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

Muhammad Yasir, Debarun Dutta, Mark DP Willcox

School of Optometry and Vision Science, University of New South Wales, Australia

Word Count: 5085

Running title: Mode of action of two antimicrobial peptides

#Corresponding author:

*Muhammad Yasir
Ph.D candidate
School of Optometry and Vision Science
University of New South Wales Australia
Tel: +61414941761
Email: m.yasir@student.unsw.edu.au
Email: yasirjri85@gmail.com

ABSTRACT

Melimine and Mel4 are chimeric cationic peptides with broad spectrum antimicrobial activity, and recent investigations have shown that they are highly biocompatible with animal model and human clinical trials. The current study examined the mechanism of action of these two antimicrobial peptides against *P. aeruginosa* with a series of investigations. Antimicrobial activities were determined by MIC and MBC. Endotoxin neutralization was determined using the LAL assay, effect on the cytoplasmic membrane was evaluated using DiSC(3)-5 and Sytox green stains, and Syto-9 and PI dyes using flow cytometry. Release of cytoplasmic materials (ATP and DNA/RNA) were determined using ATP luminescence and increase in OD_{260nm}. The ability to lyse bacteria was studied by measuring a decrease in OD_{620nm}. The MIC of the peptides remained low against *P. aeruginosa* strains, which showed efficient neutralization of LPS, indicating their role in the anti-pseudomonas and LPS binding activities. Both AMPs rapidly (starting at 30 seconds) depolarized *P. aeruginosa* cytoplasmic membrane leading to reduction in viability. Melimine was responsible for more ATP release (75%) compared to Mel4 (36%) (*P*<0.001) following two minutes exposure. For both peptides, Sytox green entered cells after five minutes of incubation. Flow cytometry demonstrated that both the AMPs permeabilized the cell membrane at 30 minutes and followed by increasing permeability. Similar results were found with DNA/RNA release experiments. Overall, melimine showed higher ability of membrane disruption, cell lysis compared to Mel4 (*P*<0.001). Knowledge regarding mechanism of action of these two AMPs would be helpful in making them as anti-pseudomonas drug.

KEYWORDS *P. aeruginosa*, Antimicrobial peptides, mechanism of action, membrane disruption

58

INTRODUCTION

60 *Pseudomonas aeruginosa* is a metabolically versatile ubiquitous Gram-negative opportunistic

61 pathogen that can cause infections in animals and plants (1). *P. aerugino*sa is responsible for

62 localized to systemic infections in humans, which can be life threatening. Over the years, *P.*

63 *aerugino*sa has become one of the most frequent causative agents of nosocomial infections,

64 associated with substantial morbidity and mortality (2). The current standards of care to treat

65 *P. aeruginosa* infections are not effective (3) as its outer membrane offers low permeability,

66 which limits the penetration of antibiotics into the bacterial cells thereby increase antibiotic

67 resistance (4, 5). Given the severity of *P. aeruginosa* infections and the limited antimicrobial

68 arsenal with which to treat them, finding new alternative antimicrobials with unique

69 mechanisms of action is urgently required (6).

70 Antimicrobial peptides (AMPs) are part of the innate immune response of living organisms

71 and have broad spectrum activity ranging from viruses to parasites at low concentrations (7).

72 AMPs are usually cationic in nature and have a varying number (from five to over a hundred)

73 of amino acids. AMPs possess multiple modes of action, rapid bacterial killing kinetics and

74 little toxicity toward human cells (8, 9). Bacteria do not easily gain resistance to AMPs due to

75 their fast killing mechanism and multiple target sites (10, 11). The mechanism of action of

76 AMPs starts by interacting with negatively charged lipopolysaccharides (LPS) in the outer

77 membranes of Gram negative bacteria (12-14) leading to a destabilization and

78 permeabilization (15). AMPs are then able to interact with the cytoplasmic membrane.

79 Several models for the interaction of AMPs with bacterial cytoplasmic membranes have been

80 proposed, such as "barrel stave" "toroidal pore" and "carpet model" (12). In all of these

81 models AMPs displace lipids, alter membrane structure and these interactions result in

82 leakage of cellular contents such as K+, ATP and DNA/RNA, ultimately resulting in cell

3

83 death (16, 17). AMPs can also act intracellularly disrupting DNA, RNA and protein synthesis

84 (18, 19).

85 Melimine (TLISWIKNKRKQRPRVSRRRRRRGGRRRR) is a cationic hybrid peptide of

86 melittin and protamine (20). It has broad spectrum activity against a wide range of Gram-

87 negative and Gram-positive bacteria (including MRSA and multi drug resistant *P.*

88 *aeruginosa*, fungi and protozoan such as *Acanthamoeba*) (20, 21). Importantly, bacteria did

89 not develop resistance against melimine when exposed at sub-MIC for 30 consecutive days

90 (20). Moreover, melimine is not cytotoxic at well above active concentrations (20,

91 21). Melimine has a random coil confirmation in aqueous environments but increases its α-

92 helical content (to 35-40%) in bacterial membrane mimetic environments. This change in

93 conformation upon binding to the membrane is widely accepted as the first step in the

94 mechanism of action of many AMPs (22). Melimine disrupts the outer membrane and rapidly

95 destroys the inner membrane potential of *P. aeruginosa* (23). Melimine retains its

96 antimicrobial activity when bound to polymers and titanium (20, 21, 24). However, in a

97 human clinical trials, melimine-coated contact lenses caused corneal staining (25).

98 A shorter sequence of melimine called Mel4 (KNKRKRRRRRGGRRRR) has been

99 designed which was shown to not result in corneal staining after being bound to contact

100 lenses (26). Mel4 is highly active against *P. aeruginosa* in solution or when immobilized on

101 surfaces (27). It is non-cytotoxic to mammalian cells *in vitro* (20, 21), animal model studies

102 in vivo and in human clinical trials (26, 28). As can be seen from the amino acid sequence of

103 both peptides, Mel4 has had several amino acids removed, including the single tryptophan in

104 melimine. Tryptophan is known to be a highly lipophilic amino acid (29), and many cationic

105 peptides contain tryptophan as an important part of their mode of action (30-32). Similarly,

106 other non-polar amino acids such as Leu and Ile can encourage peptide binding and

107 disruption of cell membranes (33). Given that both the peptides have been extensively

4

108 investigated with human clinical trials, their bactericidal mechanism is relatively unknown. In

109 addition, amino acid sequence of Mel4 is different to melimine, its mechanism to kill Gram

110 negative bacteria is completely unknown. Hence, this study examined and compared the

111 mode of action of melimine and Mel4 against *P. aeruginosa*.

112 **MATERIALS AND METHODS**

113 All experiments were run in triplicate and repeated on three separate occasions, except for

114 flow cytometry data which were obtained after two repeats. For all experiments, negative

115 controls for the effect of melimine and Mel4 were simply bacterial cells grown in their

116 absence.

117 **Synthesis of peptides**

118 Melimine and Mel4 (≥90% purity) used in the current study were synthesized by

119 conventional solid-phase peptide protocols and procured from the Auspep Peptide Company

120 (Tullamarine, Victoria, Australia).

121 **Bacterial strains**

122 Different strains of *P. aeruginosa* such as 6294 and 6206 (microbial keratitis isolates; 6294

123 an invasive strain containing the *exoS* gene and 6206 a cytotoxic strain containing the *exoU*

124 gene) (34), Paer1 (isolated from contact lens induced acute red eye, contains the *exoS* gene

125 but does not manifest the associated invasive phenotype) (34) and ATCC 19660 (isolated

126 from human septicaemia; a cytotoxic strain containing the *exoU* gene) (35) were used in the

127 current study. All these strains were obtained from stock cultures preserved at -80 °C in Brain

128 Heart Infusion (Oxoid, Basingstoke, UK) containing 25% glycerol.

129 **Bacterial cell preparation**

5

130 Bacteria were grown in Tryptic Soy Broth (TSB; Oxoid) for 12-16 h and cells were then

131 washed with phosphate buffer saline (PBS, NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.4 g/L,

132 KH_2PO_4 0.24 g/L) and diluted into the same buffer containing 1/1000 TSB to OD_{600nm} 0.05-

133 0.06 (1×10^7 colony forming units (CFU)/ml confirmed upon retrospective plate counts on TS

134 agar (Oxoid). This inoculum preparation was used in most experiments except for assessing

135 the minimum inhibitory and bactericidal concentrations and measuring the release of DiSC3-

136 5 from cells (which was performed in cells (1×10^7 CFU/ml) suspended in HEPES buffer).

137 **Minimum inhibitory and bactericidal concentrations**

138 The minimum inhibitory and minimum bactericidal concentrations of melimine and Mel4

139 were determined for all strains using a modified version of the Clinical Laboratory and

140 Standard Institute (CLSI) broth microdilution method as reported previously (36), using

141 Muller Hinton Broth (Oxoid) containing 0.01% v/v acetic acid (Sigma Aldrich, St Louis,

142 MO, USA) and 0.2% w/v bovine serum albumin (Sigma Aldrich; MHB). Bacterial cells,

143 diluted to 5×10^5 CFU ml^{-1} in MHB, were incubated with various concentrations of the

144 peptides. The MIC was set as the lowest concentration of peptides that reduced bacterial

145 numbers by ≥ 90% while the MBC was set as the lowest concentration of peptides that

146 reduced bacterial growth by >99.99% after enumeration of viable bacteria by plate count

147 method compared to bacteria grown with no antimicrobial agent.

