



Antimicrobial activity of GN peptides and their mode of action

Mojsoska, Biljana; Nielsen, Hanne Mørck; Jenssen, Håvard

Published in: **Biopolymers**

DOI: 10.1002/bip.22796

Publication date: 2016

Document Version Peer reviewed version

Citation for published version (APA):

Mojsoska, B., Nielsen, H. M., & Jenssen, H. (2016). Antimicrobial activity of GN peptides and their mode of action. *Biopolymers*, *106*(2), 172-183. https://doi.org/10.1002/bip.22796

General rights Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
 You may not further distribute the material or use it for any profit-making activity or commercial gain.
 You may freely distribute the URL identifying the publication in the public portal.

Take down policy

If you believe that this document breaches copyright please contact rucforsk@kb.dk providing details, and we will remove access to the work immediately and investigate your claim.

Antimicrobial activity of GN peptides and their mode of action

- 3 Troels Godballe¹, Biljana Mojsoska¹, Hanne M. Nielsen², Håvard Jenssen^{1,*}
- 4 ¹Dept. of Science, Systems & Models, Roskilde University, Universitetsvej 1, DK-4000 Roskilde,
- 5 Denmark.
- 6 ²Dept. of Pharmacy, Biologics, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen,
- 7 Denmark.
- 8 *Corresponding Author: *jenssen@ruc.dk* Tel +45 4674 2877

10 Abstract

Increasing prevalence of bacteria that carries resistance towards conventional antibiotics has prompted 11 the investigation into new compounds for bacterial intervention to ensure efficient infection control in 12 13 the future. One group of potential lead structures for antibiotics is antimicrobial peptides due to their characteristics as naturally derived compounds with antimicrobial activity. In this study we aimed at 14 characterizing the mechanism of action of a small set of *in silico* optimized peptides. Following 15 determination of peptide activity against E. coli, S. aureus and P. aeruginosa, toxicity was assessed 16 revealing meaningful selectivity indexes for the majority of the peptides. Investigation of the peptides 17 effect on bacteria demonstrated a rapid growth inhibition with signs of bacterial lysis together with 18 increased bacterial size. Both visual and quantitative assays clearly demonstrated bacterial membrane 19 disruption after 10 minutes for the most active peptides. The membrane disrupting effect was verified 20 by measuring the release of calcein from bacterial mimicking liposomes. This revealed the most active 21 peptides as inducers of immediate release, indicating the kinetics of membrane permeabilization as an 22 important determinant of bacterial activity. No well-defined secondary structure of the peptides could 23 be determined using CD-spectroscopy in the presence of different liposomes mixtures, implying that 24 there is no correlation between peptide secondary structure and the observed anti-bacterial and 25 cytotoxic activity for this set of peptides. In conjunction, these findings provide strong indications of 26 27 membrane disruption as the primary mechanism of bacterial growth inhibition for the tested peptides.

28

Keywords: Antibacterial host defence peptides; Membrane destabilization, Antibacterial mode of
 action; Anti-infective; b

32 Introduction

For a long time, the treatment of most infections in western countries has been considered a rather uncomplicated matter due to the effectiveness of various antibiotics. Fortunately that still applies, although the treatment of some bacterial infections has become gradually more complicated in recent years because of the increase in bacterial resistance against conventional antibiotics. This is made obvious by the fact that infectious diseases are the third leading cause of death in developed countries, despite the abundant availability of antibiotics ¹.

