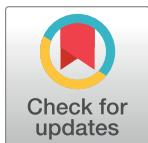


RESEARCH ARTICLE

Mode of action of the antimicrobial peptide Mel4 is independent of *Staphylococcus aureus* cell membrane permeability

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Abstract

Mel4 is a novel cationic peptide with potent activity against Gram-positive bacteria. The current study examined the anti-staphylococcal mechanism of action of Mel4 and its precursor peptide melimine. The interaction of peptides with lipoteichoic acid (LTA) and with the cytoplasmic membrane using DiSC(3)-5, Sytox green, Syto-9 and PI dyes were studied. Release of ATP and DNA/RNA from cells exposed to the peptides were determined. Bacteriolysis and autolysin-activated cell death were determined by measuring decreases in OD_{620nm} and killing of *Micrococcus lysodeikticus* cells by cell-free media. Both peptides bound to LTA and rapidly dissipated the membrane potential (within 30 seconds) without affecting bacterial viability. Disturbance of the membrane potential was followed by the release of ATP (50% of total cellular ATP) by melimine and by Mel4 (20%) after 2 minutes exposure ($p<0.001$). Mel4 resulted in staphylococcal cells taking up PI with 3.9% cells predominantly stained after 150 min exposure, whereas melimine showed 34% staining. Unlike melimine, Mel4 did not release DNA/RNA. Cell-free media from Mel4 treated cells hydrolysed peptidoglycan and produced greater zones of inhibition against *M. lysodeikticus* lawn than melimine treated samples. These findings suggest that pore formation is unlikely to be involved in Mel4-mediated membrane destabilization for staphylococci, since there was no significant Mel4-induced PI staining and DNA/RNA leakage. It is likely that the *S. aureus* killing mechanism of Mel4 involves the release of autolysins followed by cell death. Whereas, membrane interaction is the primary bactericidal activity of melimine, which includes membrane depolarization, pore formation, release of cellular contents leading to cell death.

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Introduction

S. aureus is a major cause of infections in both health care and community settings which can produce high levels of mortality and have a high economic burden on society [1, 2]. *S. aureus* can reside on the skin and in the nasal cavity of 20–50% humans [3, 4] and this poses a risk for

subsequent infections [5]. *S. aureus* is also a major cause of infections of medical devices and can cause 30–40% bacteraemia, surgical wound and implant-related infections [4, 6]. Methicillin-resistant *S. aureus* (MRSA) now causes over 50% of skin and soft tissue infections [7]. The mortality rate of *S. aureus* bacteraemia can reach up to 40% [8]. *S. aureus* associated infections are difficult to treat with currently available antibiotics [9] partly due to the increase in MRSA which are often very resistant to many different classes of antibiotics [6, 10]. To overcome these problems, new antimicrobials are needed which have unique modes of action and limited potential for resistance development.

Antimicrobial peptides (AMPs) exhibit broad spectrum antimicrobial activity against a wide range of microorganisms including bacteria, fungi, parasites and enveloped viruses at low concentrations [11–13]. AMPs are usually cationic in nature and have a varying number (from five to over a hundred) of amino acids. AMPs possess multiple modes of action, rapid bacterial killing kinetics and little toxicity toward human cells [14, 15]. Owing to the fact that these molecules exhibit multifaceted modes of action and rapidly kill bacteria, development of resistance in bacteria against AMPs is relatively rare [16, 17]. The mechanism of action of AMPs is believed to start by interacting with the negatively charged lipoteichoic acid (LTA) or teichoic acid of Gram-positive bacteria [18], through the negatively charge phosphate groups in LTA [19, 20].

The interaction with LTA is then believed to facilitate penetration of AMPs through the thick peptidoglycan layer, perhaps by the LTA acting as ladder, allowing the AMPs to reach and act on the cytoplasmic membrane [18]. AMPs disrupt phospholipid lipid bilayers of bacterial membranes by forming pores by various mechanisms called “barrel stave” or “toroidal pore” or through disintegration of lipids via the “carpet model” [21]. Cytoplasmic membrane collapse can result in leakage of cellular contents such as potassium ions, ATP and DNA/RNA which in turn may lead to cell death [22, 23]. Some AMPs translocate across the cell membrane and inhibit DNA/RNA or protein synthesis [24, 25]. AMPs can also kill Gram positive bacteria by activating cell wall bound autolytic enzymes known as autolysins [26]. LTAs, which are anchored in the cell envelope, control autolysin activity [27]. The interaction of AMPs with LTA can result in loss of regulation of autolysins which then cause autolysis by hydrolysing peptidoglycan chains and peptide bridges of murein [27].

Melimine is a cationic hybrid peptide of melittin and protamine [28]. It shows broad spectrum antimicrobial activity against Gram-negative and Gram-positive bacteria (including MRSA), fungi and protozoan such as *Acanthamoeba* without inducing resistance in bacteria [28, 29]. Melimine destroys the membrane potential of *S. aureus* [30]. Melimine, similarly to some other antimicrobial peptides such as Esculentin-1a and LL-37 [31], when covalently bound to polymers such as contact lenses inhibits initial bacterial attachment and can kill microbes [28, 29]. A shorter sequence of melimine, called Mel4, has a relatively low minimum inhibitory concentration against *S. aureus* (53.2 μM) and kills *S. aureus* when immobilized on surfaces [32]. It is non-cytotoxic to mammalian cells *in vitro* [28, 29]. Mel4 has improved ocular compatibility compared to melimine when bound to contact lenses [33]. Mel4 lacks the amino acids tryptophan, leucine and isoleucine that are present in the sequence of melimine. Tryptophan is a highly lipophilic amino acid [34] and its presence is often an important part of the activity to AMPs [35–37]. Leucine and isoleucine are hydrophobic residues which promote strong α-helix formation in AMPs which in turn results a higher degree of membrane disruption [38, 39]. Although various studies have shown that Mel4 has high bactericidal activity against staphylococci, the mode of action of Mel4 is not yet understood. Given the shorter peptide length and the unique amino acid sequence compared to melimine, an investigation of the killing mechanism of Mel4 against Gram-positive bacteria is necessary. Hence, this study was designed to evaluate the mode of action of Mel4 in comparison with melimine against *S. aureus*.

Materials and methods

Synthesis of peptides

Melimine (amino acid sequence: TLISWIKNKRKQRPRVSRRRRRGGRRR; molecular mass 3786.6) and Mel4 (KNKRKRRRRRGGRRR; 2347.8) were synthesized by conventional solid-phase peptide protocols [40, 41] and procured from the Auspep Peptide Company (Tullamarine, Victoria, Australia). The purity of the purchased peptides was ≥90%.

Bacterial strains and growth conditions

S. aureus 31 (contact lens induced peripheral ulcer isolate) [42], *S. aureus* 38 (microbial keratitis isolate) [43], and the type strain of *S. aureus*, ATCC 6538 (isolated from a “human lesion” according to the ATCC website) [44] were used in the current study. Bacteria were grown from frozen stocks overnight at 37 °C to mid-log phase in Tryptic Soy Broth (TSB; Oxoid, Basingstoke, UK) and cells were harvested following washing with phosphate buffer saline (PBS, NaCl 8 g/L, KCl 0.2 g/L, Na₂HPO₄ 1.4 g/L, KH₂PO₄ 0.24 g/L; pH 7.4) then diluted in the PBS containing 1/1000 TSB to OD_{600nm} 0.05–0.06 (1×10^7 colony forming units (CFU)/ml confirmed upon retrospective plate counts on TS agar (Oxoid)). Cells prepared this way were used in most experiments except for assessing the minimum inhibitory and bactericidal concentrations, measuring the release of DiSC3-5 from cells, and the autolytic activity assay.

Minimum inhibitory and minimum bactericidal concentration

The minimum inhibitory and minimum bactericidal concentrations of melimine and Mel4 were determined for all strains using a modified version of the Clinical Laboratory and Standard Institute (CLSI) broth microdilution method as described previously [45]. Bacterial suspensions were prepared to a final concentration of 5×10^5 CFU ml⁻¹ in Muller Hinton Broth (MHB, Oxoid) and added to wells in a sterile 96-well microtiter plate containing two-fold dilutions of each peptide with 0.01% v/v acetic acid (Sigma Aldrich, St Louis, MO, USA) and 0.2% w/v bovine serum albumin (Sigma Aldrich). The plate was incubated at 37 °C for 18 h–24 h. The MIC was set as the lowest concentration of peptides that reduced bacterial growth by ≥ 90% while the MBC was set as the lowest concentration of peptides that reduced bacterial growth by >99.99% after enumeration of viable bacteria by plate counts compared to bacteria grown without either AMP.

Interaction with LTA

Two experiments, bacterial growth in the presence of LTA and the amount of LTA in solution measured by ELISA, were performed to determine the interaction of melimine and Mel4 with LTA. Growth of *S. aureus* 38 was conducted in the presence of melimine or Mel4 following a method of Yang and Yousef [46]. *S. aureus* LTA (Sigma Aldrich; 100 µg/ml) was dissolved in MHB in wells of a 96-well microtiter plate containing *S. aureus* (5×10^5 CFU/ml) followed by addition of 100 µl of melimine or Mel4 to achieve their MICs. The OD_{600nm} was determined over time during incubation at 37 °C. A growth curve was constructed over 12 h. Controls were bacteria grown in the presence of LTA alone and bacteria grown in MHB alone (a measure of maximum bacterial growth).

A competitive ELISA using kit (My BioSource, San Diego, CA, USA) was performed to evaluate whether melimine and Mel4 could bind and neutralize LTA of *S. aureus*. LTA (200 ng/ml) was dissolved in endotoxin free water (Sigma Aldrich) with melimine or Mel4 at their MICs or MBCs as final concentrations and incubated for 1–2 h at 37 °C. Interaction of LTA

with melimine and Mel4 was assessed as an increase in OD_{450nm} compared to negative control without peptides according to manufacturer instructions.