148 **Interaction with *P. aeruginosa* lipopolysaccharide**

149 A limulus amoebocyte lysate (LAL) assay was performed to determine the interaction of

150 AMPs with lipopolysaccharides (LPS) of *P. aeruginosa* using a chromogenic assay kit (Cape

151 Cod, E. Flamouth, MA, USA). Briefly, 8×10^{-4} nmol/ml LPS from *P. aeruginosa* 10 (Sigma

152 Aldrich, St Louis, MO, USA) was dissolved with melimine and Mel4 at 1x or 2x MIC in

153 endotoxin free water (Sigma Aldrich, St Louis, MO, USA) and incubated at 37 °C for 30 min.

154   The interaction of LPS with melimine or Mel4 was assessed as the decrease in OD_{405nm},

155   following addition of the LAL reagent, compared with control (LPS in endotoxin free water)

156   without peptides.

157   **Cytoplasmic membrane disruption**

158   Three assays were performed to determine whether melimine and Mel4 could affect the

159   cytoplasmic membrane of *P. aeruginosa*. The DiSC3-5 assay was used to determine the

160   effect of the peptides on membrane potential. Two assays, Sytox Green and Propidium

161   Iodide, were conducted to determine whether the peptides could damage cytoplasmic

162   membranes and allow the stains to penetrate and bind to intracellular nucleic acids. Sytox

163   Green has a molecular mass of 213.954 g/mol and a topological polar surface area of 28.7 A^2,

164   whereas Propidium Iodide has a molecular mass of 668.087 g/mol and a topological polar

165   surface area of 55.9 A^2(37). Differences in the penetration of these two dyes through the

166   cytoplasmic membranes may be associated with different sizes of pores formed by the AMPs.

167   Cytoplasmic membrane depolarization by the AMPs was performed as described previously

168   (23) with melimine and Mel4 at 1x and 2x MIC at the final concentrations. The number of

169   viable cells were confirmed by serially diluting aliquots of bacteria in D/E neutralizing broth

170   (Remel, Lenexa, KS, USA) and plating these onto Tryptic Soy Agar (Oxoid, Basingstoke,

171   UK) containing phosphatidylcholine (0.7 g /L) and Tween 80 (5ml/L). The plates were

172   incubated at 37 ^{o}C overnight and the number of live bacteria were enumerated and expressed

173   as CFU/ml. Two positive controls of dimethyl sulfoxide (DMSO) (Merck, Billerica, MA,

174   USA) (20% v/v) in HEPES (100 µl) and sodium azide were used to depolarise the

175   cytoplasmic membranes of bacteria (38).

176   For Sytox green penetration into cells, the protocol was adopted from Li *et al.,*(39) with a few

177   modifications. Briefly, bacterial cells, aliquots (100 µl) were dispensed into wells of 96-well

7

178   plates along with 5μM Sytox green (Invitrogen, Eugene, Oregon, USA). Plates were

179   incubated for 15 minutes in the dark at room temperature and then 100 μl of melimine and

180   Mel4 were added equal to 1x or 2x as final concentrations. The increase in fluorescence was

181   measured spectrophotometrically (at an excitation wavelength of 480 nm and an emission

182   wavelength of 522 nm) every 1 minutes up to 30 minutes, and then after 150 minutes. A

183   positive control of 1% (v/v) Triton X-100 (Sigma Aldrich, St Louis, MO, USA) in PBS with

184   1/1000 TSB (100 μl) was used to disrupt the cytoplasmic membrane of bacteria.

185   Flow cytometry was used to quantify the ability of melimine and Mel4 to permeabilize

186   bacterial membranes of *P. aeruginosa* 6294 resulting in incorporation of propidium iodide

187   (PI) (Invitrogen, Eugene, Oregon, USA) into cells with compromised cell membrane.

188   Bacterial cells were stained simultaneously with SYTO9 and PI at concentrations of 7.5 μM

189   and 30 μM respectively and incubated at room temperature for 15 min. Fluorescence

190   intensities were recorded with LSRFortessa SORP Flow cytometer after addition of 1x and

191   2x MIC of melimine or Mel4 at different time points. The wavelength of green fluorescence

192   was (525/550 nm) bandpass filter for SYTO9 and a red fluorescence (610/20 nm) bandpass

193   filter for PI (40). Data were acquired and analyzed using Flowjo software (USA). Minimum

194   20000 events were recorded for each sample.

195   **Leakage of intracellular contents**

196   The leakage of ATP and DNA/RNA was measured in separate assays. Aliquots of 100 μl of

197   bacteria were incubated with melimine at the final concentrations equal to 1x or 2xMIC and

198   at 37°C for 10 min. The samples were taken at 2 min intervals and centrifuged at 9000 × g for

199   five minutes and the supernatant was kept on ice until further use. For determination of

200   internal ATP the bacterial pellets were resuspended in boiling 100 mM Tris, 4 mM EDTA pH

201   (7.4) and further incubated for 2 mins at 100 °C to lyse all the cells. The lysed cells were

202   centrifuged at 9600 × g for two minutes and the supernatant was kept on ice until further

203   analysis (41). Subsequently, both total and extracellular ATP were determined using an ATP

204   bioluminescence kit (Invitrogen, Eugene, Oregon, USA) according to manufacturer's

205   instructions.

206   The assay for measuring the loss of DNA/RNA was performed according to protocol Carson

207   *et al.,* (42) with some modification. Aliquotes (100 µl) of bacteria was incubated with

208   melimine and Mel4 at their 1x or 2x MIC and incubated at 37 $^{\circ}$C. Samples were collected at

209   different time intervals, diluted (1:10) and filtered through 0.22 µm pores (Merck,

210   Tullagreen, Ireland). The OD_{260nm} of the filtrates was measured in UV-star plate (Greiner

211   Bio-one GmbH, Frickenhausen, Germany). The results were expressed as the ratio to the

212   initial OD_{260nm}.

213   **Lysis of bacteria**

214   This experiment was adopted from the method of Carson *et al.,* (42). The bacterial lytic

215   potential of the two peptides was evaluated using two different bacterial inoculums 1×10^{8}

216   CFU/ml and 3×10^{10} CFU/ml. The smaller inoculum size was tested to see whether OD_{620nm}

217   was measurable for 1×10^{8} CFU/ml. The OD_{660nm} of bacterial suspension was adjusted to 0.1

218   to yield 1×10^{8} CFU/ml. The larger inoculum size of 3×10^{10} CFU/ml was obtained by

219   adjusting OD_{620nm} 0.3. The bacterial numbers CFU/ml were confirmed by retrospective plate

220   count. Melimine and Mel4 were added at 1x MIC and 2x MIC as final concentrations.

221   Bacterial cultures were immediately mixed and then diluted 1:1000 in TSB. The OD_{620nm} was

222   measured and additional readings were taken at 30, 60, 90, 120 minutes, 6.5 and 24 h after

223   incubating at room temperature. PBS with peptides at their respective concentrations was

224   used as blank. The results were recorded as a ratio of OD_{620nm} at each time point compared to

225   the OD_{620nm} at 0 minutes (in percentage).

226   **Statistical analyses**

227   Statistical analyses were performed using GraphPad Prism 7.02 software (GraphPad

228   Software, La Jolla, CA, USA). The effect of the different concentrations of peptides was

229   analysed using Tukey's test of multiple comparisons. Correlations between release of

230   extracellular ATP and bacterial death were examined using Pearson correlation test.

231   Statistical significance was set as $P<0.05$.

232

233 **RESULTS**

234 **Inhibitory concentrations of peptides**

235 MICs and MBCs for melimine and Mel4 against *P. aeruginosa* are shown in **Table 1.** Mel4

236 had highest bactericidal activity against *P. aeruginosa* strains 6294, 6206 and ATCC 19660

237 with a MIC of 26.6 nmol/ml. The MIC was lower for melimine against strains 6294, 6206

238 and Paer1 at 66 nmol/ml compared to strain ATCC19660. For Mel4, the MIC was lowest

239 against stains 6206, 6294 and ATCC19660 at 26.6 nmol/ml. For all strains the MBC was

240 usually 2x the MIC except for strain 6294 where the MBC for both melimine and Mel4 was

241 ≥4X the MIC while for ATCC 19660 the MBC for Mel4 was equivalent to the MIC.

242 Melimine at its lowest MBC needed 1.59×10^{12} molecules per cell to cause death whereas

243 Mel4 needed 3.2×10^{11} molecules per cell to cause cell death at its lowest MBC.

244 **Interaction with Lipopolysaccharides**

245 Both melimine and Mel4 interacted with the LPS of *P. aeruginosa* and neutralized its

246 endotoxin activity in a concentration dependent manner. At the 66 nmol/ml concentration

247 melimine significantly neutralized LPS which was evidenced by reduction of OD_{405nm} by

248 $1/6^{th}$ compared to the controls. Similarly, melimine at the concentration of 132 nmol/ml

249 neutralized LPS by $1/8^{th}$ compared to the controls. Neutralization of LPS was similar with the

250 Mel4 which reduced OD_{405nm} by half (1/2) at 26.6 nmol/ml and $1/4^{th}$ at 53.2 nmol/ml

251 compare to control.

