	Ranalexin	Lysostaphin	Inoculum	Untreated control	Ranalexin alone	Lysostaphin alone	Ranalexin with lysostaphin
MSSA isolates							
MSSA476	32	0.125	6.42 (0.02)	9.96 (0.06)	9.73 (0.19)	9.80 (0.16)	2.00 (0.00)
Newman[b]	32	0.063	5.93 (0.22)	9.94 (0.06)	9.85 (0.07)	9.82 (0.05)	2.00 (0.00)
RN4220	48	0.023	6.20 (0.08)	9.28 (0.06)	9.38 (0.04)	9.39 (0.07)	4.14 (2.51)
SH1000	32	0.063	5.94 (0.07)	9.74 (0.07)	9.73 (0.06)	9.53 (0.12)	2.00 (0.00)
MRSA isolates							
BB270[c]	16	0.063	6.32 (0.13)	9.13 (0.60)	9.48 (0.07)	9.44 (0.06)	2.00 (0.00)
MRSA4	32	0.125	5.73 (0.19)	9.84 (0.03)	9.84 (0.05)	9.87 (0.08)	2.00 (0.00)
MRSA252	48	0.125	6.49 (0.04)	9.45 (0.11)	9.36 (0.17)	9.23 (0.39)	2.00 (0.00)
VISA isolates							
3700.W	64	0.25	6.02 (0.09)	9.12 (0.07)	8.97 (0.03)	9.51 (0.19)	2.43 (0.86)
3759.V	48	0.023	6.02 (0.21)	9.95 (0.04)	9.80 (0.04)	9.90 (0.02)	2.00 (0.00)
5827	128	0.063	6.32 (0.06)	9.85 (0.08)	8.67 (0.44)	9.85 (0.06)	2.00 (0.00)
5836	64	0.5	6.42 (0.08)	9.59 (0.11)	8.72 (0.16)	9.65 (0.07)	4.19 (1.66)
Mu3	24	0.5	5.67 (0.27)	9.75 (0.08)	9.40 (0.09)	9.64 (0.04)	2.00 (0.00)
Mu50[d]	128	3	5.34 (0.43)	7.82 (0.35)	6.15 (0.13)	8.48 (0.92)	2.61 (1.22)
Lysostaphin-resistant *S. aureus* (MSSA)							
ANG133 (Δ*femAB*)	12	0.375	5.73 (0.17)	10.00 (0.05)	9.61 (0.26)	9.95 (0.09)	3.08 (1.76)
ANG144 (Δ*lyrA*)	32	0.5	6.65 (0.09)	10.03 (0.03)	10.02 (0.05)	9.72 (0.16)	2.08 (0.15)
Lysostaphin-resistant *S. aureus* (MRSA)							
ANG365 (Δ*femAB*)	4	0.125	6.27 (0.05)	9.58 (0.07)	9.59 (0.04)	9.51 (0.07)	2.19 (0.39)
ANG366 (Δ*lyrA*)	16	0.25	5.88 (0.36)	9.50 (0.13)	9.64 (0.05)	9.55 (0.10)	2.00 (0.00)
S. epidermidis	24	16	5.73 (0.06)	9.46 (0.04)	9.28 (0.27)	9.10 (0.43)	2.00 (0.00)

MSSA, meticillin-susceptible *S. aureus*; MRSA, meticillin-resistant *S. aureus*; VISA, vancomycin-intermediate *S. aureus*; CFU, colony-forming units.
[a] The detection limit was 2 $\log_{10}$ CFU/mL. $n = 4$; data given as mean (standard deviation).
[b] Parent strain of ANG133 and ANG144.
[c] Parent strain of ANG365 and ANG366 [20].
[d] Exposure was 30 h for Mu50 as this is a slow-growing strain.