Cytoplasmic membrane disruption

The effect of melimine and Mel4 on the cytoplasmic membrane of *S. aureus* was assessed in three experiments. To examine membrane depolarization, the release of the membrane potential sensitive dye DiSC3-5 was measured. Sytox Green and Propidium Iodide (PI) dyes were used to determine whether the peptides could damage the cytoplasmic membranes and allow the stains to penetrate and interact to intracellular nucleic acids. Sytox Green has a molecular mass of 213.954 g/mol and a topological polar surface area of 28.7 Å² [47] whereas PI has a molecular mass of 668.087 g/mol and a topological polar surface area of 55.9 Å² [48]. Penetration of these two dyes through compromised membrane occurs through pores and differences may indicate different pore structures/sizes created by peptides.

Cytoplasmic membrane depolarization was performed as described by Rasul *et al.*, [30]. Aliquots 100 µl bacteria labelled with 4 µM DiSC3-5 (Sigma Aldrich, St Louis, MO, USA) in HEPES and 100 µl of melimine and Mel4 at their MICs and MBCs (as final concentrations) were added to wells of 96 well microtiter plates. Increases in fluorescence were determined at 30 second intervals at an excitation wavelength of 622 nm and an emission wavelength of 670 nm over five minutes. Aliquots of bacteria were withdrawn over the time course and serially diluted in Dey-Engley (D/E neutralizing broth (Remel, Lenexa, KS, USA)) then plated on Tryptic Soy Agar (Oxoid, Basingstoke, UK) containing phosphatidylcholine (0.7 g /L) and Tween 80 (5ml/L) then incubated at 37 °C overnight. The number of live bacteria were enumerated and expressed as CFU/ml. A positive control of dimethyl sulfoxide (DMSO) (Merck, Billerica, MA, USA) HEPES (100 µl) was used to disrupt the cytoplasmic membrane potential of bacteria [49]. Initially a range of DMSO concentrations (1%, 2%, 5% and 20% (v/v)) were tested to determine which concentration gave maximum depolarization. 20% DMSO gave maximum concentration and used in the assay.

Sytox green assay was performed following the established protocol of Li *et al.*, [50]. The protocol was modified as to use TSB media instead of LB media, as TSB was used in all other experiments. Bacterial cells in PBS (100 µl) were dispensed into wells of 96-well plates along with 5µM Sytox green (Invitrogen, Eugene, Oregon, USA). Plates were incubated for 15 minutes in the dark at room temperature and then 100 µl of melimine or Mel4 were added so as to achieve their MICs and MBCs. Fluorescence was measured spectrophotometrically (at an excitation wavelength of 480 nm and an emission wavelength of 522 nm) every 5 minutes up to 30 minutes, and then after 150 minutes. Triton X-100 1% (v/v) (Sigma Aldrich, St Louis, MO, USA) in PBS with 1/1000 TSB (100 µl) was used as positive control to disrupt the cytoplasmic membrane of bacteria.

Flow cytometry was used to determine the ability of melimine and Mel4 to permeabilize the cytoplasmic membrane of *S. aureus* 38 only. Bacteria were incubated simultaneously with SYTO9 (7.5 µM) and PI (30 µM; Invitrogen, Eugene, Oregon, USA) and incubated at room temperature for 15 min. Fluorescence intensities were recorded with a LSRFortessa SORP Flow cytometer after addition of melimine or Mel4 at their MIC at different time points. The wavelength of green fluorescence was (525/550 nm) for SYTO9 and a red fluorescence (610/20 nm) for PI [51]. Data were acquired and analysed using Flowjo software (version 10.5.0, Oregon, USA). A minimum 20000 events were recorded for each sample.

Leakage of intracellular contents

Two assays, ATP release and loss of DNA/RNA. were performed to determine the leakage of intracellular contents. Bacterial suspensions (100 µl) were added to melimine and Mel4 at their

MICs and MBCs and incubated at 37°C for 10 minutes. Samples were withdrawn at two minutes intervals and centrifuged at 9000 × g for five minutes, and the supernatant removed and kept on ice until further analysis. For determination of total ATP, the bacterial pellet was resuspended in boiling 100 mM Tris, 4 mM EDTA pH (7.4) and further incubated for 2 mins at 100°C to lyse all the cells. The lysed cells were centrifuged at 9600 × g for two minutes and the supernatant was kept on ice until further use [52]. Subsequently, both total and released ATP were determined using an ATP bioluminescence kit (Invitrogen, Eugene, Oregon, USA) according to the manufacturer's instructions.

The loss of DNA/RNA was determined by following a method of Carson *et al.*, [53]. Bacteria (100 µl) were incubated at 37 °C with melimine and Mel4 at their MICs and MBCs. Samples were withdrawn at 5 minutes intervals, diluted (1:10) and filtered through 0.22 µm pores (Merck, Tullagreen, Ireland). The OD_{260nm} of the filtrates was measured in a UV-star plate (Greiner Bio-one GmbH, Frickenhausen, Germany). The results were expressed as the ratio to the initial OD_{260nm}.

Lysis of bacteria

The bacteria-lytic potential of the two peptides was evaluated using two different bacterial inoculums 1×10^8 CFU/ml (OD₆₆₀ 0.1) and 3×10^{10} CFU/ml. The smaller inoculum size was used to see whether OD_{620nm} was measurable at the concentration of cells used in all other assays. However, no measurable optical density change at 620nm was obtained, and so a larger inoculum of 3×10^{10} CFU/ml was used. The larger inoculum was obtained by adjusting OD_{620nm} to 0.3, and bacterial numbers (CFU/ml) were confirmed upon retrospective plate count. Melimine and Mel4 were added at their MICs and MBCs. Bacterial cultures were immediately mixed and then diluted 1:100 in (1/1000 TSB). The OD_{620nm} was measured and additional readings were taken at 30, 60, 90, 120 minutes, 6.5 and 24 h after incubating at room temperature. Peptides at their respective concentrations in 1/1000 TSB were used as blanks. The results were recorded as a ratio of OD_{620nm} at each time point compared to the OD_{620nm} at 0 minutes [53].

Time kill assay

Aliquots of bacterial cells (100 µl; 1×10^7 CFU/ml) were added to melimine or Mel4 at their MICs and MBCs (as final concentration) in 96 well plates and incubated at 37 °C with gentle shaking. Aliquots (100 µl) were withdrawn at specified time points, serially diluted into D/E neutralizing broth and plated onto Tryptic Soy agar containing phosphatidylcholine (0.7 g /L) and Tween 80 (5ml/L). Bacterial colonies were counted after 24 h of incubation at 37 °C. Viable bacteria were reported as log₁₀ CFU/ml. A control of bacterial suspensions without peptide was performed under the same conditions. The assay was performed in triplicate.

Release of autolysins

The presence of autolysins in supernatants of strains (*S. aureus* 31 and *S. aureus* 38) grown in the presence of melimine and Mel4 was determined spectrophotometrically by the ability of the supernatants to decrease the optical density of *Micrococcus luteus* peptidoglycan (PGN; Sigma Aldrich, St Louis, MO, USA) following a published method [54]. Bacteria were grown in MHB and harvested by washing three times with PBS. Bacteria were resuspended in the same PBS to an OD_{660nm} of 0.1 (1×10^8 CFU/ml). The bacterial suspension (100 µl) was incubated at 4X MIC of melimine or Mel4 for 5 h at 37 °C. Following centrifugation at 2000 × g for 10 minutes at 4 °C, the supernatant was recovered and filtered through 0.2 µm filter (Merck, Tullagreen, Ireland). The filtrate was saturated with 75% ammonium sulphate at 4 °C with

continuous mixing for 1 h. This mixture was centrifuged at $12000 \times g$ at 4 °C for 30 min, the supernatant was discarded, and the pellet was resuspended in 0.1 M sodium acetate buffer (pH. 6) and dialysed against four changes of buffer using Amicon ultra centrifugal filters (Merck, Millipore, Tullagreen, Carrigtwohill, IRL). The retentate was recovered and incubated with 100 µl of *Micrococcus luteus* peptidoglycan (1 mg/ml) at room temperature for 3 h. OD_{570 nm} was initially determined at 0 min and then every 30 min intervals until 3 h. Results were presented as a ratio of OD_{570 nm} at each time point versus OD_{570nm} at 0 minutes (in percentage). The retentate from the above assay was also used to detect the autolytic activity against a lawn of *Micrococcus lysodeikticus* ATCC 4698. Briefly, 10 µL of each retentate from the above assay was spotted onto a pre-seeded lawn of *M. lysodeikticus* (10^8 CFU/ml) on nutrient agar. Following incubation for 24 h at 37°C, the size of the zone of inhibition was measured. Lysozyme (5 mg/ml) used as a positive control for maximum growth inhibition. Melimine or Mel4 dissolved in PBS at 4X MIC was treated in exactly the same way but in the absence of bacteria, to test whether any of the inhibitory activity in both assays (PGN and lawn of bacteria) was due to the presence of these peptides.

Lysis of horse red blood cells

The hemolytic activities of both the peptides were determined using horse red blood cells (HRBCs; Sigma) as described previously [28]. The HRBCs were washed three times with PBS at $470 \times g$ for 5 minutes. Peptides (16.50 µM to 3407.36 µM, in PBS) were added to the washed HRBCs and incubated at 37°C for 4 h. After incubation, the cells were pelleted at $1057 \times g$ for 5 minutes, and supernatant was removed to assess the release of haemoglobin by measuring OD_{540nm}. HRBCs in PBS and HRBCs in distilled water were used as negative and positive controls, to achieve 0% and 100% lysis respectively. The relative OD of HRBCs treated with each peptide were compared to that treated with distilled water to determine the relative percentage of hemolysis. The therapeutic index of the peptides was calculated by the ratio of hemolytic activity and minimum inhibitory concentration.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 7.02 software (GraphPad Software, La Jolla, CA, USA). The effect of the different concentrations of peptides in each assay compared to controls was analysed using two-way ANOVA with Tukey's test of multiple comparisons. Correlations between release of extracellular ATP and bacterial death were examined using Pearson correlation test. Oneway ANOVA with Bonferroni test was used to analyse the hemolytic effect on horse red blood cells. Statistical significance was set as $p < 0.05$.

Results

Inhibitory concentrations of the two peptides

The MICs and MBCs for melimine or Mel4 against *S. aureus* are shown in Table 1. Both melimine and Mel4 were most active against *S. aureus* ATCC 6538. Melimine had lower MIC and MBC compared to Mel4 for all strains.

In all subsequent experiments data of the assays for *S. aureus* 38 is presented. Data for all other strains are presented in the supporting information (S1–S8 Tables). Both the AMPs displayed similar modes of action against all the strains of *S. aureus*.