252 **Membrane disruption**

253 Both melimine and Mel4 depolarized the cytoplasmic membrane of *P. aeruginosa* in a

254 concentration dependent manner. An increase in fluorescence intensity was detected as early

255 as 30 seconds after addition of either peptide to all the strains of *P. aeruginosa*. **Fig. 1a** shows

11

256   the data for *P. aeruginosa* 6294, whereas data for all other strains are available in the

257   supplementary documents. Following 150 seconds of exposure, no further increase in the

258   release of DiSC3-(5) was seen for any of the strains investigated. Fluorescein intensity was

259   higher at the MIC of melimine (66 nmol/ml) compared to the MIC of Mel4 (26.6 nmol/ml),

260   and the same trend was observed throughout the time course ($P<0.005$). This depolarization

261   of the cytoplasmic membrane was associated with 3.6 $\log_{10}$ and 4.0 $\log_{10}$ viable *P. aeruginosa*

262   6294 inhibition by melimine MIC (66 nmol/ml) and MBC (132 nmol/ml) respectively.

263   Similarly, there was 2.7 $\log_{10}$ and 3.2 $\log_{10}$ reduction by Mel4 MIC (26.6 nmol/ml) and MBC

264   (53.2 nmol/ml) (**Fig.1b**). Sodium azide also depolarized the cell membrane and released

265   DiSC3-(5) over the period of testing, but with reduced fluorescence intensity and without

266   bacterial killing. Sytox green fluorescence increased over time and was detected as early as 5

267   minutes (**Fig. 2**). The intensity of fluorescence gradually increased over 30 minutes for all the

268   peptide concentrations for all strains. However, this effect was not concentration dependent.

269   For both melimine and Mel4 no significant differences were observed between the MIC and

270   MBC. At their respective MICs, melimine allowed more Sytox green to enter cells than Mel4

271   after 150 mins ($P<0.001$). Treatment with the positive control Triton-X 100 (1%) resulted

272   emission of higher Sytox green fluorescence.

273   The membrane damaging effect of these peptides was also assessed with *P. aeruginosa* 6294

274   by the flow cytometry in the presence of the DNA intercalating dye PI. The peptides were

275   able to permeabilize the cell membrane in a concentration dependent manner, and this effect

276   was time-dependent with a higher influx of PI after 150 mins incubation (**Fig.3**). Melimine

277   permeabilized 52.5% of *P. aeruginosa* 6294 cells at its MIC at 30 min. after 150 minutes of

278   exposure, PI stained up to 90% of the cells. Although Mel4 permeabilized *P. aeruginosa*

279   membranes, permeabilization at its MIC was less compared with melimine; Mel4 damaged

280   only 17.7% cells after 30 min which increased to 46.7% after 150 min (**Fig.3**). The positive

281   control Triton-X 100 (1%) permeabilized the membrane in 18.7% cells initially which

282   increased up to 34.1% after 130 min. There was also different kinetics of cell death as it can

283   interact with both lipids as well as proteins, create pores and/or remove both lipids/proteins

284   from the membrane. with Triton-X 100. The negative control (buffer-treated) behaved as

285   expected and showed only < 1% PI-stained cells (**Fig.3**).

286   **The release of cytoplasmic contents**

287   Melimine caused 75% and 92 % of ATP release at 1x and 2x MIC concentrations after 2

288   minutes ($P<0.001$) (Figure 4). The increase of extracellular ATP directly correlated with the

289   loss of viability of *P. aeruginosa* ($R^2>0.987$). Within the first two minutes, melimine

290   decreased viability by > 3.0 $\log_{10}$. Whereas Mel4 released 36% and 44% extracellular ATP at

291   1x or 2x MIC respectively at 2 minutes (**Fig. 4**). Further incubation for 10 minutes resulted in

292   a slight increase in the release of extracellular ATP. The release of ATP was associated with

293   the reduction of >2.0 $\log_{10}$ viable bacteria. Melimine induced more leakage of ATP than Mel4

294   ($P<0.001$).

295   **Fig.5** shows the releases of DNA or RNA (260nm absorbing material) following incubation

296   with the peptides. The release of DNA or RNA of *P. aeruginosa* was first detected at 2

297   minutes time. Melimine was associated with a dose-dependent release of DNA/RNA which

298   was significantly higher than observed with the control after 5 min ($P<0.001$). On the other

299   hand, the release of DNA/RNA was not affected by the concentration of Mel4. Compared

300   with Mel4, melimine released higher amounts of DNA/RNA at all the time points observed

301   ($P<0.001$).

302   **Bacterial lysis**

13

303 When the lower cell concentration (1×10^8 CFU/ml) was used, no decrease in OD_{620nm} could

304 be seen as the optical density was very low to start with. When higher bacterial inoculum $1\times$

305 10^{10} CFU/ml was used and treated with varying concentrations of peptides over 2 hours, a

306 significant reduction in OD_{620nm} was observed (**Fig. 6**). After incubation with melimine, the

307 OD_{620nm} reduced by $25\pm12\%$ at 1x and 31 ± 3 % at 2x MIC compared to buffer treated

308 negative control (*P*<0.001). OD_{620nm} was further reduced by $37\pm03\%$ and $52\pm10\%$ with

309 1xand 2x MIC after 6 hours respectively. This trend continued over the 24 hours assay where

310 melimine had a higher bacterial-lytic effect and decreased the OD_{620nm} to more than $55\pm5\%$ at

311 both the concentrations. A similar trend was seen for Mel4 which reduced the OD_{620nm} by

312 13 ± 8 % and 21 ± 6 % at 1x and 2x MIC respectively after 2 hours (**Fig. 6**). Further reductions

313 in OD_{620nm} by $20\pm3\%$ with 1x and $48\pm5\%$ with 2x MIC was observed for Mel4 after 6.5 h

314 respectively. Similarly, OD_{620nm} decreased by $52\pm2\%$ by both the concentrations of Mel4

315 after 24 h of incubation (Fig. 6). The bacteriolytic efficiency of both melimine and Mel4 was

316 similar at their MICs (*P*=0.927) but at 2x MIC melimine reduced higher OD_{620nm} than 1x

317 MIC of Mel4 (*P*<0.004) after 24 hours of incubation. The OD_{620nm} of control cells without

318 any peptides remained unchanged over the 24 hours of the experiment.

319

320

321

14

DISCUSSION

Over last decade, melimine and Mel4 have been extensively studied *in vitro*, with 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 thoroughly investigated cationic peptides. The following **Fig. 8** summarizes the sequences of membrane interactions and bactericidal events of the two peptides against *P. aeruginosa* 6294 over the first 2 hours (120 minutes) of exposure. The effects of each of the peptides on all the strains of *P. aeruginosa* were 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. It was found that loss of viability was quicker with melimine than Mel4. Cytoplasmic membrane depolarisation was followed by ATP and DNA/RNA release from cells, which in turn was followed by permeabilization of the membrane to Sytox green dye that binds to intracellular DNA. When incubated with peptides for 24 hours 50-60% of cells were lysed.

We have found that melimine has higher bactericidal efficacy when compared to Mel4 at the studied timepoints, and this may be due to their difference in size and structure. The amino acid length required for the peptides to span bacterial cytoplasmic membranes is approximately 15–20 residues, which may vary depending on the thickness of the lipid bilayer (43-45). Mel4 has 17 amino acid, 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 affects.

15

346 Another possible reason behind the differences in the activity of the two peptides is the

347 presence of tryptophan in the sequence of melimine. Tryptophan (Trp) is known to interface

348 with lipid bilayers and can enhance peptide-membrane interactions and facilitate insertion

349 into the membrane (46, 47). A helical peptide RW-BP100 possess a Trp residue which

350 confers higher affinity and deeper insertion into bacterial membranes (48). In addition, Trp

351 has been shown to facilitate the insertion of arginine into the hydrophobic region of

352 membranes *via* cation–π interactions causing rapid membrane disruption (30). Melimine,

353 partly due to the presence of Trp, adopts a partial α helix in bio-membrane mimetic

354 environment (23). A higher helical conformation of peptides is better suited for their

355 antimicrobial activity (49). As Mel4 lacks tryptophan in its sequence, it may have less

356 affinity towards phospholipid bilayers (50). Furthermore, the amino acid sequence of Mel4

357 predicts that it would have a very low hydrophobic moment (0.039; Table 2) meaning that it

358 is less likely to be attracted within lipid bilayers (51, 52). Also, helical wheel projections of

359 melimine and Mel4 show that the hydrophobic amino acids in melimine segregate to one

360 side of the molecule, whereas as there are no hydrophobic amino acids in Mel4 that can

361 segregate (Figure 7). The lack of the non-polar amino acids Ile and Leu which can

362 encourage peptide binding and disruption of cell membranes (33) may also affect the initial

363 mode of action of Mel4. A recent study (53) has demonstrated that Mel4 does not interact

364 with lipid spheroids composed of 1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine (PC

365 18:1) or tethered lipid bilayers composed of 70% zwitterionic C20 diphytanyl-glycero-

366 phosphatidylcholine lipid and 30% C20 diphytanyl-diglyceride ether whilst melimine can

367 interact with these lipid layers. The evidence from the current study suggests that it takes

368 more time for Mel4 to interact with the bacterial membranes to the same extent as melimine

369 due to the changes in its amino acid sequence and subsequent changes in its structure. Indeed,

370 for lipid depolarisation at their respective MICs, it took Mel4 approximately 30 seconds

16

371   longer to achieve the same degree of depolarisation and 60 seconds longer to achieve the

372   same degree of killing with Mel4 compared to melimine as, evidences by the comparative

373   timeline (**Fig. 1a & 1b**). To permeabilize the cytoplasmic membrane to allow Sytox green to

374   enter cells, Mel4 at its MIC took more than 30 minutes to achieve the same degree of

375   permeabilization that melimine did at its MIC at 5 min. Confirmation of the role of Try, Ile or

376   Leu in the action of Mel4 need to be confirmed in future studies that incorporate one or more

377   of these amino acids in Mel4.