The emergence of bacteria with resistance towards conventional antibiotics is increasing at an alarming 39 rate especially in hospital settings, seriously hampering effective treatment of infections². Methicillin-40 resistant Staphylococcus aureus (MRSA) was in 2005 estimated to have caused 94 000 infections 41 leading to 19 000 deaths³ and together with other multi drug resistant (MDR) strains of *Pseudomonas* 42 aeruginosa and extended-spectrum-beta-lactamase (ESBL) producing Klebisella pneumonia and 43 *Escherichia coli*, they pose an increasing threat to public health ⁴. On top of this there has only been 44 introduced a very limited number of new antibacterial agents to the market since the 60's which 45 46 together with a rapid declining interest from medical companies into development of new antibiotics, illustrates a development that calls for concern^{2, 4}. In that perspective, it is important to pursue the 47 development of new antimicrobial agents, with a novel mode of action, to ensure that we will be able to 48 cure life-threatening infections in the future. One such group of compounds that has the potential to 49 assist in the fight against infections is antimicrobial peptides. 50

Antimicrobial peptides are a natural component of the innate immune system in a wide range of 51 organism, acting as a first line of defense against various microbes. These peptides have been isolated 52 from a great number of organisms ranging from bacteria to plants, invertebrates and vertebrates, 53 thereby demonstrating their prevalence in virtually all branches of life⁵. In relation to their abundance 54 among different kinds of organisms, the biological effect varies from direct bacterial inhibition ⁵ to 55 immune modulating⁶, proving their versatility as effector molecules. The variety in origin and 56 biological effect is reflected in the characteristics of antimicrobial peptides having a great deal of 57 diversity, in both primary and secondary structure, probably explaining their evolutionary success ⁷. 58 Even so, there are a number of structural similarities that apply for most antimicrobial peptides, usually 59

being relatively short, ranging from 12-50 amino acids, carrying a net positive charge of +2 to +9 and
 having a distinct proportion of hydrophobic residues ⁸.

The positive charge of antimicrobial peptides is generally accepted as the main feature that mediates 62 63 selectivity towards bacteria by harboring sufficient electrochemical attraction towards the negatively charged outer entities of bacterial cells to allow selective activity. As natural compounds with an 64 antimicrobial effect, antimicrobial peptides have drawn the attention of scientist to investigate their 65 potential as future antimicrobial agents. This has led to the identification of more than 1000 66 antimicrobial peptides⁹, and numerous attempts to improve activity by modifying natural antimicrobial 67 peptides or the *de novo* design of antimicrobial peptides ¹⁰ including the use of unnatural amino acid 68 analogues¹¹. Although a large number of natural and synthetic antimicrobial peptides have been 69 studied, there are still today, questions tied to the exact nature of bacterial inhibition exerted by these 70 peptides. Compiling evidence suggests that antimicrobial peptides have a plethora of effects against 71 bacteria, ranging from unspecific membrane perforation to specific inhibition of intracellular targets 72 and the bacterial inhibition exerted by some antimicrobial peptides is possibly the result of more than 73 just one isolated mechanism⁵. Although different targets have been identified, a detailed 74 characterization of the mode of action has only been carried out on a minority of peptides. To truly take 75 advantage of antimicrobial peptides and make use of their potential as lead structures for bacterial 76 77 intervention, it is therefore in that perspective that the scope of this work is to elucidate the mechanism of action of a set of different synthetic antimicrobial peptides. The antimicrobial peptides used in this 78 study were originally designed by Fjell and colleagues by the use of *in silico* screening ¹². The 10 most 79 active peptides from this optimization were selected for investigation in this study, all having a positive 80 charge of +4, uniform chain length of 9 amino acids and at least three tryptophan residues. To verify 81 the activity of the peptides, minimal inhibitory concentration (MIC) testing was carried out against E. 82 coli, P. aeruginosa and S. aureus reference strains and the two most active peptides were selected for 83 further studies to elucidate the mechanism of bacterial inhibition. Herein we demonstrate compelling 84 evidence for membrane perforation as the main contributor of bacterial inhibition together with 85 reversible metabolic disturbance at lower concentrations. 86

88 Material and methods

89 Solid phase peptide synthesis

90 The peptides were synthesized using solid phase Fmoc chemistry with amidation on the carboxyl end,

91 purified by reverse-phase HPLC using a C_{18} column (Higgins Analytical Inc. 10 μ m 250x10 mm) and a

