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Comparative mode of action of the antimicrobial peptide melimine and its derivative Mel4 against *Pseudomonas aeruginosa*

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Melimine and Mel4 are chimeric cationic peptides with broad-spectrum antimicrobial activity. They have been shown to be highly biocompatible in animal models and human clinical trials. The current study examined the mechanism of action of these two antimicrobial peptides against *P. aeruginosa*. The effect of the peptides of endotoxin neutralization, and their interactions with cytoplasmic membranes using DiSC(3)-5 and Sytox green, Syto-9 and PI dyes were analysed. Release of ATP and DNA/RNA were determined using ATP luminescence and increase in OD_{260nm}. The bacteriolytic ability of the peptides was determined by measuring decreases in OD_{620nm}. Both the peptides neutralized LPS suggesting their interaction with lipid A. Cytoplasmic membrane was disrupted within 30 seconds, which correlated with reductions in cellular viability. At 2 minutes melimine or Mel4, released 75% and 36% cellular ATP respectively ($P < 0.001$). Membrane permeabilization started 5 minutes with simultaneous release of DNA/RNA. Flow cytometry demonstrated 52% and 18% bacteria were stained with PI after 30 minutes. Overall, melimine showed higher capacity for membrane disruption compared to Mel4 ($P < 0.001$). The findings of this study have been summarized as a timeline of bactericidal activity, suggesting that the peptides permeabilized *P. aeruginosa* within 5 minutes, started lysis within 2 hours of exposure.

Pseudomonas aeruginosa is a metabolically versatile ubiquitous Gram-negative opportunistic pathogen that can cause infections in animals and plants¹. *P. aeruginosa* is responsible for localized and systemic infections in humans, which can be life threatening. *P. aeruginosa* has become one of the most frequent causative agents of nosocomial infections associated with substantial morbidity and mortality². The current standards of care to treat *P. aeruginosa* infections are not effective³ as its outer membrane offers low permeability to some antibiotics resulting in antibiotic resistance^{4,5}. Given the severity of *P. aeruginosa* infections and the limited antimicrobial arsenal with which to treat them, finding new alternative antimicrobials with unique mechanisms of action is urgently required⁶.

Antimicrobial peptides (AMPs) are part of the innate immune response of living organisms and have broad spectrum activity ranging from viruses to parasites at low concentrations⁷. 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 can be constructed to have little toxicity toward human cells^{8,9}. Bacteria do not easily gain resistance to AMPs due to their fast killing mechanism and multiple target sites^{10,11}. The mechanism of action of AMPs starts by interacting with negatively charged lipopolysaccharides (LPS) in the outer membranes of Gram-negative bacteria¹²⁻¹⁴ leading to a destabilization and permeabilization¹⁵. AMPs are then able to interact with the cytoplasmic membrane. Several models for the interaction of AMPs with bacterial cytoplasmic membranes have been proposed, such as “barrel stave”, “toroidal pore” and “carpet model”¹². In all of these models AMPs displace lipids, alter membrane structure and these interactions result in leakage of cellular contents such as K⁺, ATP and DNA/RNA, resulting in cell death^{16,17}. Some AMPs can also act intracellularly disrupting DNA, RNA and protein synthesis^{18,19}.

Melimine (TLISWIKNKRKQRPRVSRRRRRRGGRRRR) is a cationic hybrid peptide of melittin and pro-tamine²⁰. It has broad spectrum activity against a wide range of Gram-negative and Gram-positive bacteria (including MRSA and multi drug resistant *P. aeruginosa*), fungi and the protozoan such as *Acanthamoeba*^{20,21}.

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Bacterial strains	Melimine		Mel4	
	MIC nmolml ⁻¹ (µgml ⁻¹)	MBC nmolml ⁻¹ (µgml ⁻¹)	MIC nmolml ⁻¹ (µgml ⁻¹)	MBC nmolml ⁻¹ (µgml ⁻¹)
<i>P. aeruginosa</i> 6206	66 (250)	132 (500)	26.6 (62.5)	53.2 (125)
<i>P. aeruginosa</i> 6294	66 (250)	528 (2000)	26.6 (62.5)	106.5 (250)
<i>P. aeruginosa</i> Paer1	66 (250)	132 (500)	106.5 (250)	213 (500)
<i>P. aeruginosa</i> ATCC 19660	132 (500)	264 (1000)	26.6 (62.5)	26.2 (62.5)

Table 1. MIC and MBC values of melimine and Mel4 against strains of *P. aeruginosa*. MIC = minimum inhibitory concentration that inhibits the growth of $\geq 90\%$ of cells. MBC = minimum bactericidal concentration that kills $\geq 99.9\%$ of cells.

Experimental conditions	Optical Density at 405 nm (% reduction compared to positive control)*
Positive control: LPS + LAL reagent	2.47 ± 0.25 (0%)
Negative control: LPS + Endotoxin free Water	0.29 ± 0.08 (88.4 ± 3.6%)
Test: Melimine (1x MIC) + LPS + LAL Reagent	0.76 ± 0.13 (70.6 ± 5.6%)
Peptide control: Melimine (1x MIC) + LAL Reagent	0.47 ± 0.07 (81 ± 2.6%)
Test: Melimine (2x MIC) + LPS + LAL Reagent	0.46 ± 0.06 (81.5 ± 3.1%)
Peptide control: Melimine (2x MIC) + LAL Reagent	0.43 ± 0.10 (82.1 ± 6.1%)
Test: Mel4 (1x MIC) + LPS + LAL Reagent	1.30 ± 0.10 (46.7 ± 9.7%)
Peptide control: Mel4 (1x MIC) + LAL Reagent	0.73 ± 0.15 (70.5 ± 3.3%)
Test: Mel4 (2x MIC) + LPS + LAL Reagent	0.93 ± 0.21 (61.9 ± 9.0%)
Peptide control: Mel4 (2x MIC) + LAL Reagent	0.87 ± 0.23 (62.3 ± 13.4%)

Table 2. Percentage reduction in OD_{405 nm}. LPS = lipopolysaccharide; LAL = Limulus amoebocyte lysate; MIC = minimum inhibitory concentration; *All reductions in LAL activity were significantly different compared to the positive control ($P \leq 0.05$).

Importantly, bacteria did not develop resistance against melimine when exposed at sub-MIC for 30 consecutive days²⁰. Moreover, melimine is not cytotoxic at well above active concentrations^{20,21}. Melimine has a random coil conformation in aqueous environments but increases its α -helical content (to 40%) in bacterial membrane mimetic environments. This change in conformation upon binding to the membrane is widely accepted as the first step in the mechanism of action of many AMPs²². Melimine disrupts the outer membrane and rapidly destroys the inner membrane potential of *P. aeruginosa*²³. Melimine retains its antimicrobial activity when bound to polymers and titanium^{20,21,24}.