378   The membrane depolarization caused by both melimine and Mel4 was a concentration

379   dependent event. These results are in agreement with previous studies showing a

380   concentration-dependent depolarizing effect of the helical peptide LL-37 on *P. aeruginosa,*

381   and PMAP-36, GI24 and melittin on *E. coli* cell membranes (54, 55). To further confirm

382   whether membrane depolarization was a lethal event for these peptides, viable count was

383   performed. Both AMPs reduced bacterial numbers upon membrane depolarization. Higher

384   concentration of AMPs resulted in higher degrees of depolarization which in turn lead to

385   larger amounts of bacterial death. Interestingly, sodium azide depolarization of the cell

386   membrane did not affect cell viability. This suggest that membrane depolarization by itself

387   was not sufficient to cause death of *P. aeruginosa.*

388   The membrane potential is essential for bacterial replication and ATP generation (56).

389   Dissipation of the membrane potential may increase membrane permeability resulting in loss

390   of cytoplasmic contents such as ATP (57). In the present study more than 70% ATP was

391   released by melimine and approximately 35% by Mel4 following 2 minutes exposure, and the

392   amount of ATP released plateaued at after four minutes exposure to both peptides. The effect

393   of melimine is similar to the AMP porcine cathelicidin's PR-39 effect of *E. coli* which

394   induced 80% ATP leakage (58). The leakage of ATP results in depletion of intracellular ATP

395   which in turn can lead to bacterial death (56). Interestingly, over the course of the study (10

17

396   mins), the amount of ATP released from cells during exposure to Mel4 never reached the

397   amount released following exposure to melimine and also appeared to plateau indicating

398   saturation. ATP released by bacteria can be depleted in supernatants of cells, possibly by

399   hydrolysis at their cell surfaces (59). The data from the current experiments may indicate that

400   cells treated with Mel4 retain some capacity to degrade extracellular ATP.

401   The permeability of cytoplasmic membrane also resulted in Sytox green uptake into cells.

402   However, this uptake was relatively slow, and did not reach the level obtained with the

403   positive control Triton X-100. Indeed, after 30 minutes incubation the amount of fluorescence

404   due to Sytox green entering cells was equivalent to the amount that had entered cells after 10

405   minutes exposure to Triton X-100 when incubated with melimine, and <5 minutes when

406   incubated with Mel4. Furthermore, with either melimine or Mel4 it took approximately 5

407   minutes for any fluorescence due to Sytox green to be registered. The difference in the time

408   between membrane depolarization and Sytox green entering cells may be due to Sytox green

409   requiring relatively large pore sizes in bacterial cell membranes for its uptake and time to

410   intercalate with DNA. Compared with Sytox green uptake, ATP release was faster, and this

411   may be due to the different mechanisms of entry/exit through cellular membrane. For

412   example, the pore size needed for ATP to penetrate through membranes is a pore size of 1.5

413   nm (60), which is smaller than that needed for Sytox green to enter into cells. It may also take

414   a longer time of Sytox green to cross the bacterial membranes and intercalate with DNA (61,

415   62). Similar kinetics to the current study of Sytox green staining resulting from interactions

416   of the AMPs melittin and LL-37 has been reported against other Gram negative bacteria such

417   as *P. aeruginosa, E. coli* and *Salmonella typhimurium* (63).

418   To further assess whether the peptides could damage the bacterial cell membrane, PI stain

419   was used as an indicator of cells with ruptured membranes. Flow cytometry analysis

420   indicated that treatment of *P. aeruginosa* with the both AMPs enhanced uptake of PI,

421   suggesting that the bacterial cell membrane was disrupted. More than 50 % cells stained with

422   PI after exposure to melimine for 30 minutes at its MIC. However, Mel4 took longer (150

423   min) for a similar amount of the cells to stain with PI at its MIC (**Fig. 3**). The positive control

424   Triton-X resulted in less PI positive cells at this time point suggesting different rates of

425   membrane permeabilization. Therefore, the sequence of steps occurring at the membrane,

426   appears to begin with depolarization (with ATP and initial DNA/RNA leakage) and followed

427   by more significant membrane disruption resulting in Sytox green and PI influx.

428   Melimine caused a concentration-dependent release of DNA/RNA (260nm absorbing

429   material) as early as in 2 min, which is consistent with the results obtained by Minahk *et al.,*

430   (64) who demonstrated a concentration-dependent release of DNA/RNA from *Listeria*

431   *monocytogenes* after treatment with the antimicrobial peptide Enterocin CRL35 at the

432   concentrations equal to its MIC and >4X its MIC. However, Mel4 induced release of

433   DNA/RNA was dose independent. There was also a stepped release of DNA/RNA, with an

434   initial burst release 2 minutes after addition of melimine or Mel4, and then another release of

435   DNA/RNA between 60-150 minutes after addition of the peptides. The amount of DNA/RNA

436   release caused by Mel4 did not reach the level released after exposure to melimine even at

437   longer incubation times. AMPs have been shown to permeabilize bacterial membranes by

438   forming morphologically diverse pores in terms of diameter, lipid conformation surrounding

439   the pores, life span and threshold of AMPs require to stabilize the pores (65), and perhaps

440   melimine and Mel4 form morphologically diverse pores which affect release of large

441   molecules such as DNA/RNA. The two-step process of DNA/RNA release may be due to

442   damage and disintegration of DNA/RNA within the cell over the 150 minutes incubation with

443   the peptides allowing smaller lengths of DNA to exit cells. This may occur during so-called

444   bacterial apoptosis-like death, mediated via *recA,* such as occurs during antibiotic-induced

445   bacterial death (66). Examining changes in *recA* and the size of the liberated DNA/RNA in

19

446 future experiments may help understand this further. In conclusion this study has revealed a

447 comprehensive timeline of the mode of actions of melimine and Mel4 against *P. aeruginosa*

448 involved disruption of the cell membranes, efflux of its intracellular contents and lysis of

449 bacteria. It is likely that the amphipathic characteristics of melimine allowed disruption of the

450 cell membrane more rapidly than Mel4 which only had very low amphipathicity.

451 **ACKNOWLEDGEMENTS**

456

457

458

459

460

461

462

463

464

465

466

467

REFERENCES

469
470 1. Strateva T, Mitov I. 2011. Contribution of an arsenal of virulence factors to pathogenesis of Pseudomonas aeruginosa infections. *Ann of Microbiol* **61**:717-732.

472 2. Buhl M, Peter S, Willmann M. 2015. Prevalence and risk factors associated with colonization and infection of extensively drug-resistant Pseudomonas aeruginosa: a systematic review. *Expert Rev Anti Infect Ther* **13**:1159-70.

475 3. Hancock RE, Speert DP. 2000. Antibiotic resistance in Pseudomonas aeruginosa: mechanisms and impact on treatment. *Drug Resist Updat* **3:**247-255**.**

477 4. Breidenstein EB, de la Fuente-Núñez C, Hancock RE. 2011. Pseudomonas aeruginosa: all roads lead to resistance. *Tren in Microbiol* **19**:419-426.

479 5. Henrichfreise B, Wiegand I, Pfister W, Wiedemann B. 2007. Resistance mechanisms of multiresistant Pseudomonas aeruginosa strains from Germany and correlation with hypermutation. *Antimicrob Agents and Chemother* **51**:4062-4070.

482 6. Gellatly SL, Hancock REW. 2013. Pseudomonas aeruginosa : new insights into pathogenesis and host defenses. *Path Dis* **67**:159-173.

484 7. Bahar AA, Ren D. 2013. Antimicrobial peptides. *Pharmaceuticals* **6**:1543-1575.

485 8. Matsuzaki K. 2009. Control of cell selectivity of antimicrobial peptides. *Biochim Biophys Acta* **1788**:1687-1692.

487 9. Lee J-K, Park S-C, Hahm K-S, Park Y. 2013. Antimicrobial HPA3NT3 peptide analogs: placement of aromatic rings and positive charges are key determinants for cell selectivity and mechanism of action. *Biochim Biophys Acta* **1828:**443-454.