92 water/acetonitile gradient. The correct mass and purity >95% was verified using Dionex Ultimate 3000

P3 RP-UHPLC (C18 Kinetex 100 x 2.1 mm, 100 Å) electrospray ionization mass spectrometry (Finnigan

94 LTQ) (**Figure S1, Table S1**).

95

96 Minimal inhibitory concentration determination

The antimicrobial efficacy of peptides was tested using a serial dilution titration, as previously 97 described ¹³ on a panel of clinically relevant bacterial strains; i.e. E. coli (ATCC 25922), P. aeruginosa 98 PAO1 (ATCC 15692), S. aureus (ATCC 29213). Conventional antibiotics used throughout the study 99 include Ampicillin (A9518), Ciprofloxacin (17850), Tetracycline (T7660), Tobramycin (T4014) and 100 Polymyxin B (92301), all purchased from Sigma. In short, overnight cultures were grown to mid-log 101 phase before diluting to $\sim 5 \times 10^5$ CFU/mL in Mueller Hinton broth (Becton Dickinson) and 90 µL was 102 used to inoculate each well of a 96-well polypropylene plate (Cat. No. 3879, COSTAR). Then, 10 µL 103 aliquots of a 2-fold dilution series of the peptides were added and the minimal inhibitory concentration 104 (MIC) was scored as the lowest concentration that inhibited visible growth after 48 hours of incubation 105 at 37°C. 106

107

108 Hemolysis assay

Freshly drawn human blood with EDTA as anticoagulant was washed and centrifuged at 500 g for 5 minutes until the appearance of clear supernatant. A two-fold dilution series of peptides was added to 96-well polypropylene microtiter plates (COSTAR Cat. No.3879) in 75 μ L aliquots together with 50 μ L of 4 % final concentration of red blood cells (RBC) giving a total volume of 125 μ L. The plate was incubated with shaking for 1 hour at 37°C, before being centrifuged at 250 g for 5 minutes. 50 μ L of the supernatant was diluted 1:3 in sterile saline in a flat bottomed 96-well greiner plate before measuring absorbance at 414 nm, using a multi-detection microplate reader Synergy HT. 100 % lysis

116 was defined as RBC treated with 1% Triton-X 100 and sterile saline treated RBC was added for

baseline adjustments. The test range was 3.1-400 µg/mL.

118

119 MTT assay

120 Toxicity against HeLa cells was estimated by the MTT viability assay. HeLa cells were cultured in DMEM Glutamax media (32430-027, Invitrogen), media supplemented with 10% FBS and 1% 121 pen/strep using standard techniques. The cells were passaged at least 2 times before being used in the 122 assay, to ensure logarithmic growth. At 80 % confluency, cells were harvested, quantified and 10.000 123 cells/well were seeded in a 96-well microtitter plate. The cells were placed in a humidified 5% CO₂ 124 atmosphere at 37°C for 24 hours. Media was aspirated and the cells were washed with PBS w/o Ca²⁺ 125 and Mg²⁺. A mixture of peptide and DMEM media (100 µl) was added and the cells were incubated in 126 a humidified 5% CO₂ atmosphere at 37°C for 1 hour before wash with PBS with or without Ca^{2+} and 127 Mg^{2+} . MTT (50 µg) dissolved in PBS with or without Ca^{2+} and Mg^{2+} was added and the cells were 128 incubated 90 minutes in a humidified 5% CO₂ atmosphere at 37°C. MTT solution was aspirated and 129 100 µL of DMSO was added to dissolve the formazan crystals before incubating 5 minutes at 37°C. 130 The absorbance at 540 nm was read in a multi-detection microplate reader Synergy HT. Viability 131 control was assigned to samples with only HeLa cells and DMEM media and 0 % viability was 132 assigned to samples with 1 % added Triton-X-100 instead of peptides. The assay was done 3 times with 133 duplicates and results are the mean of these. Concentrations giving a 50% reduction in metabolism 134 (IC_{50}) , was calculated using equation (I) in Graphpad Prism. 135