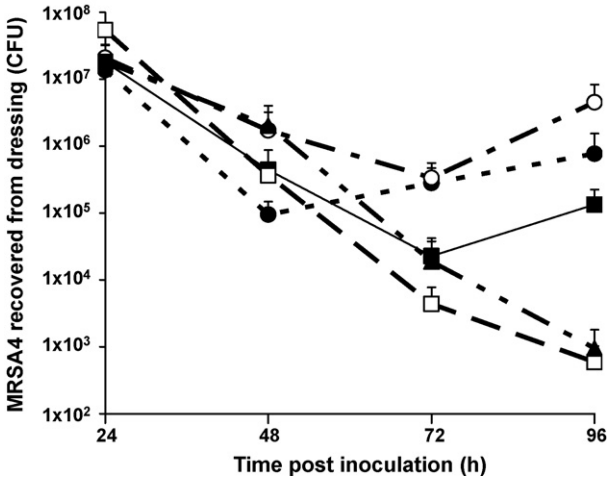

Fig. 1. Recovery of viable meticillin-resistant *Staphylococcus aureus* strain MRSA4 from gauze dressings that had been on infected wounds for 24 h periods. The first dressings removed from the wounds (at 24 h) had been treated with saline only. The dressings removed at 48, 72 and 96 h had been treated before application to the wound with phosphate-buffered saline only (●), 0.225 mg ranalexin (○), 0.0045 mg lysostaphin (■), 0.225 mg ranalexin + 0.0045 mg lysostaphin (□), or 6 mg vancomycin (▲). The limit of detection was 100 CFU/mL. Arithmetic means plotted; $n = 4$ (except for control and vancomycin groups where $n = 2$); error bars indicate +1 standard error. CFU, colony-forming units.

Body weights reduced each day in each treatment group after inoculation (data not shown), with the greatest change in the ranalexin treatment group (−16.3% at 68 h post-inoculation). Although the reduction in body weight in the combination treatment group (−10.5%) was less than the reduction in the lysostaphin only treatment group (−12.3%), this difference was not significant ($U = 23.5$; $n_1 = n_2 = 8$; $P > 0.05$). Body weights changed in the PBS and vancomycin treatment groups by −14.3% and −3.3%, respectively. In general, few adverse clinical signs were observed, but towards the end of the study (ca. 68 h) some animals showed slight piloerection (3/8 in the PBS-treated group, 7/8 in the ranalexin-treated group, 2/8 in the lysostaphin-treated group and 3/8 in the combination-treated group). In the combination treatment group, one animal developed a slightly bruised tail at 32 h. The animals' tails in the ranalexin treatment group showed slight bruising (5/8), slight swelling (1/8) and reddening (3/8), but these clinical signs were always mild.

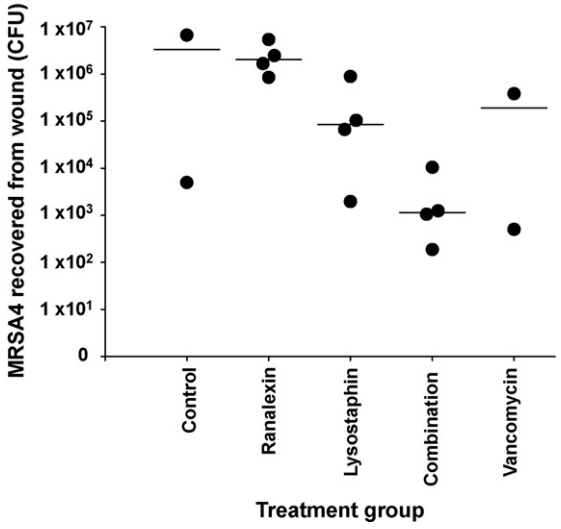

Fig. 2. Recovery of viable meticillin-resistant *Staphylococcus aureus* strain MRSA4 from infected wounds at 96 h after treatment at 24, 48 and 72 h with gauze moistened with phosphate-buffered saline only, 0.225 mg ranalexin, 0.0045 mg lysostaphin, 0.225 mg ranalexin + 0.0045 mg lysostaphin in combination, or 6 mg vancomycin. The limit of detection was 100 CFU/mL. $n = 4$ (except for control and vancomycin groups where $n = 2$); bar indicates median. CFU, colony-forming units.

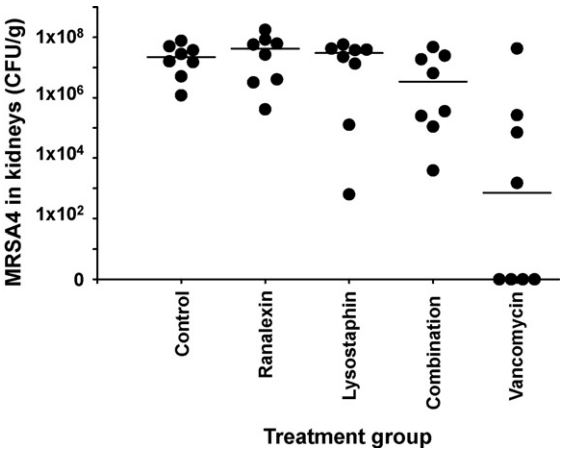

Fig. 3. Recovery of viable meticillin-resistant *Staphylococcus aureus* strain MRSA4 from infected animals' kidneys at 74 h post-inoculation after treatment (at 2, 26 and 50 h) with phosphate-buffered saline only, 12 mg/kg ranalexin, 0.1 mg/kg lysostaphin, 12 mg/kg ranalexin + 0.1 mg/kg lysostaphin, or 50 mg/kg vancomycin. The limit of detection was 100 CFU/mL. $n = 8$; bar indicates median. CFU, colony-forming units.