490 10. Altman H, Steinberg D, Porat Y, Mor A, Fridman D, Friedman M, Bachrach G. 2006. In vitro assessment of antimicrobial peptides as potential agents against several oral bacteria. *J Antimicrob Chemother* **58**:198-201.

493   11.   Seo MD, Won HS, Kim JH, Mishig-Ochir T, Lee BJ. 2012. Antimicrobial peptides

494   for therapeutic applications: a review. *Molecules* **17:**12276-86.

495   12.   Shai Y. 1999. Mechanism of the binding, insertion and destabilization of phospholipid

496   bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-

497   lytic peptides. *Biochim Biophys Acta* **1462**:55-70.

498   13.   Peschel A, Sahl H-G. 2006. The co-evolution of host cationic antimicrobial peptides

499   and microbial resistance. *Nat Rev Microbiol* **4**:529.

500   14.   Schuller F, Benz R, Sahl HG. 1989. The peptide antibiotic subtilin acts by formation

501   of voltage-dependent multi-state pores in bacterial and artificial membranes. *Eur J*

502   *Biochem* **182**:181-6.

503   15.   Tennessen JA. 2005. Molecular evolution of animal antimicrobial peptides:

504   widespread moderate positive selection. *J Evol Biol* **18**:1387-94.

505   16.   Abee T. 1995. Pore-forming bacteriocins of Gram-positive bacteria and self-

506   protection mechanisms of producer organisms. *FEMS Microbiol Lett* **129**:1-9.

507   17.   Pag U, Oedenkoven M, Sass V, Shai Y, Shamova O, Antcheva N, Tossi A, Sahl H-G.

508   2008. Analysis of in vitro activities and modes of action of synthetic antimicrobial

509   peptides derived from an α-helical 'sequence template'. *J. Antimicrob Chemother.*

510   **61**:341-352.

511   18.   Brogden KA. 2005. Antimicrobial peptides: pore formers or metabolic inhibitors in

512   bacteria? *Nat Rev Microbiol* **3**:238-50.

513   19.   Straus SK, Hancock RE. 2006. Mode of action of the new antibiotic for Gram-

514   positive pathogens daptomycin: comparison with cationic antimicrobial peptides and

515   lipopeptides. *Biochim Biophys Acta* **1758**:1215-23.

516   20.   Willcox M, Hume E, Aliwarga Y, Kumar N, Cole N. 2008. A novel cationic-peptide

517   coating for the prevention of microbial colonization on contact lenses. *J Appl*

518   *Microbiol* **105**:1817-1825.

519   21.   Dutta D, Cole N, Kumar N, Willcox MDP. 2013. Broad Spectrum Antimicrobial

520   Activity of Melimine Covalently Bound to Contact Lenses. *Inves Ophthalm Visual*

521   *Science* **54**:175-182.

522   22.   Gao G, Cheng JT, Kindrachuk J, Hancock RE, Straus SK, Kizhakkedathu JN. 2012.

523   Biomembrane interactions reveal the mechanism of action of surface-immobilized

524   host defense IDR-1010 peptide. *Chem Biol* **19**:199-209.

525   23.   Rasul R, Cole N, Balasubramanian D, Chen R, Kumar N, Willcox MDP. 2010.

526   Interaction of the antimicrobial peptide melimine with bacterial membranes. *Int J of*

527   *Antimicrob Agents* **35**:566-572.

528   24.   Chen RX, Willcox MDP, Ho KKK, Smyth D, Kumar N. 2016. Antimicrobial peptide

529   melimine coating for titanium and its in vivo antibacterial activity in rodent

530   subcutaneous infection models. *Biomaterials* **85**:142-151.

531   25.   Dutta D, Ozkan J, Willcox MDP. 2014. Biocompatibility of Antimicrobial Melimine

532   Lenses: Rabbit and Human Studies. *Optom and Vis Sci* **91**:570-581.

533   26.   Dutta D, Zhao T, Cheah KB, Holmlund L, Willcox MDP. 2017. Activity of a

534   melimine derived peptide Mel4 against Stenotrophomonas, Delftia, Elizabethkingia,

535   Burkholderia and biocompatibility as a contact lens coating. *Cont Lens Ant Eye*

536   **40**:175-183.

537   27.   Dutta D, Kumar N, DP Willcox M. 2016. Antimicrobial activity of four cationic

538   peptides immobilised to poly-hydroxyethylmethacrylate. *Biofouling* **32:**429-438.

539 28. Dutta D, Kamphuis B, Ozcelik B, Thissen H, Pinarbasi R, Kumar N, Willcox MDP.

540   2018. Development of Silicone Hydrogel Antimicrobial Contact Lenses with Mel4

541   Peptide Coating. *Optom Vis Sci* **95**:937-946.

542 29. Mishra AK, Choi J, Moon E, Baek K-H. 2018. Tryptophan-Rich and Proline-Rich

543   Antimicrobial Peptides. *Molecules* **23**:815.

544 30. Chan DI, Prenner EJ, Vogel HJ. 2006. Tryptophan-and arginine-rich antimicrobial

545   peptides: structures and mechanisms of action. *Biochimic Biophys Acta* **1758**:1184-

546   1202.

547 31. Bi X, Wang C, Ma L, Sun Y, Shang D. 2013. Investigation of the role of tryptophan

548   residues in cationic antimicrobial peptides to determine the mechanism of

549   antimicrobial action. *J Appl Microbiol* **115**:663-672.

550 32. Zhu X, Ma Z, Wang J, Chou S, Shan A. 2014. Importance of tryptophan in

551   transforming an amphipathic peptide into a Pseudomonas aeruginosa-targeted

552   antimicrobial peptide. *PLoS one* **9**:e114605.

553 33. Saint Jean KD, Henderson KD, Chrom CL, Abiuso LE, Renn LM, Caputo GA. 2018.

554   Effects of Hydrophobic Amino Acid Substitutions on Antimicrobial Peptide

555   Behavior. *Prob Antimicro Proteins* **10**:408-419.

556 34. Zhu H, Conibear TC, Bandara R, Aliwarga Y, Stapleton F, Willcox MD. 2006. Type

557   III secretion system-associated toxins, proteases, serotypes, and antibiotic resistance

558   of Pseudomonas aeruginosa isolates associated with keratitis. *Curr Eye Res* **31**:297-

559   306.

560 35. Pillar CM, Hazlett LD, Hobden JA. 2000. Alkaline protease-deficient mutants of

561   Pseudomonas aeruginosa are virulent in the eye. *Curr Eye Res* **21:730-9.**

24

562   36.   Wiegand I, Hilpert K, Hancock REW. 2008. Agar and broth dilution methods to

563   determine the minimal inhibitory concentration (MIC) of antimicrobial substances.

564   *Nat Protoc* **3**:163-175.

565   37.   Anonymous. 2018. https://pubchem.ncbi.nlm.nih.gov/compund/. Accessed 10-April

566   2018.

567   38.   te Winkel JD, Gray DA, Seistrup KH, Hamoen LW, Strahl H. 2016. Analysis of

568   antimicrobial-triggered membrane depolarization using voltage sensitive dyes. *Front*

569   *Cell and Dev Biol* **4**:29.

570   39.   Li L, Shi Y, Su G, Le G. 2012. Selectivity for and destruction of Salmonella

571   typhimurium via a membrane damage mechanism of a cell-penetrating peptide

572   ppTG20 analogue. *Int J of Antimicrob Agents* **40**:337-343.

573   40.   Orman MA, Brynildsen MP. 2013. Establishment of a method to rapidly assay

574   bacterial persister metabolism. *Antimicrob Agents and Chemother* **57**:4398-4409.

575   41.   Veldhuizen EJ, Schneider VA, Agustiandari H, Van Dijk A, Tjeerdsma-van

576   Bokhoven JL, Bikker FJ, Haagsman HP. 2014. Antimicrobial and immunomodulatory

577   activities of PR-39 derived peptides. *PLoS One* **9**:e95939.

578   42.   Carson CF, Mee BJ, Riley TV. 2002. Mechanism of action of Melaleuca alternifolia

579   (tea tree) oil on Staphylococcus aureus determined by time-kill, lysis, leakage, and

580   salt tolerance assays and electron microscopy. *Antimicrob Agents and Chemother*

581   **46**:1914-1920.

582   43.   Beevers AJ, Dixon AM. 2010. Helical membrane peptides to modulate cell function.

583   *Chem Soci Rev* **39**:2146-2157.

584   44.   MacKenzie KR. 2006. Folding and stability of α-helical integral membrane proteins.

585   *Chem Rev* **106**:1931-1977.

586   45.   Popot J-L, Engelman DM. 2000. Helical membrane protein folding, stability, and

587   evolution. *Ann Rev of Biochem* **69**:881-922.

588   46.   Chan DI, Prenner EJ, Vogel HJ. 2006. Tryptophan- and arginine-rich antimicrobial

589   peptides: Structures and mechanisms of action. *Biochim Biophys Acta* **1758**:1184-

590   1202.