Table 1. MIC and MBC values of melimine and Mel4 against *S. aureus*.

Bacterial strains	Melimine		Mel4	
	MIC (μM)	MBC (μM)	MIC (μM)	MBC (μM)
<i>S. aureus</i> 31	33.01	66.02	106.48	212.96
<i>S. aureus</i> 38	33.01	66.02	106.48	212.96
<i>S. aureus</i> ATCC 6538	16.50	16.50	53.24	53.24

MIC = minimum inhibitory concentration that kills $\geq 90\%$ of the positive control

MBC = minimum bactericidal concentration that kills $\geq 99.99\%$ of bacteria.

<https://doi.org/10.1371/journal.pone.0215703.t001>

Interaction with LTA

Melimine at its MIC (33.01 μM) and Mel4 at its MIC (66.02 μM) inhibited the growth of *S. aureus* (Fig 1) with $\text{OD}_{600\text{ nm}} < 0.2$ after 14 hours of growth. However, in the presence of LTA *S. aureus* could grow with significant increases in $\text{OD}_{600\text{nm}}$ starting after 6 h ($p < 0.001$; Fig 1) with both peptides. At 6 h, the $\text{OD}_{600\text{nm}}$ was not different between melimine-LTA and Mel4-LTA ($p = 0.601$). However, at 8 h, the $\text{OD}_{600\text{nm}}$ with Mel4-LTA was significantly higher ($p = 0.005$) than melimine-LTA. The presence of LTA alone did not affect the growth of *S. aureus* and growth pattern was similar as in MHB alone.

In the ELISA assay at their MICs, melimine neutralized 1.2 ± 0.1 ng LTA/nmol and Mel4 neutralized 1.1 ± 0.1 ng LTA/nmol ($p > 0.999$). Similarly, at their bactericidal concentrations they neutralized 0.8 ± 0.1 ng/nmol and 0.6 ± 0.1 ng/nmol respectively ($p > 0.777$).

Membrane disruption

Melimine depolarized the cytoplasmic membrane of *S. aureus* in a predominantly concentration independent manner. An increase in fluorescence intensity was detected as early 30 seconds after addition of either peptide to all strains of *S. aureus*. The increase in fluorescence was continuous until at 180 sec and became constant after this time until the last observation at 300 sec (Fig 2). The fluorescein intensity was not statistically different at the MIC and MBC of melimine ($p > 0.999$). However, at its MBC Mel4 yielded a significantly higher fluorescence than at its MIC at 60 to 90 sec ($p < 0.05$) and after this time the degree of depolarization was similar for both the concentrations. This depolarization of the cytoplasmic membrane was not directly associated with the bacterial death as only $< 0.5 \log_{10}$ bacteria were affected with both the peptides at either concentration (Fig 2).

The fluorescence of Sytox green increased over time only with melimine and no fluorescence was detected with Mel4. Melimine at its MIC or MBC permeabilized the cell membrane and the fluorescence increased significantly after 10 minutes of exposure compared to buffer treated controls ($p < 0.026$) (Fig 3). The intensity of fluorescence gradually increased over 150 minutes for both the concentrations of melimine against all strains, but no significant difference was observed between the MIC and MBC ($p > 0.999$). Treatment with the positive control Triton-X 100 resulted emission of higher Sytox green fluorescence compared to either peptides within 10 minutes ($p < 0.001$).

The membrane damaging effect of these peptides was also assessed with *S. aureus* 38 only by flow cytometry in the presence of the DNA intercalating dye PI and the membrane permeable dye Syto-9 (Fig 4). After 30 minutes exposure to melimine at its MIC, 22.2% of cells had high levels of Syto-9 staining but low levels of PI, 66.31% of cells had high levels of both Syto-9 and PI staining and 10.1% of cells had high levels of PI but low levels of Syto-9 staining. The total percentage of cells with high levels of PI staining after 30 minutes exposure to melimine

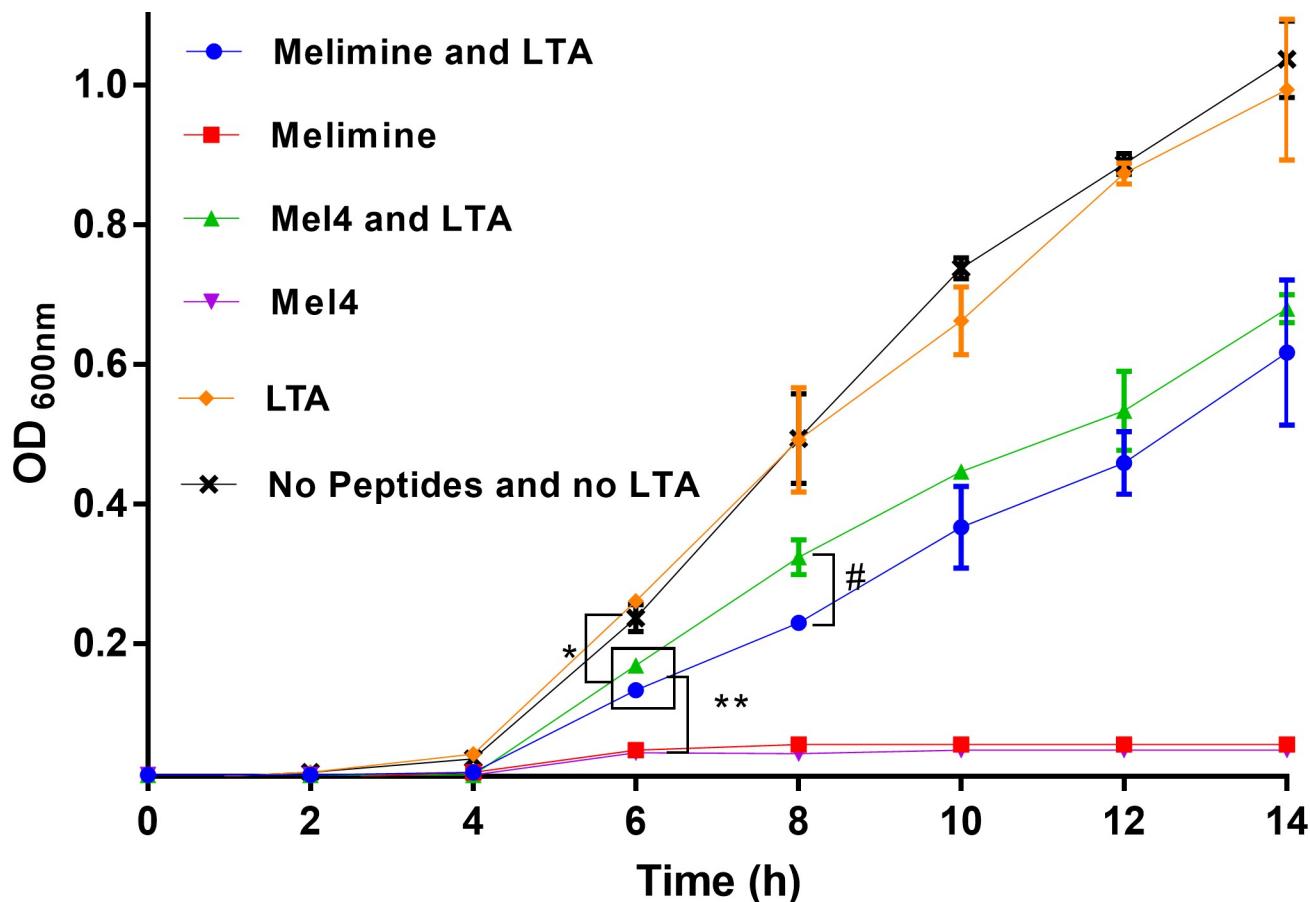


Fig 1. Growth of *S. aureus* in the presence and absence of LTA. The antibacterial effect of melimine and Mel4 was significantly reduced by addition of LTA compared to peptides alone after 6 h of incubation. The LTA alone had higher effect than LTA with any peptides at 6 h. Mel4-LTA increased OD_{600 nm} more than melimine-LTA at 8 h. Error bars represents the means (\pm SD) of three independent experiments performed in triplicate. * represent $p \leq 0.001$, ** $p \leq 0.0001$ and # $p = 0.005$.

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was 76.41%. Whilst after 150 minutes the total number of PI stained cells remained approximately the same at 80.4%, the proportions had changed with 34.2% of cells having high levels of PI staining and low levels of Syto-9 staining and 46.2% having high levels of both Syto-9 and PI staining. The time course of staining with Mel4 at its MIC was different to melimine. After 30 minutes incubation, 55.3% of cells had high levels of Syto-9 staining but low levels of PI, 32.8% of cells had high levels of both Syto-9 and PI staining and 3.58% of cells had high levels of PI but low levels of Syto-9 staining. The total percentage of cells with high levels of PI staining after 30 minutes exposure to melimine was 36.4%. After 150 minutes the total number of PI stained cells remained approximately the same at 41.6%, as did the relative proportions with 3.91% of cells having high levels of PI staining and low levels of Syto-9 staining and 37.7% having high levels of both Syto-9 and PI staining. However, when looking at the flow cytometry readout (Fig 4) there did appear to be a general increase in the percentage of double stained cells, but the gating did not bin cells with sufficiently fine detail. There was a difference in the kinetics of cell death with the positive control Triton-X 100 (1%), which after 30 minutes stained 16.34% cells with high PI and increased up to 24.94% after 150 min incubation. With buffer treated (negative control) cells > 86% were stained with high levels of Syto-9 and low levels of PI even after 150 minutes incubation (Fig 4).