A shorter sequence of melimine called Mel4 (KNKRKRRRRRGGRRRR) has been designed which was shown to have improved ocular compatibility when bound to contact lenses²⁵. Mel4 is highly active against *P. aeruginosa* in solution or when immobilized on surfaces²⁶. It is noncytotoxic to mammalian cells *in vitro*^{20,21}, in animal model studies and in human clinical trials^{25,27}. Mel4 has had several amino acids removed from melimine's sequence, including the single tryptophan in melimine. Tryptophan is known to be a highly lipophilic amino acid²⁸, and many cationic peptides contain tryptophan as an important part of their mode of action^{29–31}. Similarly, other non-polar amino acids such as Leu and Ile can encourage peptide binding and disruption of cell membranes³². Although Mel4 have been extensively investigated with human clinical trials, its bactericidal mechanism is unknown. Hence, this study examined and compared the mode of action of melimine and Mel4 against *P. aeruginosa*.

Results

Inhibitory concentrations of peptides. MICs and MBCs for melimine and Mel4 against *P. aeruginosa* are shown in Table 1. Mel4 had a low MIC of 26.6 nmol/ml against *P. aeruginosa* strains 6294, 6206 and ATCC 19660. Melimine had its lowest MIC 66 nmol/ml against 6294, 6206 and Paer1. For all strains the MBC was usually 2x the MIC except for strain 6294 where the MBC for both melimine and Mel4 was $\geq 4x$ the MIC while for ATCC 19660 the MBC for Mel4 was equivalent to the MIC. Melimine at its lowest MBC needed 1.59×10^{12} molecules per cell to cause death whereas Mel4 needed 3.2×10^{11} molecules per cell to cause cell death at its lowest MBC.

Interaction with Lipopolysaccharides. The LAL test was used to assess the ability of the AMPs to interact and neutralize the LPS. Both melimine and Mel4 inhibited LPS's ability to stimulate amoebocyte lysate in a dose-dependent manner as demonstrated in Table 3. At 1x MIC, melimine significantly neutralized LPS and reduced OD_{405 nm} by 71% compared to the positive control ($P < 0.001$). At 2x MIC melimine neutralized approximately 82% LPS (Table 2). Compared with melimine, less neutralization of LPS activity in the LAL assay was achieved with Mel4 while decreasing the OD_{405 nm} by 47% at 1x MIC and 62% at 2x MIC ($P < 0.001$). None of the tested peptides induced Limulus proenzyme activation when incubated in the absence of LPS.

Membrane disruption. Both melimine and Mel4 rapidly within 30 seconds depolarized the cytoplasmic membrane of *P. aeruginosa* in a concentration dependent manner. Figure 1A shows the data for *P. aeruginosa*

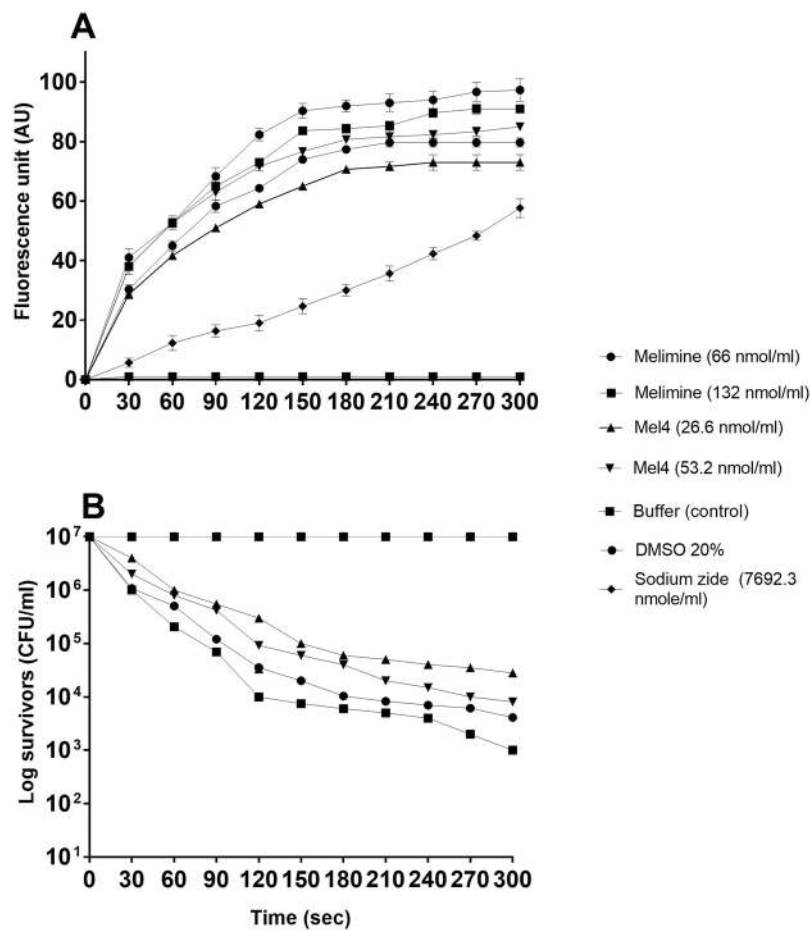


Figure 1. Cytoplasmic membrane depolarization of *P. aeruginosa* 6294 by melimine and Mel4, as assessed by release of the membrane potential-sensitive dye DiSC3-(5) measured spectroscopically at 622_{nm} to 670_{nm} excitation and emission wavelength, and corresponding bacterial survival as determined by plate counts. Data presented as means (\pm SD) of three independent repeats in triplicate cells. NB, addition of sodium azide to cells did not result in any cell death compared to controls.

Peptides	Molecular mass	Number of amino acids	Net charge	Hydro-phobicity <H> [*]	Hydrophobic moment < μ H> [*]	Polar residue (number)	Non-polar residue (number)
Melimine	3786.6	29	+16	-0.250	0.222	23	6
Mel4	2347.8	17	+14	-0.846	0.039	17	0

Table 3. Properties of melimine and Mel4 peptides. ^{*}The properties were predicted using online software <http://heliquet.ipmc.cnrs.fr/cgi-bin/ComputParams.py>.