591   47.   Yau W-M, Wimley WC, Gawrisch K, White SH. 1998. The preference of tryptophan

592   for membrane interfaces. *Biochemistry* **37**:14713-14718.

593   48.   Torcato IM, Huang Y-H, Franquelim HG, Gaspar D, Craik DJ, Castanho MA,

594   Henriques ST. 2013. Design and characterization of novel antimicrobial peptides, R-

595   BP100 and RW-BP100, with activity against Gram-negative and Gram-positive

596   bacteria. *Biochimic Biophys Acta* **1828**:944-955.

597   49.   Blondelle SE, Houghten RA. 1991. Hemolytic and antimicrobial activities of the

598   twenty-four individual omission analogs of melittin. *Biochemistry* **30**:4671-4678.

599   50.   Kleinschmidt JH. 2003. Membrane protein folding on the example of outer membrane

600   protein A of Escherichia coli. *Cell and Mol Life Sci* **60**:1547-1558.

601   51.   Dathe M, Wieprecht T, Nikolenko H, Handel L, Maloy WL, MacDonald DL,

602   Beyermann M, Bienert M. 1997. Hydrophobicity, hydrophobic moment and angle

603   subtended by charged residues modulate antibacterial and haemolytic activity of

604   amphipathic helical peptides. *FEBS Lett* **403**:208-12.

605   52.   Pérez-Payá E, Houghten RA, Blondelle SE. 1995. The role of amphipathicity in the

606   folding, self-association and biological activity of multiple subunit small proteins. *J

607   Biol Chem* **270**:1048-1056.

608   53.   Berry T, Dutta D, Chen R, Leong A, Wang H, Donald WA, Parviz M, Cornell B,

609   Willcox M, Kumar N, Cranfield CG. 2018. The lipid membrane interactions of the

610   cationic antimicrobial peptide chimeras melimine and cys-melimine. *Langmuir*

611   doi:10.1021/acs.langmuir.8b01701.

612   54.   Zhu X, Shan A, Ma Z, Xu W, Wang J, Chou S, Cheng B. 2015. Bactericidal

613   efficiency and modes of action of the novel antimicrobial peptide T9W against

614   Pseudomonas aeruginosa. *Antimicrob Agents and Chemother* **59**:3008-3017.

615   55.   Lv Y, Wang J, Gao H, Wang Z, Dong N, Ma Q, Shan A. 2014. Antimicrobial

616   properties and membrane-active mechanism of a potential α-helical antimicrobial

617   derived from cathelicidin PMAP-36. *PloS One* **9**:e86364.

618   56.   Suh SJ, Shuman J, Carroll LP, Silo-Suh L. 2016. BEEP: An assay to detect bio-

619   energetic and envelope permeability alterations in Pseudomonas aeruginosa. *J

620   Microbiol Meth* **125:**81-6.

621   57.   Nan YH, Bang J-K, Shin SY. 2009. Design of novel indolicidin-derived antimicrobial

622   peptides with enhanced cell specificity and potent anti-inflammatory activity.

623   *Peptides* **30**:832-838.

624   58.   Veldhuizen E, Schneider V, Agustiandari H, van Dijk A, Tjeerdsma-van Bokhoven J.

625   2014. Antimicrobial and Immunomodulatory Activities.

626   59.   Mempin R, Tran H, Chen C, Gong H, Kim Ho K, Lu S. 2013. Release of extracellular

627   ATP by bacteria during growth. *BMC Microbiol* **13**:301.

628   60.   Okuda K-i, Zendo T, Sugimoto S, Iwase T, Tajima A, Yamada S, Sonomoto K,

629   Mizunoe Y. 2013. Effects of bacteriocins on methicillin-resistant Staphylococcus

630   aureus biofilm. *Antimicrob Agents and Chemother* 00888-13.

631   61.   Rausch JM, Marks JR, Rathinakumar R, Wimley WC. 2007. β-Sheet Pore-Forming

632   Peptides Selected from a Rational Combinatorial Library: Mechanism of Pore

633   Formation in Lipid Vesicles and Activity in Biological Membranes. *Biochemistry*

634   **46**:12124-12139.

635   62.   Rathinakumar R, Walkenhorst WF, Wimley WC. 2009. Broad-spectrum antimicrobial

636   peptides by rational combinatorial design and high-throughput screening: the

637   importance of interfacial activity. *J Americ Chem Soci* **131:**7609-7617.

638   63.   Rajasekaran G, Kim EY, Shin SY. 2017. LL-37-derived membrane-active FK-13

639   analogs possessing cell selectivity, anti-biofilm activity and synergy with

640   chloramphenicol and anti-inflammatory activity. *Biochim Biophys Acta* **1859**:722-

641   733.

642   64.   Minahk CJ, Farías ME, Sesma F, Morero RD. 2000. Effect of enterocin CRL35 on

643   Listeria monocytogenes cell membrane. *FEMS Microbiol lett* **192**:79-83.

644   65.   Li J, Koh J-J, Liu S, Lakshminarayanan R, Verma CS, Beuerman RW. 2017.

645   Membrane active antimicrobial peptides: translating mechanistic insights to design.

646   Frontiers in neuroscience 11:73.

647   66.   Dwyer DJ, Camacho DM, Kohanski MA, Callura JM, Collins JJ. 2012. Antibiotic-

648   induced bacterial cell death exhibits physiological and biochemical hallmarks of

649   apoptosis. *Mol Cell* **46**:561-572.

650

651

652

653

654

655

656

657 Table 1. MIC and MBC values of melimine and Mel4 against strains of *P. aeruginosa*

Bacterial strains	Melimine		Mel4	
	MIC nmolml^{-1} (µgml^{-1})	MBC nmolml^{-1} (µgml^{-1})	MIC nmolml^{-1} (µgml^{-1})	MBC nmolml^{-1} (µgml^{-1})
P. aeruginosa 6206	66 (250)	132 (500)	26.6 (62.5)	53.2 (125)
P. aeruginosa 6294	66 (250)	528 (2000)	26.6 (62.5)	106.5 (250)
P. aeruginosa Paer1	66 (250)	132 (500)	106.5 (250)	213 (500)
P. aeruginosa ATCC 19660	132 (500)	264 (1000)	26.6 (62.5)	26.2 (62.5)

658 MIC= minimum inhibitory concentration that inhibits the growth of ≥90% of cells

659 MBC = minimum bactericidal concentration that kills ≥99.9% of cells.

660

661

662

663

664

665

666

667

668

669

670

671

672

673

674

675

676   Table 2. Properties of melimine and Mel4 peptides.

Peptides	Molecular mass	Number of amino acids	Net charge	Hydro-phobicity <H>*	Hydrophobic moment <µH>*	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

677   *The properties were predicted using online software http://heliquest.ipmc.cnrs.fr/cgi-bin/ComputParams.py

678

679

680

681

682

683

684

685

686

687

Figure legends:
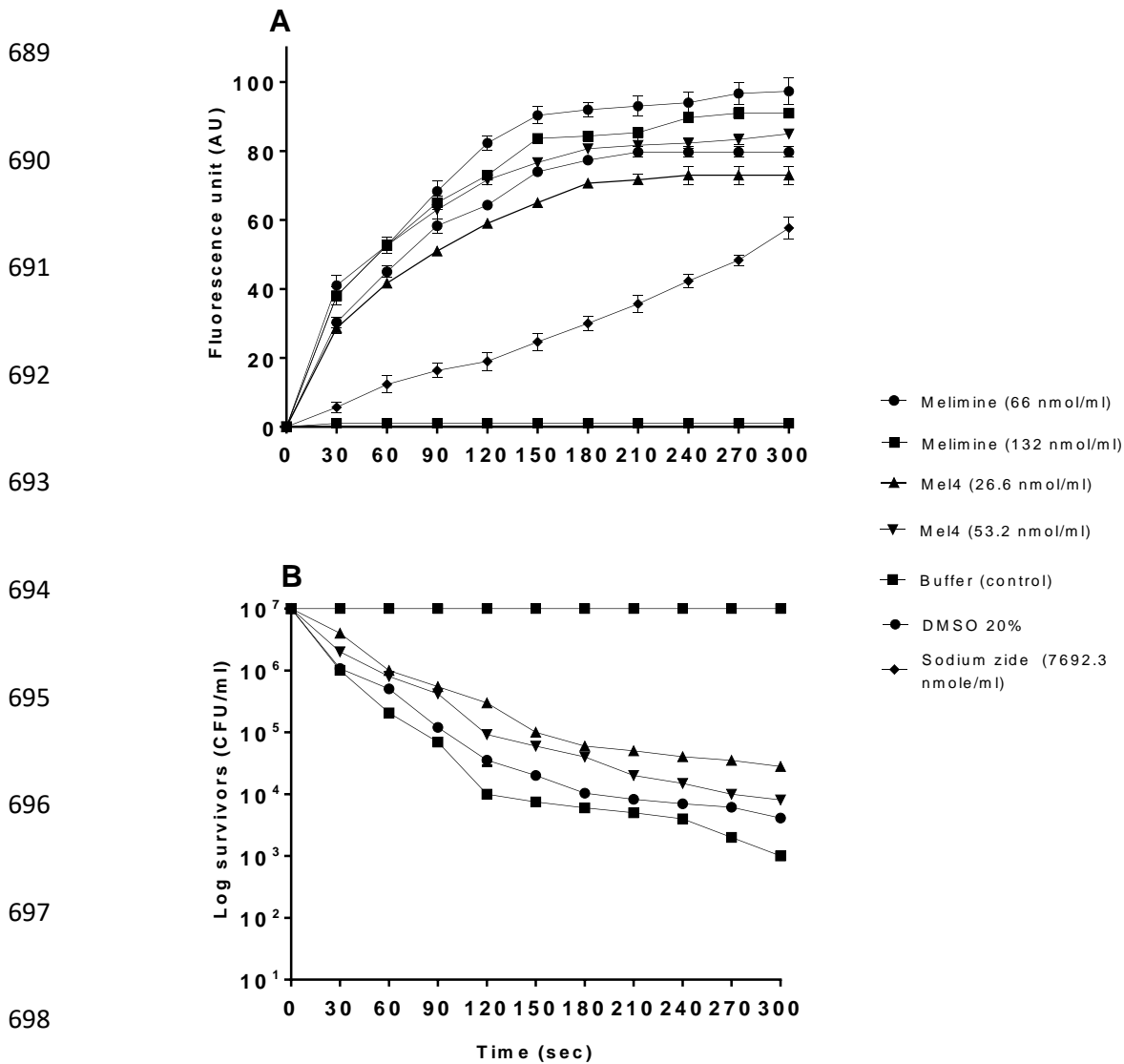
Fig. 1 Cytoplasmic membrane depolarization of *P. aeruginosa* 6294 by melimine and Mel4,