¹³⁶ Y =
$$\frac{100}{1+10^{((Log/C 50÷ X) \cdot HillSlope)}}$$

(Eq. I)

137

139 Killing kinetics

Colony forming units (CFU) counts were monitored for 180 minutes, following peptide exposure at 140 concentration corresponding to 1×, 2× and 4×MIC. Overnight culture of E. coli ATCC 25922 was 141 142 diluted with fresh Mueller Hinton broth and re-grown to mid-log phase before diluting to a turbidity of OD_{600} 0.1 and loading 90 µL into wells of a flat bottomed 96-well Greiner plate containing 10 µL of 143 peptides solution. Growth control was assigned to wells without peptide. The content of individual 144 plates was collected and plated out in duplicate on LB agar plates. Between extractions, the microtitter 145 plate was sealed and placed at 37°C. LB agar plates were incubated for 18 hours at 30°C and colonies 146 were counted. The presented results are the mean of 3 independent experiments. 147

The effect of peptides on bacterial growth was investigated by monitoring optical density (OD) 148 149 following peptide exposure. Overnight culture of E. coli ATCC 25922 was diluted with fresh Mueller Hinton broth and re-grown to mid-log phase before diluting to a turbidity of OD₆₀₀ 0.1. A microtiter 150 plate was filled with 10 µL of peptide or antibiotic solution per wells, before loading 90 µL of bacterial 151 suspension. For both peptides and antibiotics, concentrations corresponding to $1 \times -$, $2 \times -$ and $4 \times MIC$ 152 were used. OD₆₀₀ was measured, with a multi-detection microplate reader Synergy HT at 37°C, every 8 153 154 minutes for 5 hours with shaking. The readings were baseline corrected against wells with only Mueller Hinton broth. Comparison between a standard spectrophotometer, with a cuvette length of 1 cm, and 155 the readings obtained from the plate reader showed a difference with 5-fold lower readings from the 156 plate reader (data not shown). In order to make readings meaningful, in relation to the correlation 157 158 between optical density and concentration of bacterial cells, the readings from the plate reader was multiplied with a factor of 5. 159

160 The effect on both bacterial DNA content and change in cell size after exposure with peptides was 161 monitored over 180 minutes by flow cytometry. An overnight culture of *E. coli* ATCC 25922 was 162 diluted with fresh Mueller Hinton broth and re-grown to mid-log phase before diluting to a turbidity of 163 $OD_{600} 0.1$. A total of 90 µL of this bacterial suspension was loaded into a flat bottomed 96-well Greiner 164 plate for peptide and antibiotic samples and 100 µL for control samples. After extraction of the zero 165 sample, 10 µL of peptide solution corresponding to 1× and 4× the MIC concentration was added for a 166 total volume of 100 µL. Two wells were loaded for each treatment to ensure enough bacteria for the

flow cytometry analysis. Immediately after extraction the samples were put on ice and centrifuged at 10 167 000 g for 5 minutes at 4°C and resuspended in 100 µL 10 mM Tris HCl pH 7.4 before fixing with 1000 168 µL 77% ethanol. The following day, the samples were centrifuged at 10 000 g for 5 minutes before 169 170 gently removing the supernatant and adding 140 µL staining solution (90 µg/mL Mitramycin and 20 µg/mL ethidium bromide in 10 mM Tris pH 7.4, 10 mM MgCl₂). The samples where run on an A10 171 Bryte Flow Cytometer and when possible, 20 000 events were included in the analysis.