6294, whereas data for all other strains are available in the Supplementary Information. Following 150 seconds of exposure, no further depolarisation (increase in the release of DiSC3-(5)) occurred for any of the strains. More depolarisation occurred with the higher median MIC of melimine (66 nmol/ml) compared to the median MIC of Mel4 (26.6 nmol/ml), and the same trend was observed throughout the time course ($P < 0.005$). This depolarization of the cytoplasmic membrane was associated with $>3.6 \log_{10}$ reduction in colony forming units of *P. aeruginosa* 6294 for melimine at its MIC (66 nmol/ml) or MBC (132 nmol/ml). Similarly, there was $>2.7 \log_{10}$ reduction in bacterial numbers by Mel4 at its MIC (26.6 nmol/ml) and MBC (53.2 nmol/ml) (Fig. 1B). The control substance sodium azide also depolarized the cell membrane and released DiSC3-(5) but without bacterial killing.

Sytox green fluorescence, a measure of permeabilization of the cytoplasmic membrane, increased over time and its release was significantly different to the negative control after 5 minutes incubation with both AMPs ($P < 0.005$) (Fig. 2). The permeabilization gradually increased over 30 minutes for all the peptide concentrations for all strains. However, this effect was not concentration dependent for either peptide as no significant differences were observed between the MIC and MBC. At their respective MICs, melimine allowed more Sytox green to enter cells than Mel4 after 150 mins ($P < 0.001$). Treatment with the positive control Triton-X 100 resulted maximum membrane permeabilization.

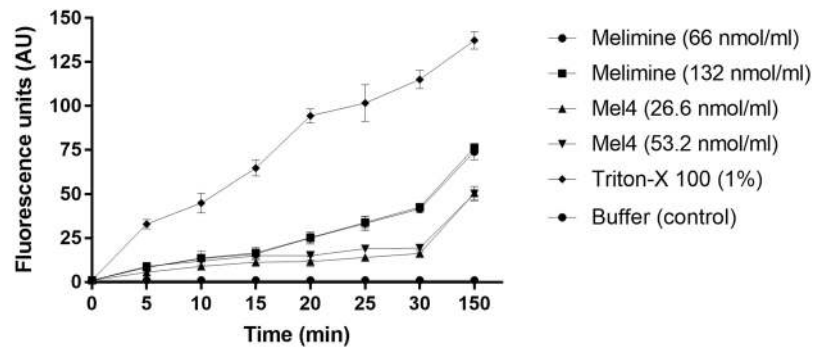


Figure 2. Cytoplasmic membrane permeability of *P. aeruginosa* 6294 by melimine and Mel4 at different concentrations. Fluorescence due to binding of Sytox green fluorescent probe with DNA was measured spectroscopically at 480_{nm} to 522_{nm} excitation and emission wavelength. Data presented as means (\pm SD) of three independent repeats in triplicate.

The membrane damaging effect of these peptides was also assessed with *P. aeruginosa* 6294 by the flow cytometry in the presence of the DNA intercalating dye Propidium Iodide, which is only permeable to cells with damaged membranes, and Syto 9 which can enter intact cells. The peptides were able to permeabilize the cell membrane in a concentration independent manner, but this effect was time-dependent, and similarly to experiments with Sytox green, maximum permeabilization occurred after 150 mins incubation (Fig. 3). For melimine at its MIC, after 30 mins exposure 10% of cells were not stained, 15.2% were stained with only Syto 9, 74.8% showed membrane permeabilization (22.3% of cells were double stained, 52.5% were stained only with Propidium Iodide). After 150 minutes of exposure, 97.5% of the cells had permeabilized membranes (7.8% double stained, 89.7% stained with only Propidium Iodide). Although Mel4 permeabilized *P. aeruginosa* membranes, permeabilization and the kinetics was different compared with melimine. After 30 min exposure to Mel4 at its MIC 5.2% of cells were not stained, 64.7% were stained with only Syto 9, and 30.1% of cells had permeabilized membranes (12.4% double stained, 17.7% cells stained only with Propidium Iodide). After 150 minutes exposure, 1.2% of cells were not stained, 26.4% were stained with Syto 9 only, and 72.3% of cells had permeabilized membranes (25.6% double stained, 46.7% stained only with Propidium Iodide; Fig. 3). The positive control Triton-X 100 showed different kinetics to both peptides. After 30 minutes exposure 46.1% of cells were not stained, 27.7% were stained with only Syto 9, 7.6% were double stained and 18.7% were stained with only Propidium Iodide. After 150 min exposure, 60% of cells were unstained, 3.1% were stained only with Syto 9, 2.4% were double stained and 34.1% were stained with only Propidium Iodide. The negative control (buffer-treated) behaved as expected and <7% permeabilized (Fig. 3).

The release of cytoplasmic contents. Melimine released 75% and 92% of total cellular ATP at 1x and 2x its MIC after 2 minutes ($P < 0.001$) (Fig. 4). The increase of extracellular ATP directly correlated with the loss of viability of *P. aeruginosa* ($R^2 > 0.987$). Within the first two minutes, melimine decreased viability by $>3.0 \log_{10}$. Exposure to Mel4 released 36% and 44% extracellular ATP at 1x or 2x its MIC after 2 minutes (Fig. 4). Further incubation for 10 minutes resulted in a slight increase in the release of extracellular ATP. The release of ATP was associated with the reduction of $>2.0 \log_{10}$ viable bacteria. Melimine induced more leakage of ATP than Mel4 ($P < 0.001$).

Figure 5 shows the releases of DNA/RNA (260 nm absorbing material) following incubation with the peptides. The release of DNA/RNA of *P. aeruginosa* was significantly ($P < 0.001$) increased compared to the negative control after 2 minutes incubation with either peptide. Melimine was associated with a dose-dependent release of DNA/RNA but the release of DNA/RNA was not affected by the concentration of Mel4. Compared with Mel4, melimine released higher amounts of DNA/RNA at all time points ($P < 0.001$).