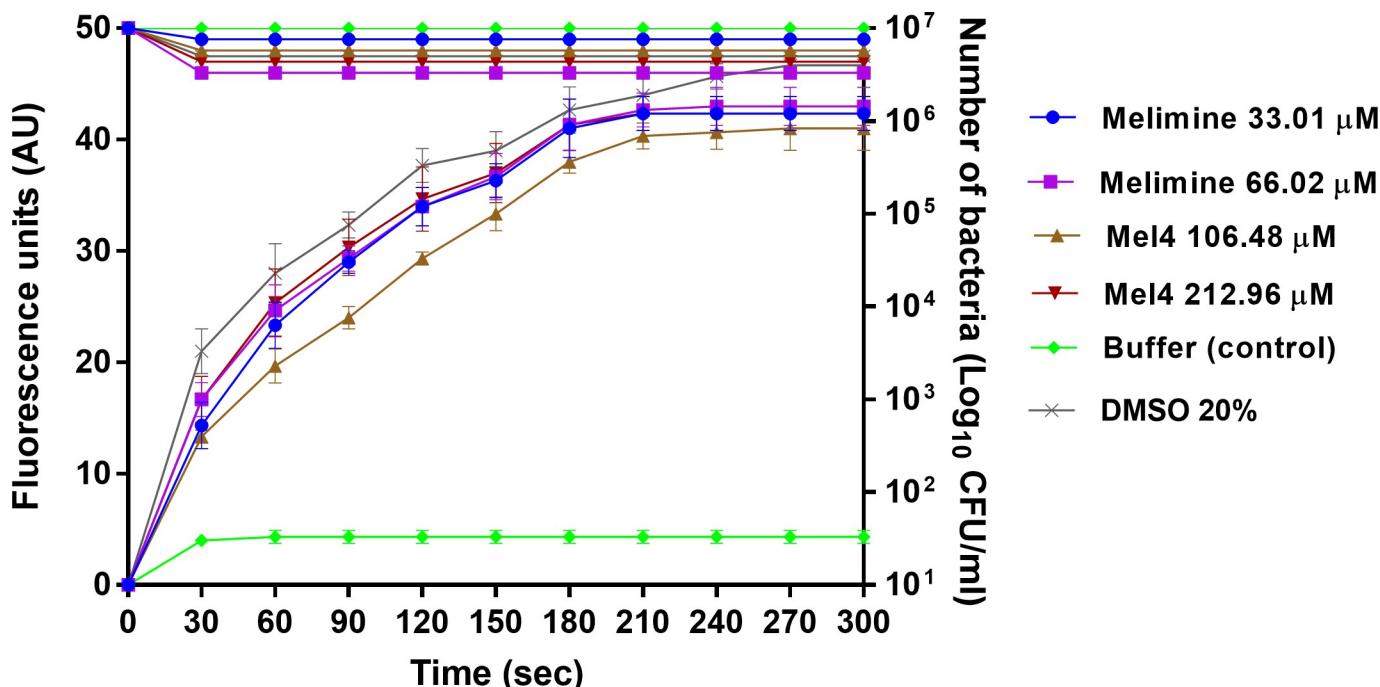


Fig 2. Cell membrane depolarization. Cell membrane depolarization of *S. aureus* 38 as assessed with the release of membrane potential-sensitive dye DiSC3-(5) during treatment with peptides, measured spectroscopically at 622_{nm} to 670_{nm} excitation and emission wavelengths, and corresponding bacterial death as determined by plate counts. Each symbol represents data curve for increase in fluorescence (on the left y axis) and for reduction in bacterial count CFU/ml (on the right y axis). Data are presented as means (\pm SD) of three independent repeats in triplicate. DMSO (20%) was used as a positive control and buffer used as a negative control.

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The release of cytoplasmic contents

Melimine at its MIC or MBC released 49% and 51% of total cellular ATP respectively after 2 minutes of exposure ($p<0.001$) (Fig 5) and this amount of ATP did not increase after the initial burst at 2 minutes. Within the first two minutes, melimine decreased live bacteria by approximately 1.4 log₁₀ at its MIC and 1.5 at its MBC, although this was not statistically different to each other and there was no further reduction in bacterial numbers to the end of the assay period (10 minutes). Compared with melimine, Mel4 released significantly ($p>0.999$) less ATP at its MIC (19%) and MBC (21%) after 2 min (Fig 5), with no increase up to 10 minutes. There were < 0.5 log₁₀ viable bacteria after 2 minutes incubation with Mel4 at its MIC or MBC, with no further cell death over the remaining 8 minutes of the assay. Incubation with melimine released higher amounts of ATP and produced more bacterial death ($p<0.001$) than Mel4 at the MICs or MBCs.

Release of DNA/RNA (260nm absorbing materials) is shown in Fig 6. Incubation with melimine resulted in release of DNA/RNA from *S. aureus* in time dependent but dose independent manner. Although increase in optical density first happened at 5 minutes with melimine but it was not significant ($p\geq0.083$). A significant increase in the optical density happened after 10 minutes exposure with increases of 1.7 times and 2.0 times at MIC and MBC respectively ($p<0.001$). Moreover, optical density increased 4.8 times at MIC and 5.1 times at MBC after 150 minutes. On the hand, incubation with Mel4 did not increase optical density at its MICs or MBCs over the course of 150 minutes of the assay ($p\geq0.9945$).

Bacterial lysis

At low bacterial concentrations (1×10^8 CFU/ml), both the peptides showed no effect on optical density. This was probably due to bacterial concentration was too low to detect any change

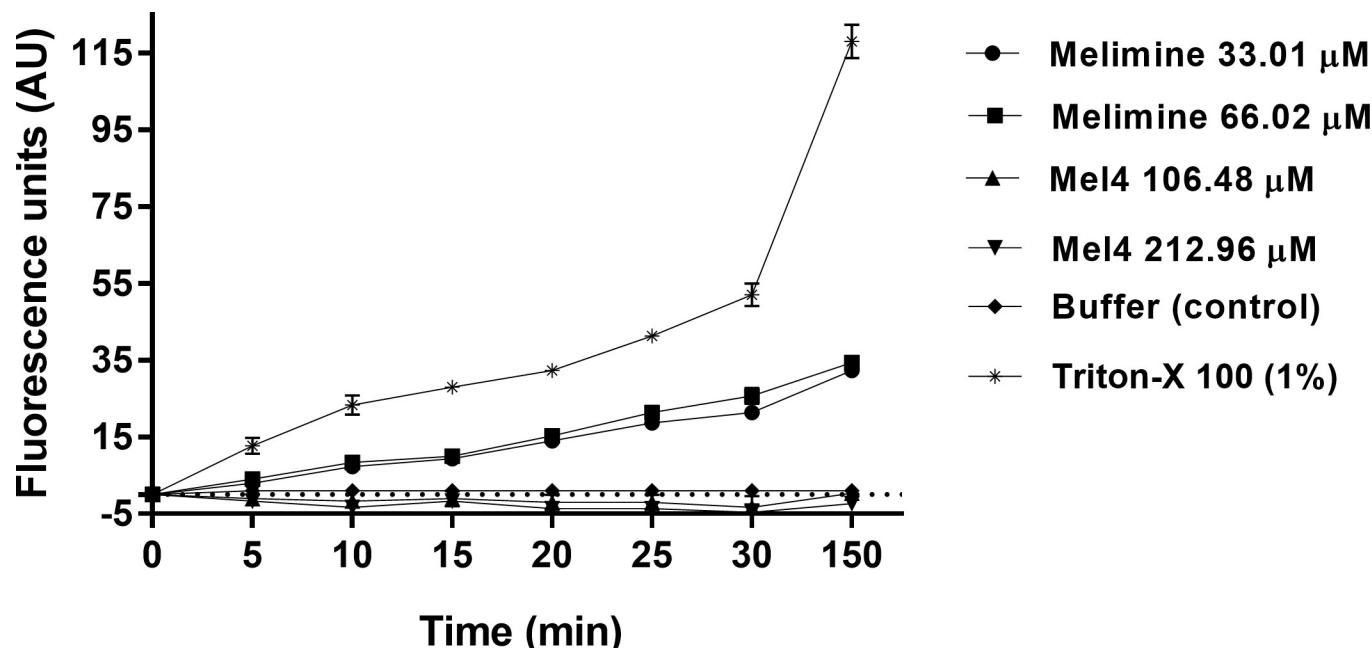


Fig 3. Cell membrane permeabilization. Cell membrane permeability of *S. aureus* 38 was assessed with as the increase in fluorescence intensity due to interaction of Sytox green dye with DNA (measured spectrophotically at 480_{nm} excitation and 522_{nm} emission wavelengths) at their MICs and MBCs of melimine and Mel4. Data are presented as means (\pm SD) of three independent repeats in triplicate. Triton-X 100 (1%) was used as a positive control and buffer only as a negative control.

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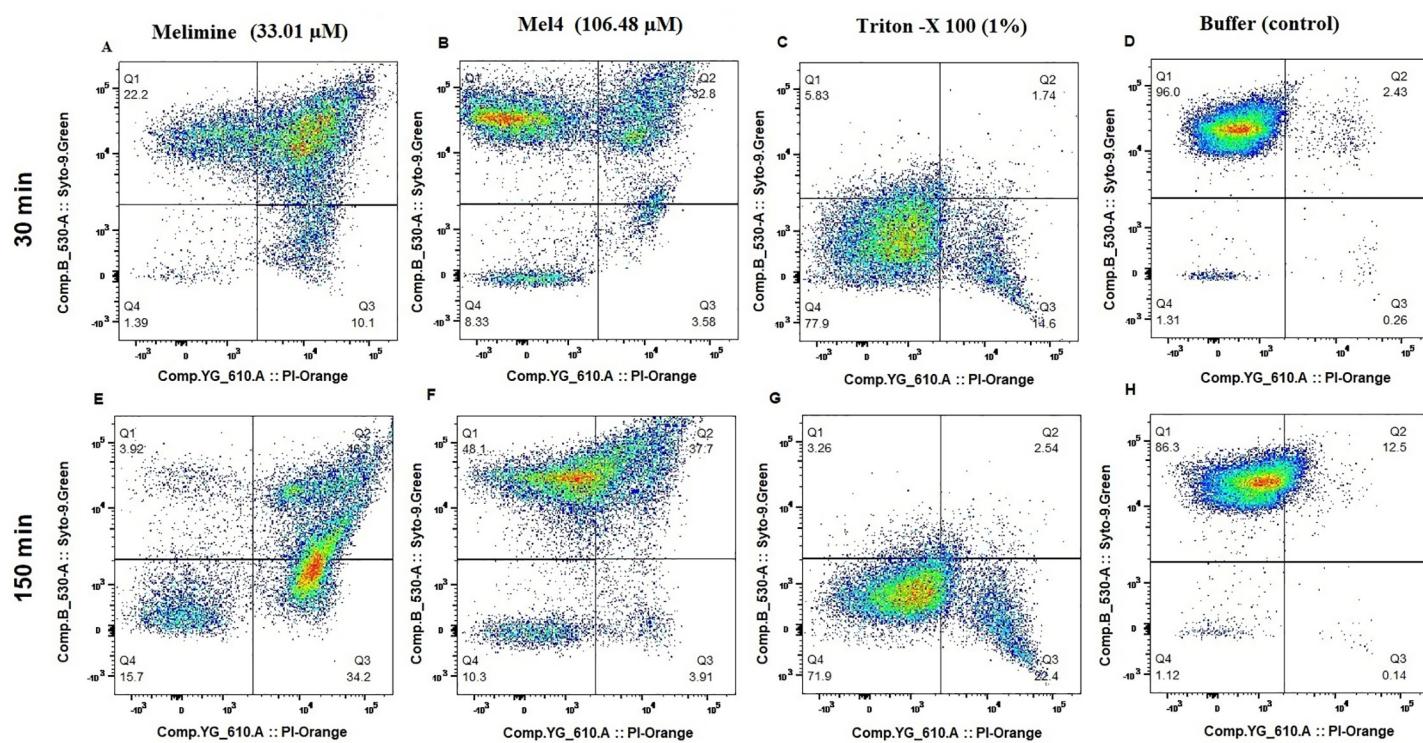


Fig 4. Flow cytometry. Cell membrane permeabilization of *S. aureus* 38 as determined with Syto-9 (membrane permeable) and Propidium Iodide (membrane impermeable stains) dyes using Flow cytometry.