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.

706

707

712
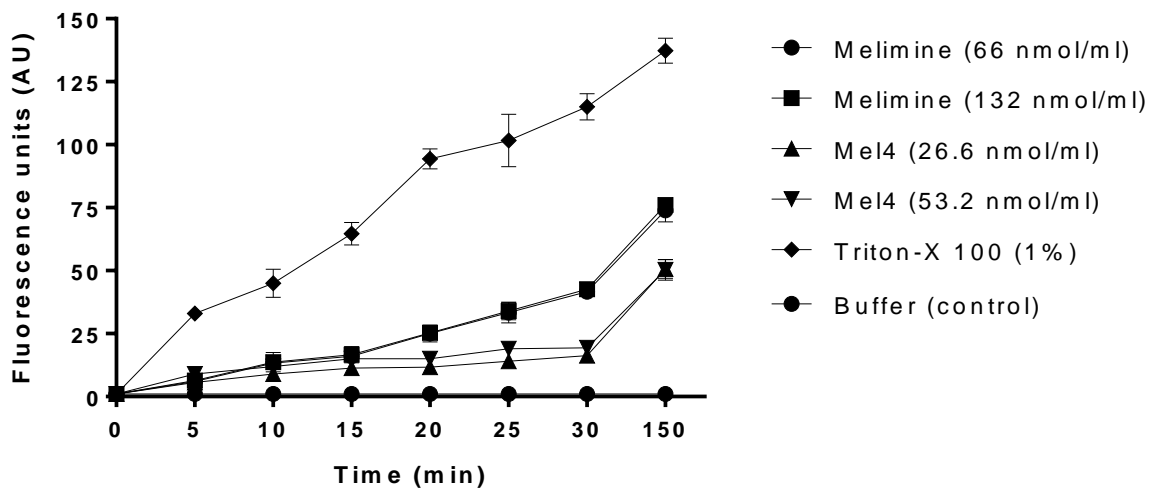
Fig. 2 Cytoplasmic membrane permeability of *P. aeruginosa* 6294 by melimine and Mel4 at different concentrations. Fluorescence due to binding of Sytox green fluorescent probe with DNA was measured spectroscopically at 480_{nm} to 522_{nm} excitation and emission wavelength. Data presented as means (±SD) of three independent repeats in triplicate.

717

718

719

720

721

722
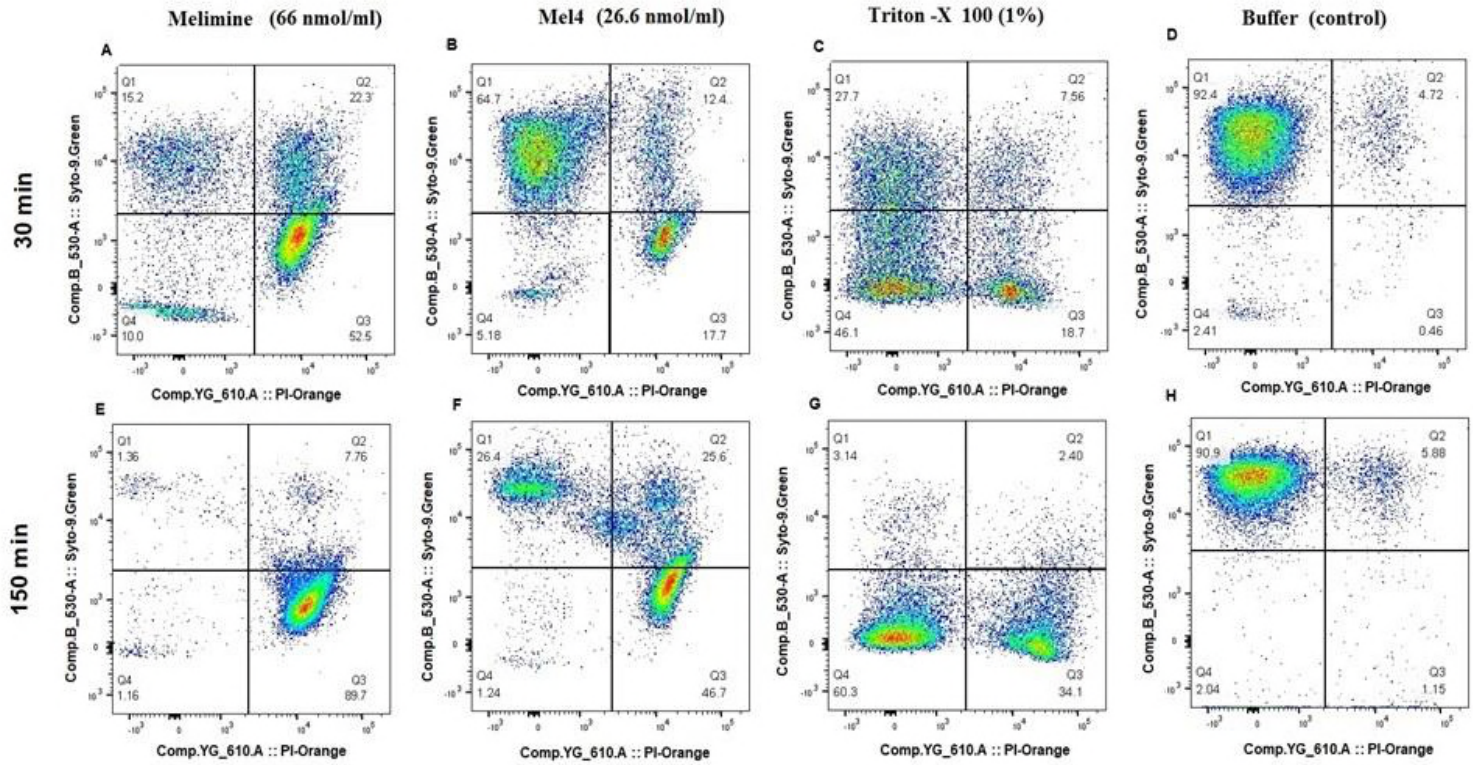
Fig. 3 Membrane permeabilization of *P. aeruginosa* 6294 produced by melimine and Mel4 at their MICs determined by flow cytometry with Syto 9 (membrane permeable) and Propidium Iodide (membrane impermeable) stains.

726

727

728

729

730

731

732

733

734

735

736

737

738

739

740

741

742

743

744
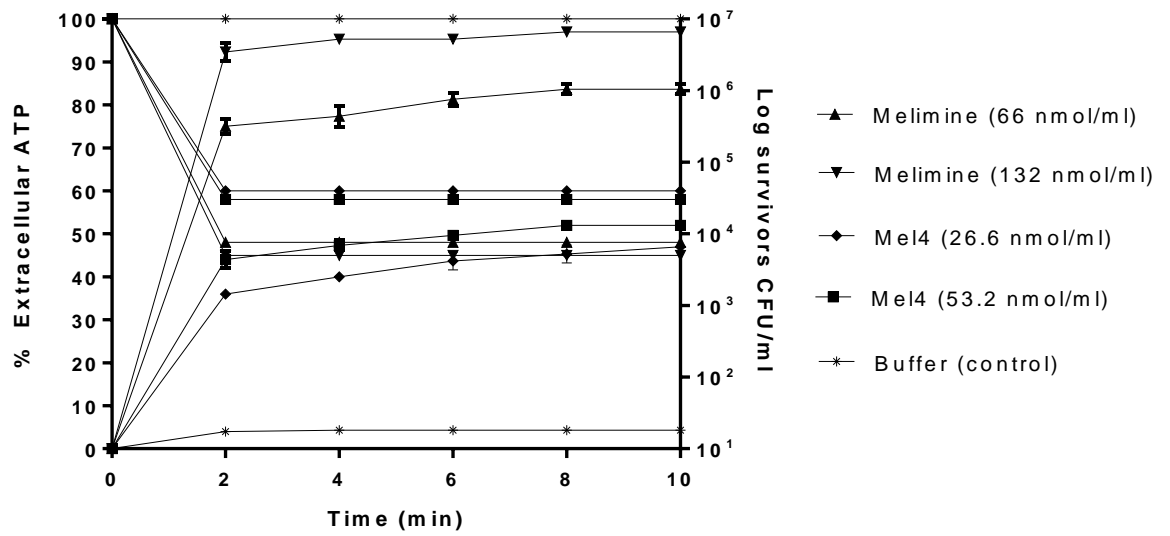
745 **Fig. 4** The effect of melimine and Mel4 on ATP release from *P. aeruginosa* 6294 at MIC and

746 two times MIC concentrations and the corresponding change in the number of viable cells.