Bacterial lysis. When the lower cell concentration of cells (1×10^8 CFU/ml) was used, no decrease in OD_{620nm} could be seen as the optical density was very low to start with. When 1×10^{10} CFU/ml was used and treated with varying concentrations significant reductions in OD_{620nm} was observed (Fig. 6). After incubation with melimine for 2 hours, the OD_{620nm} was significantly reduced by $25 \pm 12\%$ at 1x MIC and $31 \pm 3\%$ at 2x MIC compared to at time zero ($P < 0.001$). OD_{620nm} was further reduced by $37 \pm 03\%$ and $52 \pm 10\%$ with 1x and 2x MIC after 6.5 hours respectively. After 24 hours incubation melimine had lysed $>55 \pm 5\%$ of cells at both concentrations. A similar trend was seen for Mel4 which reduced the OD_{620nm} by $13 \pm 8\%$ and $21 \pm 6\%$ at 1x and 2x MIC respectively after 2 hours ($P < 0.015$). Further reductions in OD_{620nm} by $20 \pm 3\%$ with 1x and $48 \pm 5\%$ with 2x MIC was observed after 6.5 h. Similarly, OD_{620nm} decreased by $52 \pm 2\%$ by both the concentrations of Mel4 after 24 hours of incubation ($P < 0.004$) (Fig. 6). The bacteriolytic efficiency of both melimine and Mel4 was similar at their MICs ($P = 0.927$) and 2x MICs ($P > 0.999$) but 2x MIC of melimine caused more lysis than 1x MIC of Mel4 ($P < 0.004$) after 24 hours of incubation. The OD_{620nm} of cells in the absence of peptides remained unchanged over the 24 hours of the experiment.

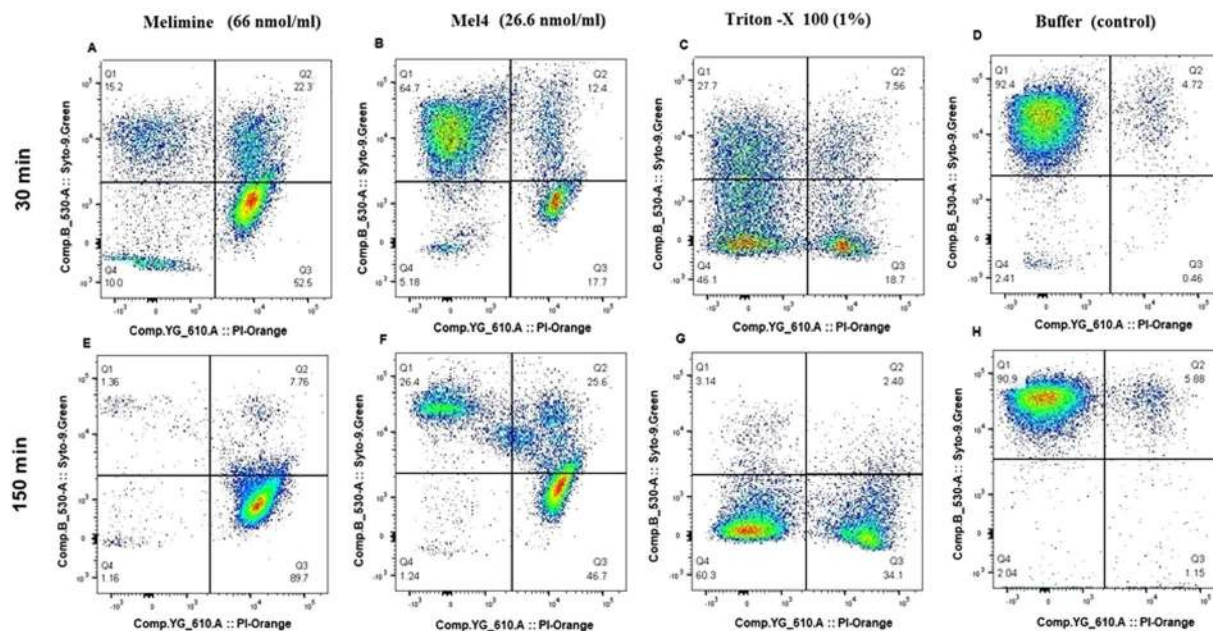


Figure 3. Membrane permeabilization of *P. aeruginosa* 6294 produced by melimine and Mel4 at their MICs determined by flow cytometry with Syto 9 (membrane permeable) and Propidium Iodide (membrane impermeable) stains.

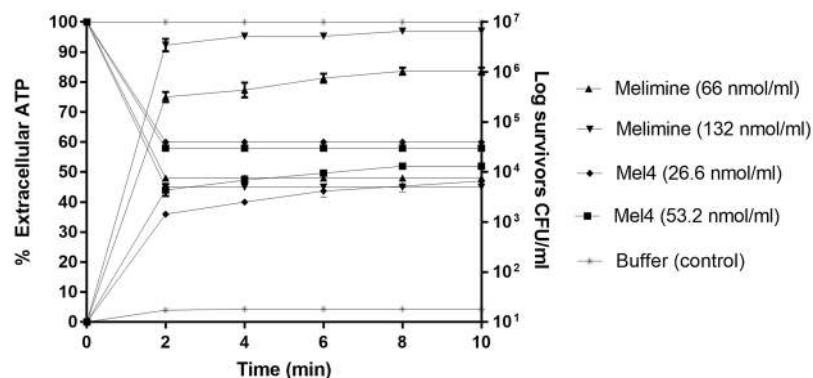


Figure 4. The effect of melimine and Mel4 on ATP release from *P. aeruginosa* 6294 at MIC and two times MIC and the corresponding change in the number of viable cells. Data presented as means (\pm SD) of three independent repeats in triplicate compared with buffer-treated control.

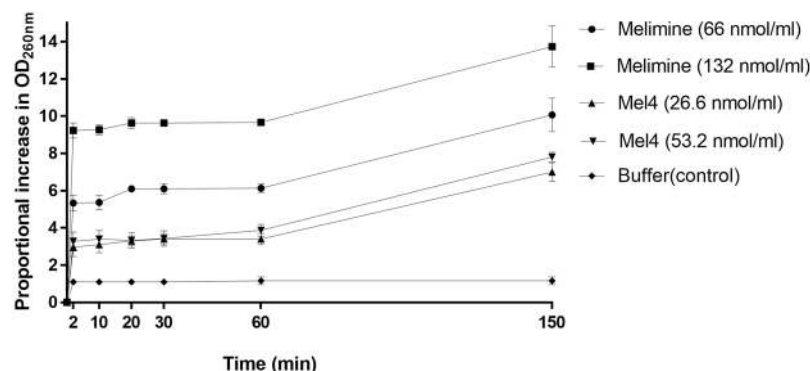


Figure 5. Increase of DNA/RNA from *P. aeruginosa* 6294 due to action of melimine and Mel4 at MIC and two times MIC determined spectroscopically at OD_{260nm} . Data presented as means (\pm SD) of three independent repeats in triplicate compared with buffer-treated control.