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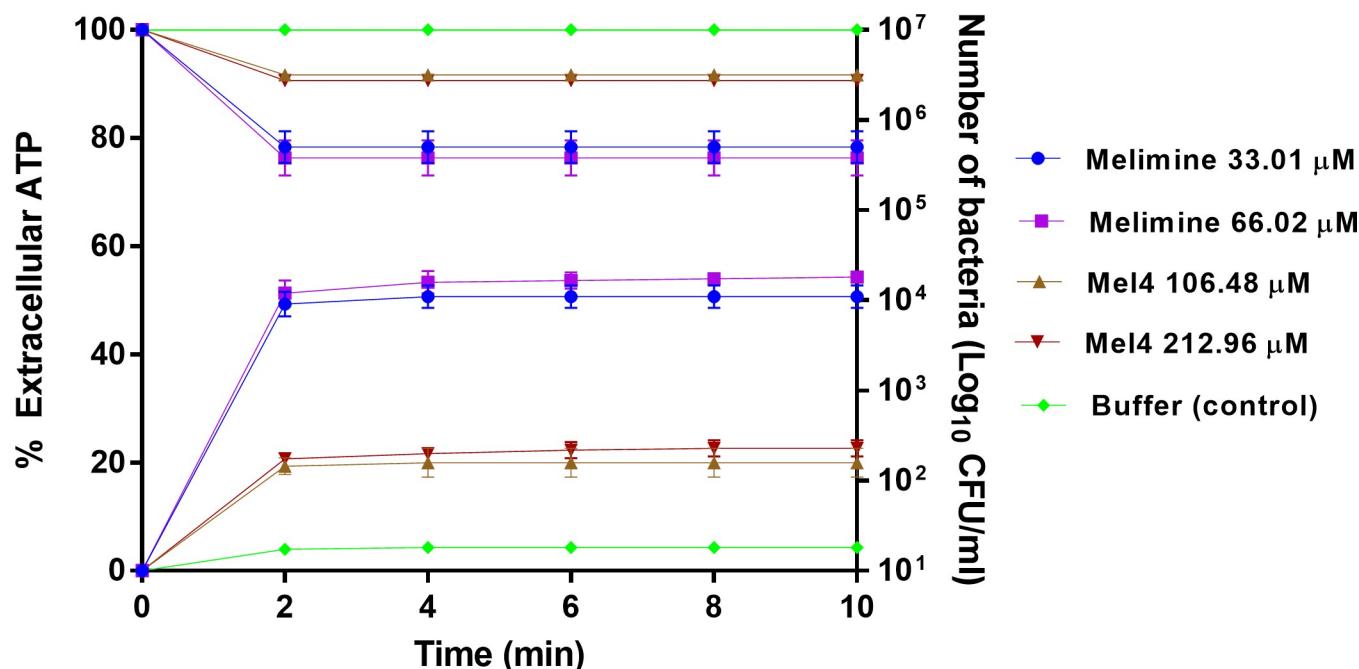


Fig 5. Leakage of ATP. The leakage of cellular ATP (in percentage) from *S. aureus* 38 after treatment with MIC or MBC of each peptide shown at X axis. The corresponding change in the number of live cells (CFU/ml) estimated through viable count displayed at Y axis. Each symbol represents data curve for increase in fluorescence (on the left y axis) and for reduction in bacterial count CFU/ml (on the right y axis). Errors bars represent the means (\pm SD) of three independent experiments in triplicate compared with buffer-treated control.

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in optical density. However, when higher inoculum size of 1×10^{10} CFU/ml was used and treated with various concentration of peptides, a significant reduction in optical density was

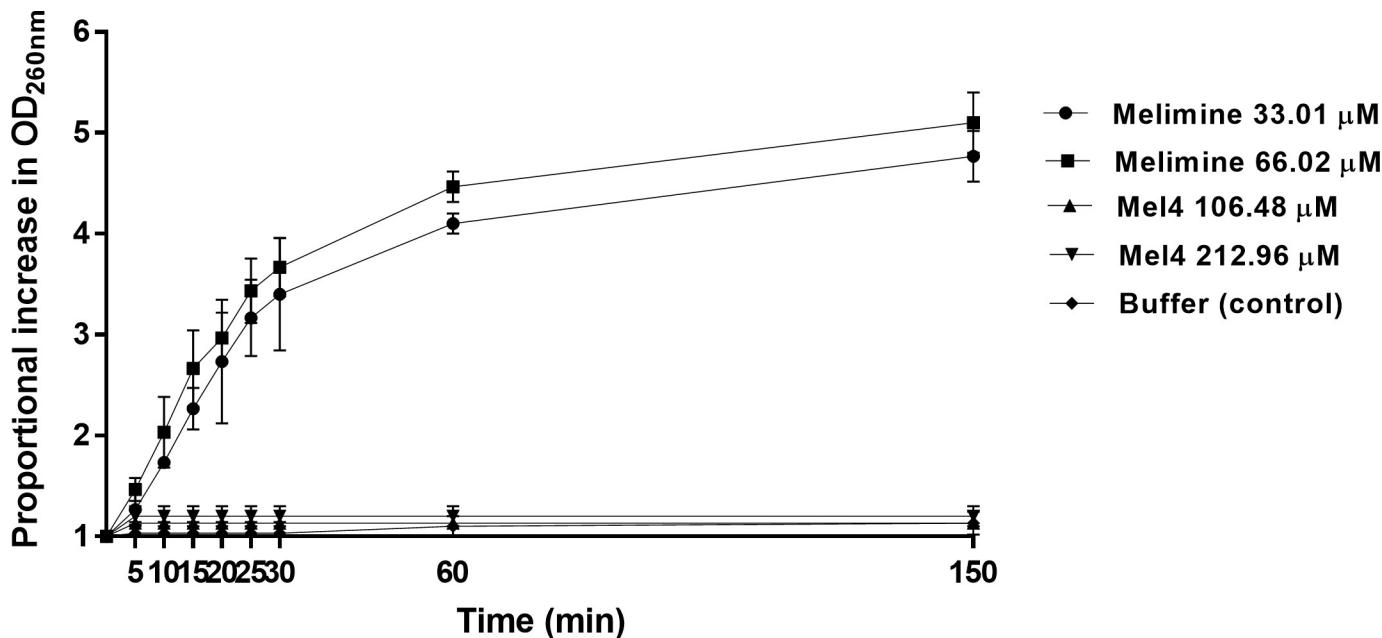


Fig 6. Release of DNA/RNA. The release of UV absorbing materials (DNA/RNA) from *S. aureus* 38 determined spectrophotically at OD_{260nm} after incubation with MIC or MBC of melimine and Mel4. Data are presented as means (\pm SD) of three independent repeats in triplicate compared with buffer-treated control.

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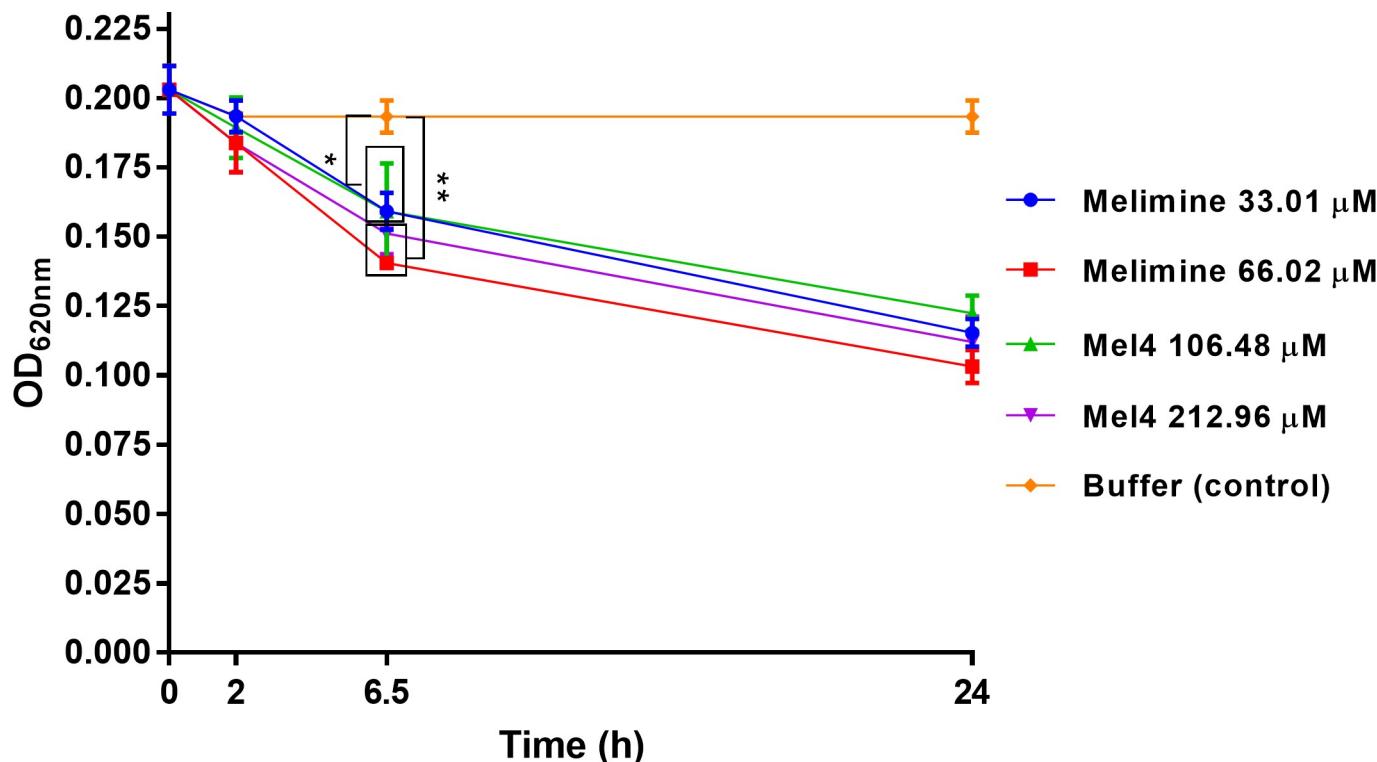