4. Discussion

In combination, ranalexin with lysostaphin reduces the burden of MRSA in infected wounds and treatment dressings to a greater extent than treatment with each component of the combination alone. This is the first demonstration that an AMP administered in combination with lysostaphin can achieve enhanced antibacterial activity *in vivo* compared with either component alone. The combination of ranalexin with lysostaphin synergistically kills MSSA, MRSA, VISA and lysostaphin-resistant strains of *S. aureus* and a clinical isolate of *S. epidermidis*. This combination is effective in human serum and reduces the bacterial burden in a mouse model of systemic MRSA infection.

In contrast to a previous report [12], ranalexin and combination treatments containing ranalexin were found to be toxic to erythrocytes and Vero cells *in vitro* at concentrations close to those needed for potent bactericidal activity *in vitro*. However, perhaps more importantly, only mild signs of toxicity were detected in the mouse systemic model, whilst there was no evidence for toxicity in the rabbit wound model. Moreover, other studies have shown ranalexin to be non-toxic at efficacious antibacterial concentrations *in vivo* [13,14], suggesting that the highly effective combination of ranalexin with lysostaphin could still have potential medical applications.

Topically applied agents are important in the prevention and management of wound infections. Compared with systemic antibiotics, topical therapies benefit from lower toxicity and higher local concentrations of the antibacterial agent, especially where the blood supply to the wound is poor [21]. Presently, all available antibacterial dressings contain broad-spectrum agents, but such agents and non-specific biocides can actually impair wound healing processes [22,23]. Moreover, broad-spectrum agents may kill microorganisms that live harmlessly on the skin or in the wound and provide niche protection against opportunistic pathogens [24]. A dressing containing ranalexin with lysostaphin could be used as a prophylactic to reduce the chances of contracting an infection, as its narrow spectrum of activity should not affect the normal skin flora but would prevent staphylococci from colonising the dressing itself or the wound area. Alternatively, the

dressing could be applied once an infection has become established. Also, wound exudate would not be expected to interfere with the combination's efficacy, as it remains effective in human serum and wound exudate contains similar components to serum [25].

Combination therapies greatly reduce the opportunity for resistant strains to be selected provided that two mechanisms of action are used by the drugs in question [26]. Furthermore, the narrow spectrum of the ranalexin with lysostaphin treatment reduces the opportunity for resistance to emerge in the normal bacterial flora, which is a mechanism recognised for the emergence and spread of resistance to pathogens [27]. Importantly, the present study shows that in combination ranalexin with lysostaphin is synergistically bactericidal against *S. aureus* strains with reduced susceptibility to lysostaphin, which is often characterised by mutation of the *fem* or *lyrA* genes [16,28]. *S. aureus* mutants with reduced susceptibility to ranalexin have not been found [29] and are thought to be rare due to its non-specific mode of action. The combination of ranalexin with lysostaphin was effective and synergistic against VISA isolates but an assessment is required for its effectiveness against further important strains, including community-acquired MRSA (CA-MRSA).

Additional medications are urgently required for MRSA owing to the emergence of resistance to the 'last resort' antibiotic, vancomycin [4], as well as the newly introduced agents daptomycin [30], linezolid [31], quinupristin/dalfopristin [32] and teicoplanin [33]. Concerns have been raised regarding the safety of lysostaphin with respect to inducing adverse immunological reactions; however, these responses seem unapparent even after prolonged or systemic application [34–38]. Hence, considering its efficacy, the combination of ranalexin with lysostaphin may be useful in the prevention and treatment of drug-resistant-*S. aureus* infections, especially for topical application in a gel [11] or incorporated into a wound dressing. That AMPs can enhance the antibacterial effectiveness of lysostaphin *in vivo* highlights a new avenue of research in the fight against drug-resistant staphylococci.

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