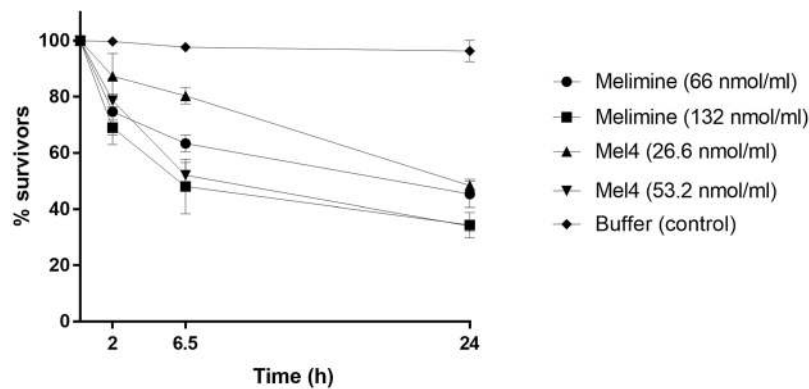


Figure 6. Lysis of *P. aeruginosa* 6294 by melimine and Mel4 at MIC and two times MIC measured spectroscopically at OD_{620nm}. Data presented as means (\pm SD) of three independent repeats in triplicate compared with buffer-treated control.

Discussion

Over last decade, melimine and Mel4 have been extensively studied *in vitro*, and in animal models and human clinical trials. This study for the first time compared and contrasted the timeline of the antimicrobial mechanism of actions of these two cationic peptides. The effects of either peptide on all the strains of *P. aeruginosa* was similar. Both peptides were able to significantly neutralise the endotoxic activity of LPS. Both peptides could depolarize the cytoplasmic membrane, and this was associated with rapid loss of cell viability. Loss of viability occurred more rapidly with melimine than Mel4. Cytoplasmic membrane depolarization was followed by ATP and DNA/RNA release from cells and permeabilization of the membrane. When incubated with peptides for 24 hours 50–60% of cells were lysed.

Usually the LAL assay generally does not reveal specific mechanism of interaction of AMPs with LPS components. However, there are number of studies which have used the LAL assay to study the interaction and hypothesized the possible way of AMPs interaction with individual components of LPS. The neutralization of LAL activity has been used previously to study the interaction of AMPs LL-37, LF-33 and HNPs-1–4 with the LPS^{33,34}. The LAL assay is believed to be neutralized due to the binding of peptides to the lipid A portion of endotoxin³⁴. The lipid A portion of endotoxin is responsible for the activation of LAL^{35,36}. More specifically, using the lipopeptides LF12 and polymyxin B it has been suggested that the interaction between peptides and LPS may be due to binding of the cationic peptides to phosphate groups on the LPS and lipid A portion³⁷. Furthermore, hydrophobic interactions between the acyl chain of the lipopeptides and the lipid chains of LPS may also occur³⁷. Mel4 has no hydrophobic residues, and melimine is only very weakly hydrophobic (Table 3) (Fig. 7), Mel4 and melimine probably interact with LPS predominantly through the phosphate groups on lipid A. Melimine killed more cells compared to Mel4 at each timepoint. This may be due to their different sizes and structure. The amino acid length required for peptides to span bacterial cytoplasmic membranes is approximately 15–20 residues^{38–40}. Mel4 is composed of 17 amino acids, just within the membrane spanning length, whereas melimine has 29 amino acid which probably allows it to easily span the cytoplasmic membrane of *P. aeruginosa*. The shorter length of the Mel4 peptide may mean that it takes longer to penetrate through the outer membrane or start to interact with the inner membrane of *P. aeruginosa* in order to kill the bacteria, or it needs to orientate itself more effectively into the membrane to begin to exert its effects. Also, tryptophan (Trp) in melimine's amino acid sequence which is not in Mel4 may have effects. Trp is known to interface with lipid bilayers and can enhance peptide-membrane interactions and facilitate peptide insertion into membranes^{29,41}. A helical peptide RW-BP100 possess a Trp residue which confers higher affinity and deeper insertion into bacterial membranes⁴². In addition, Trp facilitates the insertion of arginine, which is in high mounts in melimine, into the hydrophobic region of membranes *via* cation- π interactions causing rapid membrane disruption²⁹. Melimine, partly due to the presence of Trp, adopts a partial α helix in bio-membrane mimetic environments²³. A higher helical conformation of peptides is better suited for their antimicrobial activity⁴³. As Mel4 lacks tryptophan in its sequence, it may have less affinity towards phospholipid bilayers⁴⁴. Furthermore, the amino acid sequence of Mel4 predicts that it would have a very low hydrophobic moment (0.039; Table 3) meaning that it is less likely to be attracted within lipid bilayers^{45,46}. Also, helical wheel projections of melimine and Mel4 show that the hydrophobic amino acids in melimine segregate to one side of the molecule, whereas as there are no hydrophobic amino acids in Mel4 that can segregate (Fig. 7). The presence of non-polar amino acids such as Ile and Leu can encourage peptide binding and disruption of cell membranes³² and their absence in Mel4 compared to melimine may also affect the initial mode of action of Mel4. Compared to melimine, Mel4 has been shown to be 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 diphanyl-glycero-phosphatidylcholine lipid and 30% C20 diphanyldiglyceride ether⁴⁴. The evidence from the current study suggests that it takes more time for Mel4 to interact with the bacterial membranes to the same extent as melimine which may be due to the changes in its amino acid sequence and subsequent changes in its structure. Indeed, for lipid depolarisation at their respective MICs, it took Mel4 approximately 30 seconds longer to achieve the same degree of depolarisation and 60 seconds longer to achieve the same degree of killing compared to melimine (Fig. 1A,B). To permeabilize the cytoplasmic membrane to allow Sytox green to enter cells,

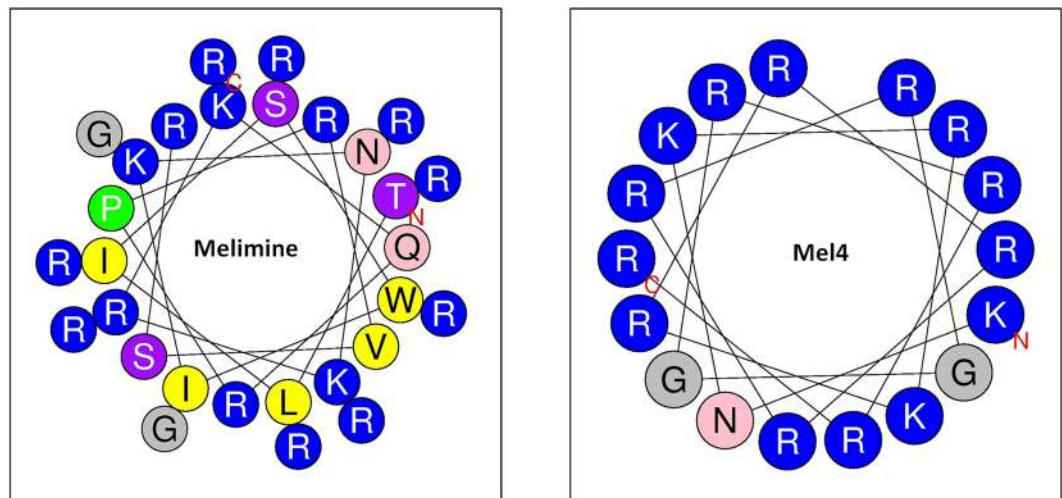


Figure 7. Helical wheel projection of melimine and Mel4. Positive charged residues are represented in blue circles, uncharged residues are in grey circles, polar residues in pink circles and hydrophobic residues in yellow circles.