Fig 7. Bacterial lysis. Bacterial lysis of *S. aureus* 38 was determined spectroscopically as decrease in OD_{620nm} after incubation with MIC and MBC of melimine and Mel4. Data are shown as means (\pm SD) of three independent repeats in triplicate compared with buffer-treated control. * represent $p < 0.001$ and ** $p < 0.0001$.

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observed at 6.5 hours (Fig 7). At this time point, melimine reduced optical density by 21±1% at its MIC and 31±4% at its MBC compared to buffer treated control ($p < 0.001$) (Fig 7). Melimine lysed more than 42% cells after 24 h. A similar trend was observed for Mel4 which reduced optical density by 22±3% and 25±2% at its MICs and MBCs respectively after 6.5 h (Fig 7). After 24 h of incubation, Mel4 caused lysis of more than 40% cells. Over 24 hours the bacterial-lytic efficiency of both melimine and Mel4 was similar and no significant difference was observed ($p \geq 0.851$). The optical density of control cells (buffer treated) remained unchanged over the 24 h of the experiment.

The killing kinetics of AMPs was determined by exposing *S. aureus* to their MIC and MBC. Melimine reduced the number of cells by $1.1 \log_{10}$ at its MIC and $1.2 \log_{10}$ CFU/ml at its MBC after 2 h of incubation (Fig 8). At its MIC, after 2 hours melimine continued to reduce the numbers of live cells, but the rate of reduction diminished after 12 hours. The initial rate of reduction from 0–2 h was $6.6 \text{ CFU } \log_{10}/\text{ml/hour}$, from 2–12 hours was $5.9 \text{ CFU } \log_{10}/\text{ml/hour}$ and from 12–24 h was $2.9 \text{ CFU } \log_{10}/\text{ml/hour}$. At its MBC, the initial rate of reduction was $6.7 \text{ CFU } \log_{10}/\text{ml/hour}$, then from 2–24 h the rate remained constant at $5.7 \text{ CFU } \log_{10}/\text{ml/hour}$. Mel4 at its MIC did not significantly reduce bacterial viability within the first 2 h. The rate of reduction from 2–12 h was $5.6 \text{ CFU } \log_{10}/\text{ml/hour}$ and $3.8 \text{ CFU } \log_{10}/\text{ml/hour}$ from 12–24 h. At its MBC the rate of reduction was also negligible initially (0–2 h), however, from 2–24 h the rate of reduction increased to $5.5 \text{ CFU } \log_{10}/\text{ml/hour}$. Killing rate of melimine from 2–24 was significantly higher than Mel4 ($p < 0.001$). The bacterial viability remained unaffected in control (buffer treated) over 24 h of the experiment.

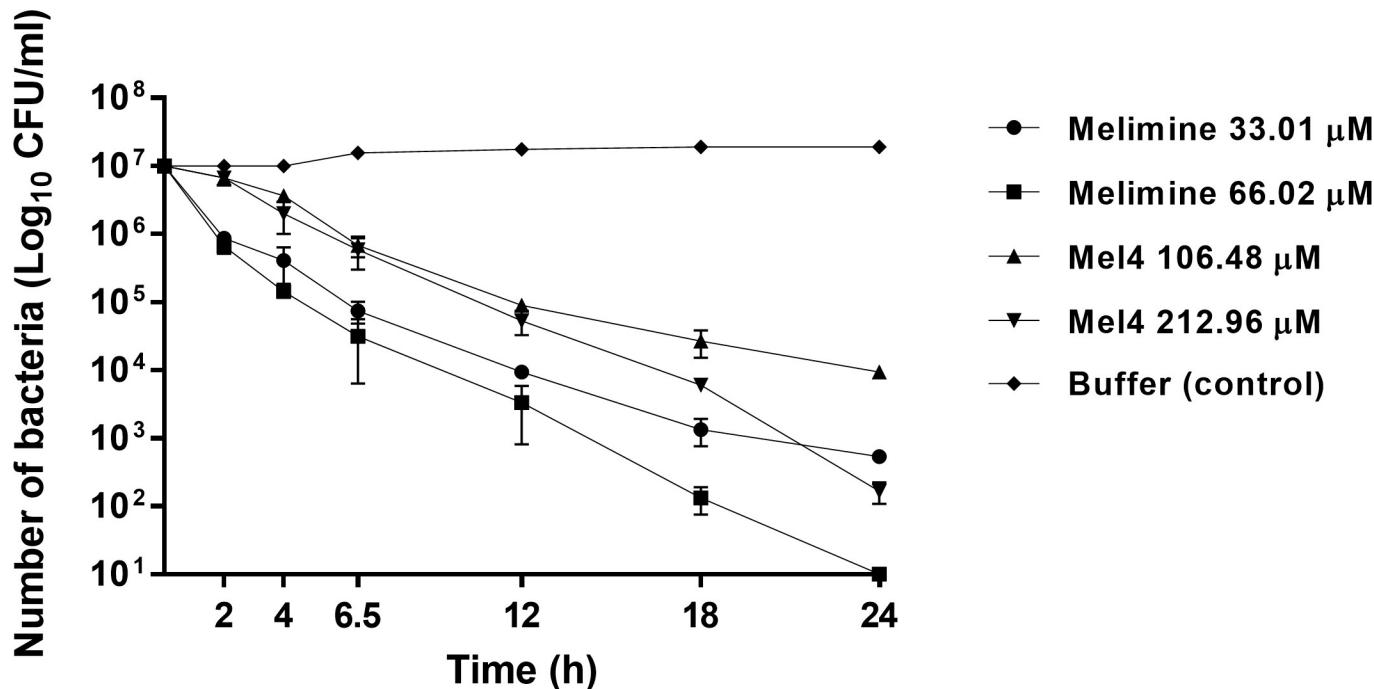


Fig 8. Bacterial killing kinetics. Time kill kinetics of melimine and Mel4 against *S. aureus* 38. Aliquots analyzed at different time intervals for viable count showed that peptides decrease the bacterial viability (\log_{10} CFU/ml) with different rates compared to buffer treated (negative control).

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Autolytic activity

Autolytic activity of peptide-treated *S. aureus* supernatants was tested using PGN as the substrate. The supernatant of Mel4 treated *S. aureus* had a higher reduction of OD_{570nm} of PGN suspension than melimine treated or controls (bacteria grown in the absence of peptides) (Fig 9A). The supernatants of Mel4 treated *S. aureus* resulted in a decrease in PGN density of 17 ±3% which was significantly more compared with melimine treated (8±2%; $p = 0.004$) and controls (7±1%, $p < 0.001$) at 60 min ((Fig 9A)). The reduction in PGN density caused by melimine or buffer supernatants was not significantly different at each time point ($p = 0.999$). The rate of PGN degradation for Mel4 was 0.28% OD₅₇₀/min up to 60 mins and then there was no more activity from 60–180 minutes. The rate for lysozyme was 0.7% OD₅₇₀/min within first 30 minutes. Lysozyme produced higher PGN hydrolysis, decreasing OD 21±4% at 30 min. The autolysis of culture supernatants was also examined using a pre-seeded lawn of *Micrococcus lysodeikticus*. Melimine or Mel4 suspended in buffer and treated in the same way as culture supernatants of *S. aureus* did not produce any zone of inhibition, showing that the peptides must either have been destroyed or removed during processing. The supernatant from Mel4 treated cells produced a zone of inhibition of 8±1 mm (Fig 9IIA) whereas for melimine and buffer treated cells smaller zones of inhibition of 5±1 mm were produced (Fig 9IA). The positive control lysozyme formed largest 12±2 mm zone of inhibition.