747 Data presented as means (±SD) of three independent repeats in triplicate compared with

748 buffer-treated control.

749

754
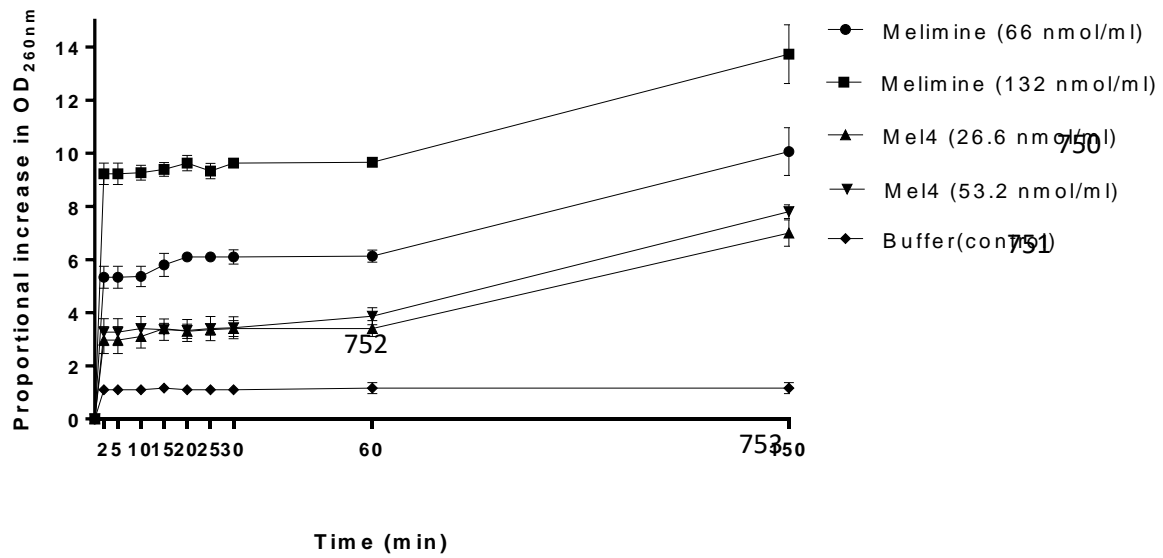
Fig. 5 Increase of DNA/RNA from *P. aeruginosa* 6294 due to action of melimine and Mel4 at MIC and two times MIC concentrations determined spectroscopically at OD_{260nm}. Data presented as means (±SD) of three independent repeats in triplicate compared with buffer-treated control.
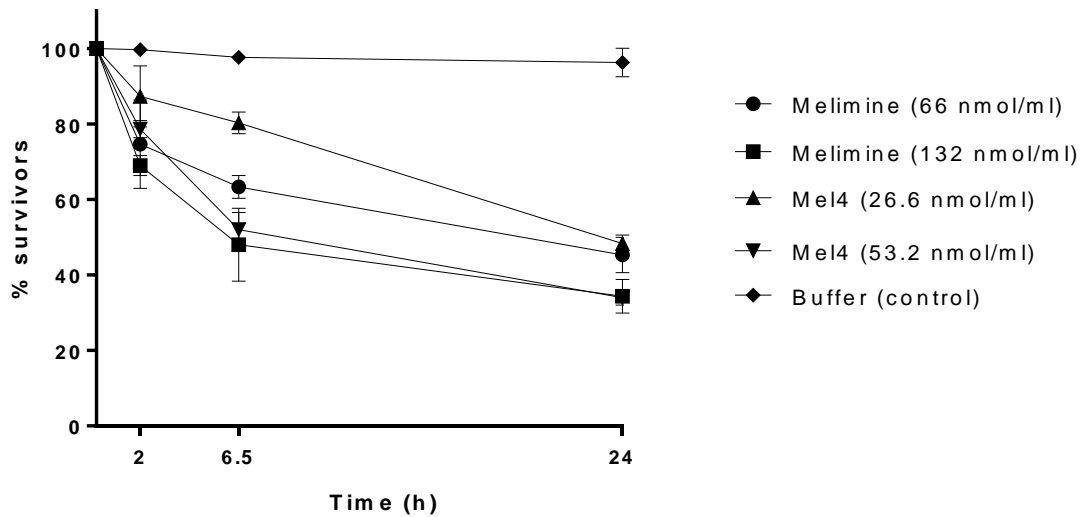
759

760

761

762

763

764

765

766

767

768

769

770

771

Fig. 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 (±SD) of three independent

repeats in triplicate compared with buffer-treated control.

775
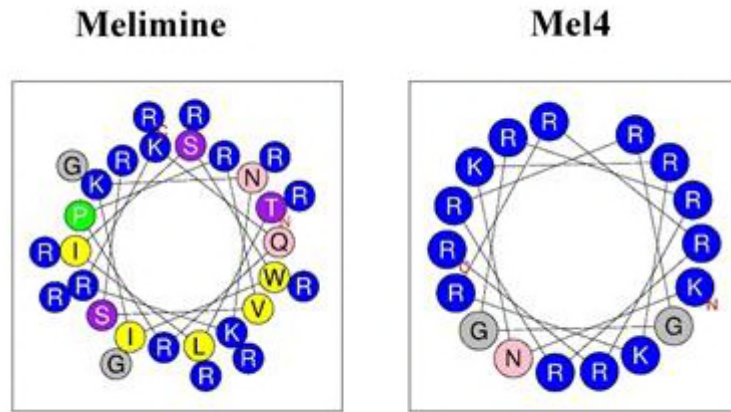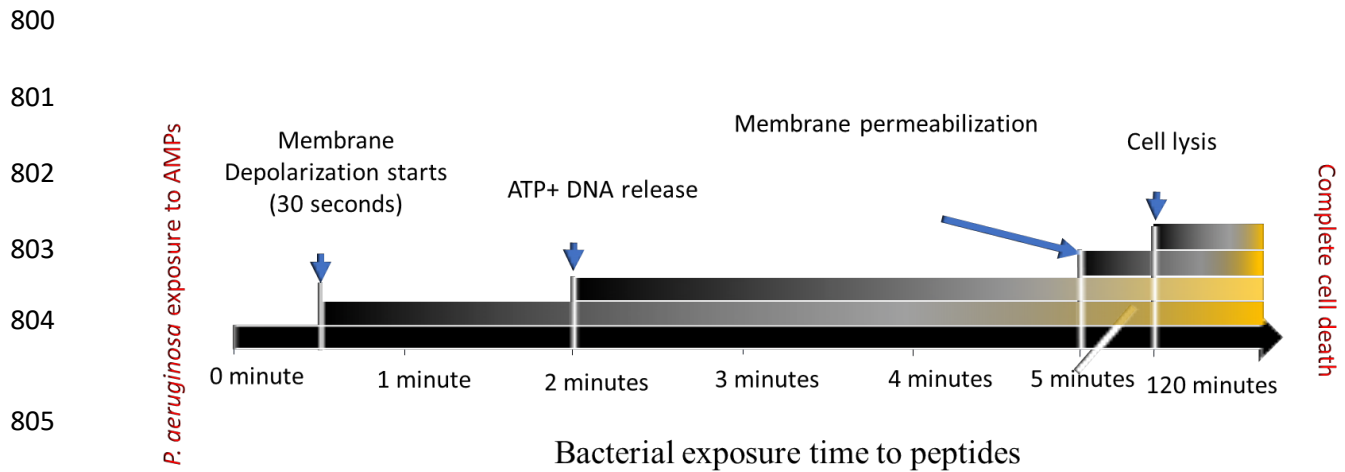
776

777
778
779

780

781

782

783

784

785 **Fig. 7** Helical wheel projection of melimine and Mel4. Positive charged residues are

786 represented in blue circles, uncharged residues are in grey circles, polar residues in pink

787 circles and hydrophobic residues in yellow circles.

788

789

790

791

792

793

794

795

796

797

798

799

800

801

Fig. 8 Timeline of bacterial killing by melimine and Mel4. Both AMPs started cell membrane depolarization at 30 seconds followed by ATP and DNA/RNA release at 2 minutes. Cell membrane permeabilization happened at 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.