Mel4 at its MIC took more than 30 minutes to achieve the same degree of permeabilization that melimine did at its MIC at 5 min. Confirmation of the role of Try, Ile or Leu in the action of Mel4 needs to be confirmed in future studies that incorporate one or more of these amino acids in Mel4.

The membrane depolarization caused by both melimine and Mel4 was a concentration dependent event. These results are in agreement with previous studies showing a concentration-dependent depolarizing effect of the helical peptide LL-37 on *P. aeruginosa*, and PMAP-36, GI24 and melittin on *E. coli* cell membranes^{47,48}. To further confirm whether membrane depolarization was a lethal event for these peptides, viable count was performed. Both AMPs reduced bacterial numbers upon membrane depolarization. Higher concentration of AMPs resulted in higher degrees of depolarization which in turn led to larger amounts of bacterial death. Interestingly, sodium azide depolarization of the cell membrane did not affect cell viability. This suggest that membrane depolarization by itself was not sufficient to cause death of *P. aeruginosa*.

The membrane potential is essential for bacterial replication and ATP generation⁴⁹. Dissipation of the membrane potential may increase membrane permeability resulting in loss of cytoplasmic contents such as ATP⁵⁰. In the present study more than 70% of total cellular ATP was released by melimine and approximately 35% by Mel4 following 2 minutes exposure, and the amount of ATP released plateaued after 4 minutes exposure to both peptides. The effect of melimine is similar to porcine cathelicidin's PR-39 effect on *E. coli* which induced 80% ATP leakage⁵¹. The leakage of ATP results in depletion of intracellular ATP which in turn can lead to bacterial death⁵². Interestingly, over the course of the study (10 mins), the amount of ATP released from cells during exposure to Mel4 never reached the amount released following exposure to melimine and also appeared to plateau indicating saturation. ATP released by bacteria can be depleted in supernatants of cells, possibly by hydrolysis at their cell surfaces⁵². The data from the current experiments may indicate that cells treated with Mel4 retain some capacity to degrade extracellular ATP.

The permeability of cytoplasmic membrane also resulted in Sytox green uptake into cells. However, this uptake was relatively slow, and did not reach the level obtained with the positive control Triton X-100. Indeed, after 30 minutes incubation the amount of fluorescence due to Sytox green entering cells was equivalent to the amount that had entered cells after 10 minutes exposure to Triton X-100 when incubated with melimine, and <150 minutes when incubated with Mel4. Furthermore, with either melimine or Mel4 it took approximately 5 minutes for any fluorescence due to Sytox green to be registered. The difference in the time between membrane depolarization and Sytox green entering cells may be due to Sytox green requiring relatively large pore sizes in bacterial cell membranes for its uptake and time to intercalate with DNA. Compared with Sytox green uptake, ATP release was faster, and this may be due to the different mechanisms of entry/exit through cellular membrane. For example, the pore size needed for ATP to penetrate through membranes is a pore size of 1.5 nm⁵³, which may be smaller than that needed for Sytox green to enter into cells. It may also take a longer time of Sytox green to cross the bacterial membranes and intercalate with DNA^{54,55}. The differences in the apparent rate of ATP release compared to Sytox green entry into cells may also be due to differences in the assay sensitivities. Similar kinetics to the current study of Sytox green staining resulting from interactions of the AMPs melittin (a parent peptide of melimine and Mel4) and LL-37 has been reported against other Gram-negative bacteria such as *P. aeruginosa*, *E. coli* and *Salmonella typhimurium*⁵⁶.

To further assess whether the peptides could damage the bacterial cell membrane, Propidium Iodide stain was used as an indicator of cells with ruptured membranes. Flow cytometry analysis indicated that treatment of *P. aeruginosa* with the both AMPs enhanced uptake of PI, suggesting that the bacterial cell membrane was disrupted. More than 50% cells stained with PI after exposure to melimine for 30 minutes at its MIC. However, Mel4 took longer (150 min) for a similar amount of the cells to stain with PI at its MIC (Fig. 3). The

positive control Triton-X resulted in less PI positive cells at this time point suggesting different rates of membrane permeabilization. Therefore, the sequence of steps occurring at the membrane, appears to begin with depolarization and followed by more significant membrane disruption (Sytox green and Propidium Iodide) influx which occur approximately at the same time as ATP and initial DNA/RNA leakage. The apparent differences in kinetics of Triton X100 with Sytox green and Propidium Iodide may be due to differences in types of pores that need to be formed to allow penetration of the two dyes into the bacteria, or interactions of the dyes with Triton X100.

Melimine caused a concentration-dependent release of DNA/RNA (260 nm absorbing material) as early as in 2 minutes, which is consistent with the results obtained by Minahk *et al.*⁵⁷ who demonstrated a concentration-dependent release of DNA/RNA from *Listeria monocytogenes* after treatment with the antimicrobial peptide Enterocin CRL35 at the concentrations equal to its MIC and >4x its MIC. However, Mel4 induced release of DNA/RNA was dose independent. The dose independence of the Mel4 release of DNA/RNA may possibly have occurred due to aggregation of Mel4. However, we have shown that a variant of Mel4 containing a cysteine residue at its N-terminus (cys-mel4) does not dimerise in solution⁴⁴ and this would be even more likely that Mel4 to dimerise given that cysteine residues can form disulphide bonds. Furthermore, we have previously shown that Mel4's parent analogue melimine does not aggregate in PBS or distilled water using AFM. The high positive charges of melimine and Mel4 would mean dimerization is not electrostatically favourable. There was also a stepped release of DNA/RNA, with an initial burst release 2 minutes after addition of melimine or Mel4, and then another release of DNA/RNA between 60–150 minutes after addition of the peptides. The amount of DNA/RNA release caused by Mel4 did not reach the level released after exposure to melimine even at longer incubation times. AMPs have been shown to permeabilize bacterial membranes by forming morphologically diverse pores in terms of diameter, lipid conformation surrounding the pores, life span and threshold of AMPs require to stabilize the pores⁵⁸, and perhaps melimine and Mel4 form morphologically diverse pores which affect release of large molecules such as DNA/RNA. The two-step process of DNA/RNA release may be due to damage and disintegration of DNA/RNA within the cell over the 150 minutes incubation with the peptides allowing smaller lengths of DNA to exit cells. This may occur during so-called bacterial apoptosis-like death, mediated via *recA*, such as occurs during antibiotic-induced bacterial death⁵⁹. Examining changes in *recA* and the size of the liberated DNA/RNA in future experiments may help understand this further. Eventually, both peptides result in complete cellular lysis of *P. aeruginosa*. Furthermore, whilst there were some differences in the MIC and MBC between strains, both peptides behaved in a similar manner to all four strains of *P. aeruginosa* (see Supplementary Data), indicating that there were no differences in the interaction whether the strains were of the invasive or cytotoxic genotype or phenotype, and the only effect of MIC or MBC differences were to the amount of peptides that were needed to kill cells, not to their mechanism of action.