Lysis of horse red blood cells

Melimine and Mel4 exhibited dose dependent hemolytic effects against HRBCs. Melimine started to hemolys HRBCs (6%) at 264.08 μM. Significant lysis (17%) occurred at higher 518.16 μM concentration. ($p < 0.001$; Fig 10). Compared with melimine, Mel4 showed less hemolysis at their corresponding MICs. The first appreciable hemolysis of HRBCs by Mel4

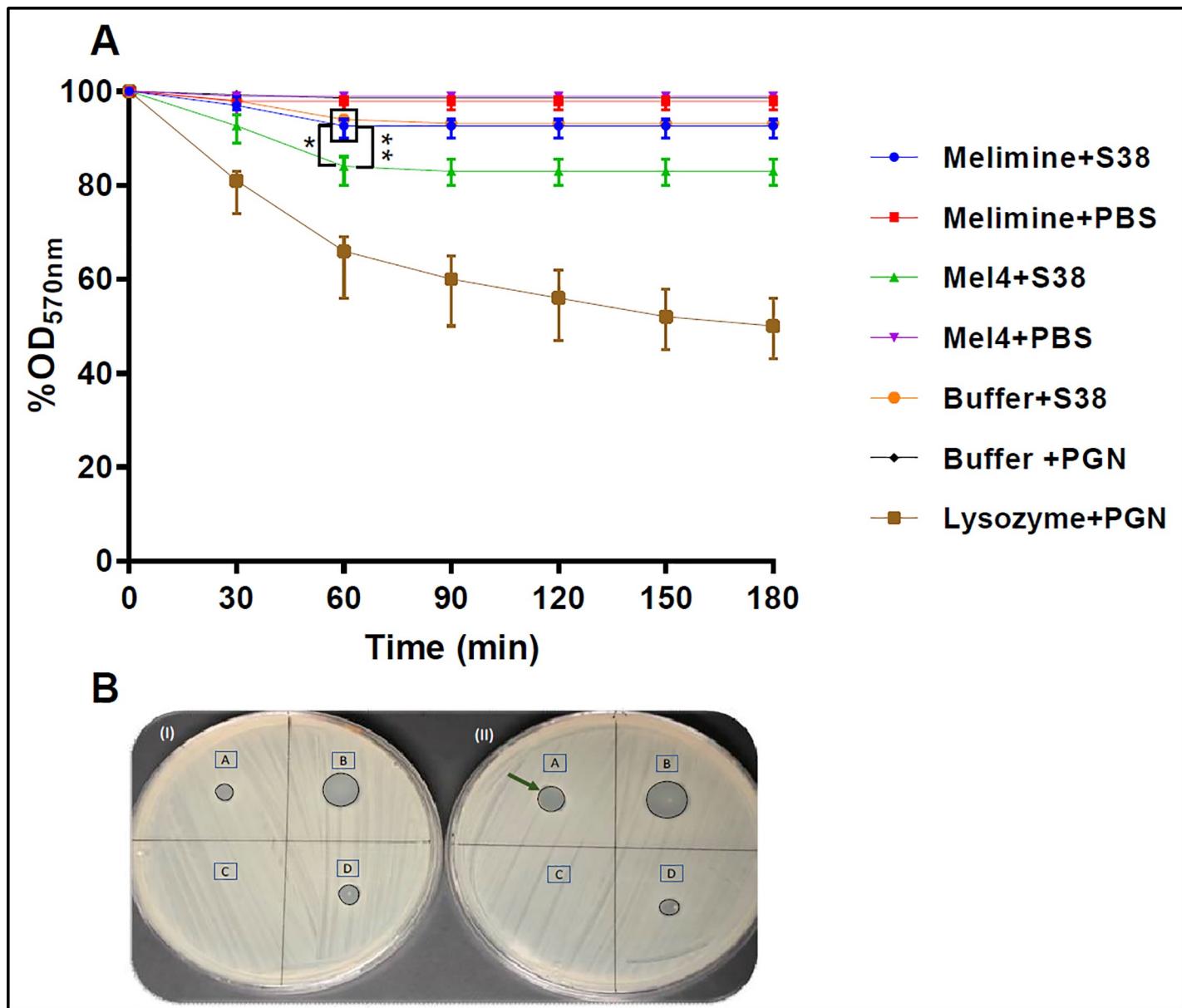


Fig 9. Detection of autolysins release. (A) Release of autolysins was determined by measuring decrease in OD_{570nm} of PGN suspensions following treatment with cell-free supernatants of melimine and Mel4 treated *S. aureus* 38 (S38). Cell-free supernatants from Mel4 treated *S. aureus* resulted in a greater degradation of PGN than those from melimine or buffer treated *S. aureus*. Data presented as means (\pm SD) of three independent repeats in triplicate. * represent $p \leq 0.004$ compared to melimine treated bacteria and ** $p < 0.001$ compared to buffer control (B) Release of autolysins was also monitored by determining the zone of inhibition (arrow) of cell-free supernatants against *Micrococcus lysodeikticus* ATCC 4698 after (I) melimine and (II) Mel4 treatment. IA = supernatant from melimine treated *S. aureus* 38, IIA = supernatant from Mel4 treated *S. aureus*, IB and IIB zones produced by lysozyme (5 mg/ml), IC no zone produced when melimine was incubated with buffer alone, IIC Mel4 incubated with buffer alone, ID and IID *S. aureus* incubated without addition of melimine or Mel4.

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(5%) was observed at 1703.68 μ M ($p < 0.041$). However, exposure to 3407.36 μ M resulted in slightly higher lysis (6%) of HRBCs ($p < 0.022$). The therapeutic index (that is hemolytic concentration/MIC) was 16 for both melimine and Mel4.

Discussion

The current study demonstrated that the mode of action of *S. aureus* killing by Mel4 and its precursor melimine were different. A previous study had shown that 30 minutes incubation

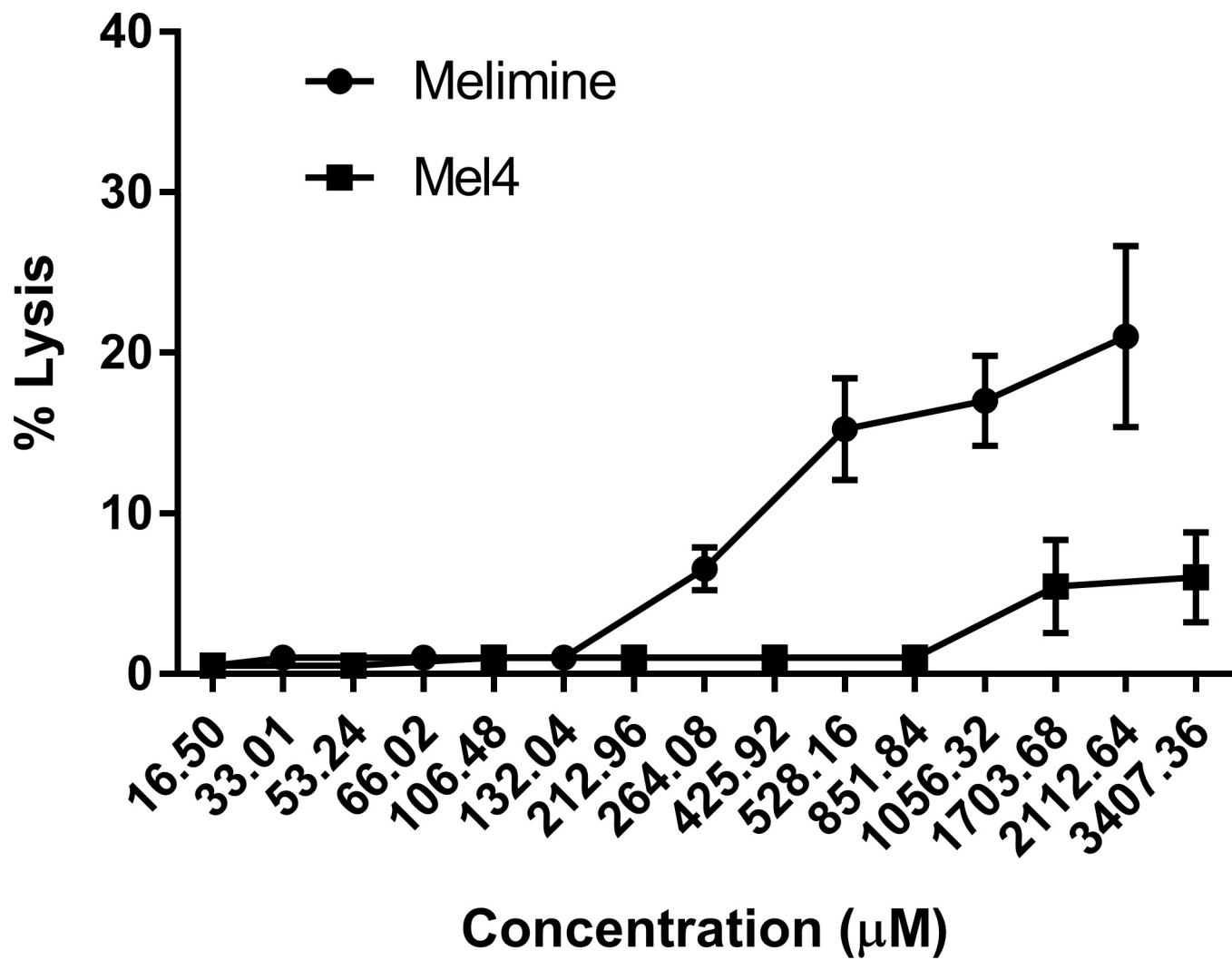


Fig 10. Hemolysis of horse erythrocytes. Hemolysis was determined as increase in $\text{OD}_{540\text{nm}}$ after incubation with increasing concentration of the melimine and Mel4. Errors bars represents the means ($\pm\text{SD}$) of three independent experiments performed at three separate occasion in triplicate.

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with melimine can distort *S. aureus* cells, induce bleb formation and accumulation of cell debris [30], as well as being able to permeabilise the cytoplasmic membrane of *S. aureus* in a concentration independent manner [30]. The previous study also showed that melimine was only able to produce $<1 \log_{10}$ reduction in numbers of *S. aureus* cells when incubated for up to five minutes. The current study demonstrated similar effects, but extended this to demonstrate that melimine could interact with LTA of *S. aureus* and produce breakage in the cytoplasmic membrane of *S. aureus* that resulted in leakage of intracellular contents (ATP and DNA/RNA) eventually leading to cell lysis and death. Melimine was also able to liberate a small amount of autolysins from the staphylococcal cells. Whilst Mel4 could also interact with LTA and depolarise the cytoplasmic membrane. It had a weaker effect on producing pores which was evidenced by negligible Sytox green intake by cells, minimum disruption to cytoplasmic membrane compared to melimine and minimum propidium iodide staining. Based on the flow cytometry data it is suggested that it can cause transient cell membrane permeability. In addition, less ATP was released from Mel4-treated cells and no DNA/RNA was released

over 150 minutes. However, both the peptides eventually resulted in cell lysis and death after 24 hours exposure. Mel4-treated cells released greater amount of autolysins compared to melimine-treated ones. It is probable that the main killing mechanism of Mel4 was release of autolysins from LTA in cell walls along with membrane depolarization. Live bacteria produce a proton gradient outside the cell membrane, and the protons can accumulate in the cell walls and protonate the D-Alanine ester linkages of LTA [55]. The protonation of D-Ala ester linkages lowers the pH of the cell wall which in turn suppress the activity of autolysins bound to LTA [55]. Upon dissipation of the membrane potential, deprotonation of the D-Ala ester linkages of LTA raises the pH of the cell wall resulting in activation of autolysins [55, 56]. Moreover, the membrane embedded glycolipid anchor of LTA plays an important role in its inhibitory effect on autolysins [57]. Antimicrobial peptides cause the delocalization of the glycolipid anchor and subsequently release autolysins by disrupting the cell membrane [57]. Therefore, the membrane depolarization caused by melimine and Mel4 may lead to LTA deprotonation and release of LTA which in turn liberates autolysins.

There are substantial differences in the amino acid compositions of Mel4 and melimine which contains 17 and 29 amino acids respectively. The lack of tryptophan in the sequence of Mel4 may be important in the differences in modes of action. Tryptophan (Trp) is known to interact with lipid bilayers, can enhance peptide-membrane interactions and facilitate insertion of peptides into the membrane [35, 58]. Trp and Arginine (Arg) together are known to promote stable and deeper insertion into the cell wall of Gram-positive bacteria [59]. Trp facilitates the insertion of Arg into hydrophobic regions of membranes via cation–π interactions causing rapid membrane disruption [35]. May be due to the presence of Trp, melimine can adopt a partial α helix in bio-membrane mimetic environment [30]. The antimicrobial activity of AMPs is often attributed to this helical conformation [60]. Mel4 lacks tryptophan and also has a low hydrophobic moment (0.039), meaning that it is less likely to be attracted within lipid bilayers [61]. Mel4 is unable to interact with lipid spheroids composed of 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (PC 18:1) or tethered lipid bilayers composed of 70% zwitterionic C20 diphentanyl-glycero-phosphatidylcholine lipid and 30% C20 diphentanyl-diglyceride but melimine can interact with these lipid layers [62].

Both melimine and Mel4 could interact with LTA. This interaction is likely due to their positive charge and the negative charge of LTA given by its phosphate groups. Melimine has an overall positive charge of +15, whilst Mel4 has +14 [32], and this small difference may partly explain some of the different effects of their interactions with LTA. At their MICs, there were 3 times more molecules of Mel4 present than melimine yet the inhibition of bacterial growth was not different, perhaps indicating a reduced ability of Mel4 to interact with LTA. In the ELISA assay at their MICs or MBCs melimine neutralized approximately the same number of LTA molecules as Mel4, further indicating a reduced Mel4-LTA interaction. However, this was not the case with the amount of autolysis observed after incubation of *S. aureus* cell supernatants with *Micrococcus* cells. LTA and teichoic acids bind to staphylococci cell wall and regulate the autolysins activity [63, 64], and addition of AMPs liberate autolysins [65].

A cytoplasmic membrane potential is essential for bacterial replication and ATP synthesis [66]. Dissipation of the membrane potential can increase membrane permeability resulting in loss of ATP [67]. Depletion of intracellular ATP can result in loss of viability of bacteria [66]. ATP leakage was associated with an increase of $1.5 \log_{10}$ *S. aureus* bacterial death within 2 min with melimine. However, release of ATP did not correlate with bacterial death with Mel4. Melimine reduced bacterial viability in the ATP assay but not in the membrane depolarization assay at same time point. This effect may be due to the presence of 100 mM KCl in the bacterial suspension media of the membrane depolarization assay. KCl can increase the MIC of AMPs against *S. aureus* [68]. The amount of ATP released by Mel4 plateaued after four minutes

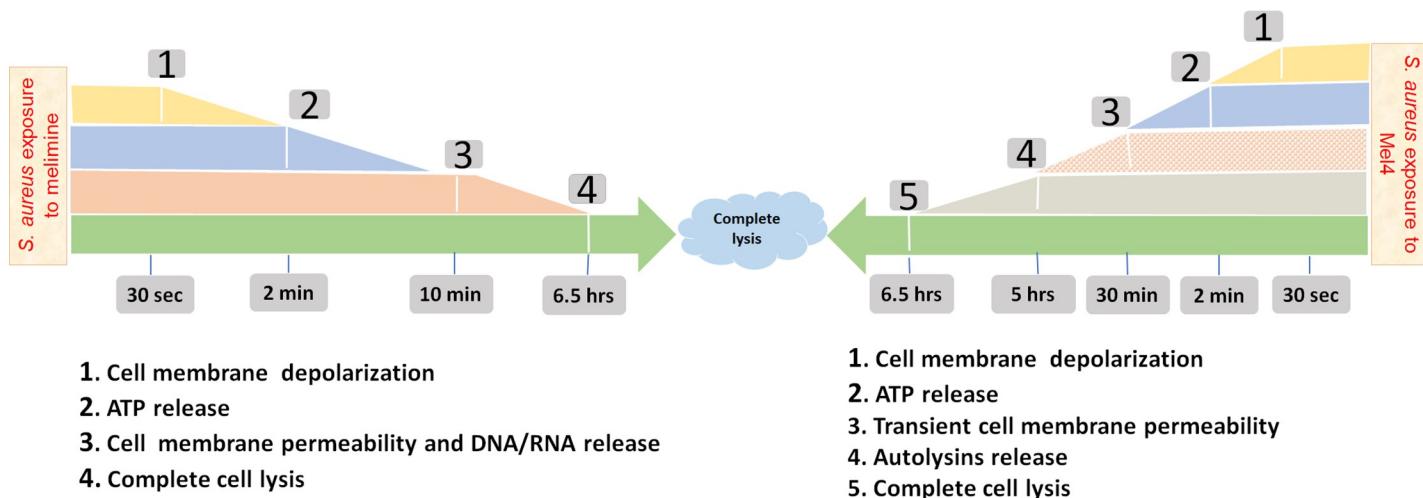


Fig 11. Timeline of melimine or Mel4 interacting with *S. aureus*. Both AMPs induced cell membrane depolarization after 30 seconds exposure to the staphylococci followed by release of ATP after 2 minutes. Cell membrane permeabilization occurred with melimine only after 10 minutes with near simultaneous release of DNA/RNA. Mel4 caused transient cell membrane after 30 minutes with no release of DNA/RNA. Mel4 showed autolysins induced killing after 5 hours. Complete bacterial lysis started after 6.5 hours of incubation with both the peptides.

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exposure and never reached to the level of melimine until 10 minutes exposure. ATP may be depleted in the supernatant of Mel4 treated cells possibly by hydrolysis at their cell surfaces. Mel4 released small amounts of ATP without permeabilizing the cell membrane. ATP being a smaller molecule needs only a pore size of 1.5 nm to leak out from compromised membranes [69]. Thus, pores size produced by Mel4 in the cell membrane are enough for ATP release as they do not need well defined pores in the membrane to escape from bacteria.

Both melimine and Mel4 exhibited low hemolytic activity. The concentration at which both the peptides started lysis of HRBCs was 16 times higher than their MICs. These results are comparable with our previous published data where melimine produced 9% hemolysis of sheep red blood cells at 20 times higher concentration than its MIC [28]. Compared to Mel4, the slightly higher hemolytic activity of melimine at similar concentration may be related to higher hydrophobicity, amphipathicity and helicity [70]. Melimine contains the hydrophobic amino acids leucine, Isoleucine and tryptophan that can cause hemolysis of erythrocyte [71], whereas Mel4 lacks all these hydrophobic amino acids is less hydrophobic in turn showed less hemolytic activity against horse erythrocytes.

Conclusions

It is likely that the amphipathic characteristics of melimine allowed disruption of the cell membranes and pore formation that resulted in ATP and DNA release and ultimately cell death. However, Mel4 showed less interaction with cell membranes and its killing of *S. aureus* was more likely due to activation autolysins along with minimum membrane disruption. A comprehensive timeline of the mechanism of action of melimine and Mel4 against *S. aureus* is summarized in Fig 11.

Supporting information

S1 Table. Cell membrane depolarization. Cell membrane depolarization as assessed by the release of the membrane potential sensitive dye DiSC3-5, measured spectroscopically at 622_{nm} excitation and 670_{nm} emission wavelengths. Data are presented as means (\pm SD) of three

independent repeats performed in triplicate.
(PDF)

S2 Table. Reduction in number of live bacteria during treatment with the peptides. The decrease in the number of viable bacteria (CFU/ml) that occurred at the same time as measuring changes to their membrane depolarization. Data are presented as means (\pm SD) of three independent repeats performed in triplicate.
(PDF)

S3 Table. Cell membrane permeabilization caused by the two peptides. Cell membrane permeabilization was assessed as the increase in fluorescence intensity due to interaction of Sytox green dye with DNA (measured spectrally at 480_{nm} excitation and 522_{nm} emission wavelengths). Data are presented as means (\pm SD) of three independent repeats performed in triplicate.

(PDF)

S4 Table. Leakage of ATP from cells upon addition of the two peptides. The leakage of cellular ATP (in percentage) from bacteria after treatment with 1X and 2X MIC of each peptide. Data are presented as means (\pm SD) of three independent repeats performed in triplicate.
(PDF)

S5 Table. Reduction in number of live bacteria during treatment with peptides that occurred in the ATP assay. Data are presented as means (\pm SD) of three independent repeats performed in triplicate.
(PDF)

S6 Table. Release of DNA/RNA from cells upon addition of peptides. The release of UV absorbing materials (DNA/RNA) was determined spectrally at OD_{260nm} after treatment with 1X and 2X MIC of peptides. Data are presented as means (\pm SD) of three independent repeats performed in triplicate.
(PDF)

S7 Table. Bacterial lysis after addition of peptides. Bacterial lysis was determined as the decrease in OD_{620nm} (in percentage) after treatment with 1X and 2X MIC of peptides. Data are presented as means (\pm SD) of three independent repeats performed in triplicate.
(PDF)

S8 Table. Hydrolysis of peptidoglycan after addition of cell-free supernatants of cells that had been treated with peptides. The hydrolysis of peptidoglycan (PGN) was determined by measuring decrease in OD_{570nm}. Data are presented as means (\pm SD) of three independent repeats performed in triplicate.
(PDF)

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Formal analysis: Muhammad Yasir.

Methodology: Muhammad Yasir.

Supervision: Debarun Dutta, Mark D. P. Willcox.

Writing – original draft: Muhammad Yasir.

Writing – review & editing: Muhammad Yasir, Debarun Dutta, Mark D. P. Willcox.

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