In conclusion this study has revealed a comprehensive timeline of the mode of actions of melimine and Mel4 against *P. aeruginosa* which involves disruption of the cell membranes, efflux of its intracellular contents and lysis of bacteria. Figure 8 summarizes the sequence of events that occurs after incubation of *P. aeruginosa* cells with either peptide over 2 hours. It is likely that the amphipathic characteristics of melimine allowed disruption of the cell membrane more rapidly than Mel4 which only had very low amphipathicity.

Materials and Methods

All experiments were run in triplicate and repeated on three separate occasions, except for flow cytometry data which were obtained after two repeats. For all experiments, negative controls for the effect of melimine and Mel4 were simply bacterial cells grown in their absence.

Synthesis of peptides. Melimine and Mel4 ($\geq 90\%$ purity) used in the current study were synthesized by conventional solid-phase peptide protocols^{60,61} and procured from the Auspep Peptide Company (Tullamarine, Victoria, Australia).

Bacterial strains. Different strains of *P. aeruginosa* such as 6294 and 6206 (microbial keratitis isolates; 6294 an invasive strain containing the *exoS* gene and 6206 a cytotoxic strain containing the *exoU* gene⁶²), Paer1 (isolated from contact lens induced acute red eye, contains the *exoS* gene but does not manifest the associated invasive phenotype⁶²) and ATCC 19660 (isolated from human septicaemia; a cytotoxic strain containing the *exoU* gene⁶³) were used in the current study. All these strains were obtained from stock cultures preserved at -80°C in Brain Heart Infusion (Oxoid, Basingstoke, UK) containing 25% glycerol.

Bacterial cell preparation. Bacteria were grown in Tryptic Soy Broth (TSB; Oxoid) for 12–16h and cells were then washed with phosphate buffer saline (PBS, NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.4 g/L, KH_2PO_4 0.24 g/L) and diluted into the same buffer containing 1/1000 TSB to $\text{OD}_{600\text{nm}}$ 0.05–0.06 (1×10^7 colony forming units (CFU)/ml confirmed upon retrospective plate counts on TS agar (Oxoid). This inoculum preparation was used in most experiments except for assessing the minimum inhibitory and bactericidal concentrations which used cells in Muller Hinton broth and measuring the release of DiSC3–5 from cells which was performed in cells (1×10^7 CFU/ml) suspended in HEPES buffer.

Minimum inhibitory and bactericidal concentrations. 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 reported previously⁶⁴, using Muller Hinton Broth (Oxoid) containing 0.01% v/v acetic acid (Sigma Aldrich, St Louis, MO, USA) and 0.2% w/v bovine serum albumin (Sigma Aldrich; MHB). Bacterial cells, diluted to 5×10^5 CFU ml^{-1} in MHB, were

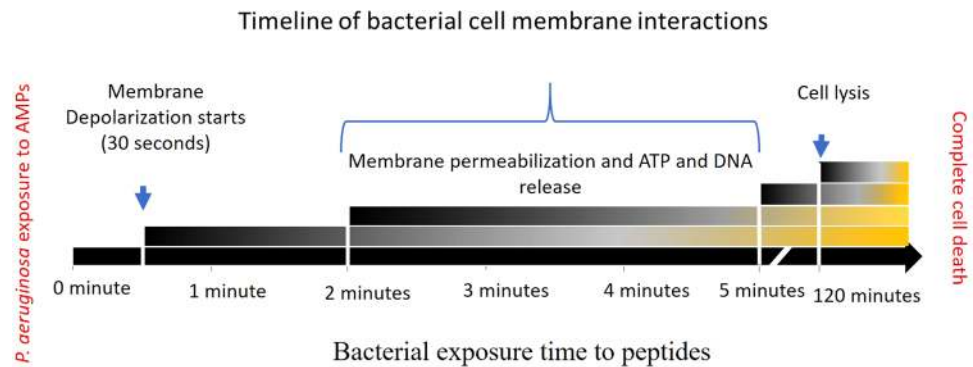


Figure 8. Timeline of *P. aeruginosa* killing by melimine and Mel4. Both AMPs started cell membrane depolarization at 30 seconds. Cell membrane permeabilization, release of ATP and DNA/RNA happened between 2 to 5 minutes of exposure. Complete bacterial lysis started at 120 minutes of incubation with peptides. All the events started at same time point for both AMPs but intensity of event to occur for melimine was higher than Mel4 at each studied time point.

incubated with various concentrations of the peptides. The MIC was set as the lowest concentration of peptides that reduced bacterial numbers by $\geq 90\%$ 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 count method compared to bacteria grown with no antimicrobial agent.

Interaction with *P. aeruginosa* lipopolysaccharide. A limulus amoebocyte lysate (LAL) assay was performed to determine the interaction of AMPs with lipopolysaccharides (LPS) of *P. aeruginosa* using a chromogenic assay kit (Cape Cod, E. Falmouth, MA, USA). Briefly, 8×10^{-4} nmol/ml LPS from *P. aeruginosa* 10 (Sigma Aldrich, St Louis, MO, USA) was dissolved with melimine and Mel4 at 1x or 2x MIC in endotoxin free water (Sigma Aldrich, St Louis, MO, USA) and incubated at 37°C for 30 minutes. The interaction of LPS with melimine or Mel4 was assessed as the decrease in $\text{OD}_{405\text{nm}}$ following addition of the LAL reagent, compared with control (LPS in endotoxin free water) without peptides in (percentage) using the formula.

$$\frac{(\text{OD}_{405\text{nm}} \text{ of Positive Control}) - (\text{OD}_{405\text{nm}} \text{ of Samples})}{(\text{OD}_{405\text{nm}} \text{ of Positive Control})} \times 100$$

Cytoplasmic membrane disruption. Three assays were performed to determine whether melimine and Mel4 could affect the cytoplasmic membrane of *P. aeruginosa*. The DiSC3-5 assay was used to determine the effect of the peptides on membrane potential. Two assays, Sytox Green and Propidium Iodide, were conducted to determine whether the peptides could damage cytoplasmic membranes and allow the stains to penetrate and bind to intracellular nucleic acids. Sytox Green has a molecular mass of 215.025 g/mol and a topological polar surface area of 28.7 \AA^2 (<https://pubchem.ncbi.nlm.nih.gov/compound/46863923>)⁶⁵, whereas Propidium Iodide has a molecular mass of 668.087 g/mol and a topological polar surface area of 55.9 \AA^2 (<https://pubchem.ncbi.nlm.nih.gov/compound/104981>)⁶⁶. Differences in the penetration of these two dyes through the cytoplasmic membranes may be associated with different sizes of pores formed by the AMPs.

Cytoplasmic membrane depolarization by the AMPs was performed as described previously²³ with melimine and Mel4 at 1x and 2x MIC at the final concentrations. The number of viable cells were confirmed by serially diluting aliquots of bacteria in D/E neutralizing broth (Remel, Lenexa, KS, USA) and plating these onto Tryptic Soy Agar (Oxoid, Basingstoke, UK) containing phosphatidylcholine (0.7 g/L) and Tween 80 (5 ml/L). The plates were incubated at 37°C overnight and the number of live bacteria were enumerated and expressed as CFU/ml. Two positive controls of dimethyl sulfoxide (DMSO) (Merck, Billerica, MA, USA) (20% v/v) in HEPES (100 μl) and sodium azide were used to depolarize the cytoplasmic membranes of bacteria⁶⁷.

For Sytox green penetration into cells, the protocol was adopted from Li *et al.*⁵⁸ with a few modifications. Briefly, bacterial cells, aliquots (100 μl) were dispensed into wells of 96-well plates along with 5 μM Sytox green (Invitrogen, Eugene, Oregon, USA) as final concentrations. Plates were incubated for 15 minutes in the dark at room temperature and then 100 μl of melimine and Mel4 were added equal to 1x or 2x as final concentrations. The increase in fluorescence was measured spectrophotometrically (at an excitation wavelength of 480 nm and an emission wavelength of 522 nm) every 1 minutes up to 30 minutes, and then after 150 minutes. A positive control of 1% (v/v) Triton X-100 (Sigma Aldrich, St Louis, MO, USA) in PBS with 1/1000 TSB (100 μl) was used to disrupt the cytoplasmic membrane of bacteria.

Flow cytometry was used to quantify the ability of melimine and Mel4 to permeabilize bacterial membranes of *P. aeruginosa* 6294 resulting in incorporation of propidium iodide (PI) (Invitrogen, Eugene, Oregon, USA) into cells with compromised cell membrane. Bacterial cells were stained simultaneously with SYTO9 and PI at concentrations of 7.5 μM and 30 μM respectively and incubated at room temperature for 15 min. Fluorescence intensities were recorded with LSRFortessa SORP Flow cytometer after addition of 1x and 2x MIC of melimine or Mel4 at different time points. The wavelength of green fluorescence was (525/550 nm) bandpass filter for SYTO9

and a red fluorescence (610/20 nm) bandpass filter for PI⁶⁸. Data were acquired and analyzed using Flowjo software (USA). Minimum 20000 events were recorded for each sample.

Leakage of intracellular contents. The leakage of ATP and DNA/RNA was measured in separate assays. Aliquots of 100 μ l of bacteria were incubated with melimine at the final concentrations equal to 1x or 2x MIC and at 37 °C for 10 minutes. The samples were taken at 2 min intervals and centrifuged at 9000 \times g for 5 minutes and the supernatant was kept on ice until further use. For determination of internal ATP the bacterial pellets were 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 \times g for two minutes and the supernatant was kept on ice until further analysis⁵¹. Subsequently, both total and extracellular ATP were determined using an ATP bioluminescence kit (Invitrogen, Eugene, Oregon, USA) according to manufacturer's instructions.

The assay for measuring the loss of DNA/RNA was performed according to protocol Carson *et al.*⁶⁹ with some modification. Aliquots (100 μ l) of bacteria was mixed with melimine and Mel4 at their 1x or 2x MIC and incubated at 37 °C. Samples were collected at different time intervals, diluted (1:10) and filtered through 0.22 μ m pores (Merck, Tullagreen, Ireland). The OD_{260nm} of the filtrates was measured in UV-star plate (Greiner Bio-one GmbH, Frickenhausen, Germany). The results were expressed as the ratio to the initial OD_{260nm}.

Lysis of bacteria. This experiment was adopted from the method of Carson *et al.*⁶⁹. The bacterial lytic potential of the two peptides was evaluated using two different bacterial inoculums 1 \times 10⁸ CFU/ml and 3 \times 10¹⁰ CFU/ml. The smaller inoculum size was tested to see whether OD_{620nm} was measurable for 1 \times 10⁸ CFU/ml. The OD_{620nm} of bacterial suspension was adjusted to 0.1 to yield 1 \times 10⁸ CFU/ml. The larger inoculum size of 3 \times 10¹⁰ CFU/ml was obtained by adjusting OD_{620nm} 0.3. The bacterial numbers CFU/ml were confirmed by retrospective plate count. Melimine and Mel4 were added at 1x MIC and 2x MIC as final concentrations. Bacterial cultures were immediately mixed and then diluted 1:1000 in TSB. The initial 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. PBS with peptides at their respective concentrations was used as blank. The results were recorded as a ratio of OD_{620nm} at each time point compared to the OD_{620nm} at 0 minutes (in percentage).

Statistical analyses. Statistical analyses were performed using GraphPad Prism 7.02 software (GraphPad Software, La Jolla, CA, USA). Neutralization of LPS was estimated using one-way ANOVA with Tukey's test. The time dependent effect of different concentrations of peptides 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. Statistical significance was set as P < 0.05.

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Author Contributions

M.Y. designed the study, performed the experiments, analysed the data and wrote the manuscript. D.D. supervised and helped M.Y. in analysing data and edited the article. M.W. planned the project, developed the theoretical framework, edited the article.

Additional Information

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