173

172

Membrane depolarization assay 174

The LIVE/DEAD® BacLight [™] Bacterial Viability Kit (L7012, Invitrogen), was applied to peptide 175 treated E. coli ATCC 25922, to investigate a visual indication of the membrane permeability 176 capabilities of the peptides. Overnight culture of E. coli ATCC 25922 was diluted with fresh Mueller 177 Hinton broth and re-grown to mid-log phase before diluting to a turbidity of OD_{600} 0.1. Then 10 µL of 178 peptide solution corresponding to a final test concentration of 1× and 4×MIC was added to a flat 179 bottomed polystyrene Greiner plate before loading 90 µL of bacteria suspension. 100 µL of bacterial 180 suspension was used as a control. Each well corresponded to one time point for each individual 181 treatment. The plate was placed at 37°C and the samples were extracted at the indicated time points and 182 immediately put on ice. The samples were then centrifuged at 10 000g for 5 minutes, and the pellet 183 resuspended in 100 µL 0.9% ice cold NaCl, to remove interfering media components. Staining solution 184 (1 µL) was added and incubated for 15 minutes in the dark, before loading to 1% agarose slips on 185 microscope slides. Staining solution was prepared by adding 5 µL of 3.3 mM Syto-9 dye in DMSO 186 187 (Compound A) and 5 µL of 20 mM Propidium iodide in DMSO (Compound B) to 20 µL of sterile water. Samples were inspected with a Leica DM5000B microscope using a mercury lamp. A filter cube 188 with excitation filters of 436, 495 and 580 nm and emission filters of 460, 535 and 630 nm was used to 189 be able to detect both live and dead bacteria. 190

To verify the findings from microscope pictures with an assay that could characterize the membrane 191 permeability of the entire bacterial population in the samples, the Live/Dead quantification was 192 193 performed. The assay was essentially performed according to the manufacturer's instructions. After establishing a standard curve from known ratios of isopopyl killed and live bacteria an overnight 194

culture of E. coli ATCC 25922 was diluted with fresh Mueller Hinton broth and re-grown to mid-log 195 196 phase before diluting to a turbidity of OD_{600} 0.1. The bacterial solution and peptide solution was mixed in a 9:1 ratio (vol/vol) in a 96-well polypropylene plate (Cat. No. 3879, COSTAR) and incubated at 197 198 37°C. At the indicated timepoints the content of individual wells was extracted, put on ice and then pelleted at 11 000 g for 8 minutes before being resuspended in 0.9% NaCl and placed on ice again. The 199 resuspended bacterial suspension was then added to individual wells of flat bottomed 96-well 200 polystyrene plate and mixed with 100 uL staining solution before incubation in the dark for 15 minutes 201 202 and subsequent measurement of fluorescence on a multi-detection microplate reader Synergy HT. 203 Green fluorescence was excited at 485 nm and the emission detected at 528 nm, whereas the red fluorescence was excited at 530 nm and detected at 645 nm. Percentage of living cells was obtained by 204 using equation (II) using the standard curve made from known ratios of live cells. The staining solution 205 was prepared by adding 3 µL of both compound A and B, for each 1 mL of distilled sterilized water 206 207 and stored in the dark.

208

% live cells =
$$\frac{Green \, fluorescence}{Red \, fluorescence} \cdot \frac{1}{0.0627}$$

209 Liposome preparation

Large unilamellar vesicles (LUVs) was prepared by mixing POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-210 phosphocholine) and POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol) and in a molar ratio 211 of 3:7 as a simple mimic of bacterial membranes and POPC:POPG:Cholesterol was mixed in a molar 212 ratio of 5:2:3 as a simple mimic of mammalian membrane. POPC (770557), POPG (770257) and 213 Cholesterol were all purchased from Avanti Lipids (Alabaster, Alabama). The lipid mixture was 214 concentrated on a rotary evaporator and washed 3 times with 99.9 % ethanol to remove residual 215 organic solvents. The lipid mixture was then dissolved in 4 mL HEPES buffer (10 mM HEPES, 150 216 217 mM KCl, 0.03 mM CaCl₂, 0.01 mM EDTA, pH 7.4) with 20 mM calcein (C0875, Sigma), for calcein 218 containing liposomes and 10 mM Tris pH 7.4 for empty liposomes used for CD spectroscopy, followed by thorough mixing and sonication for 5 minutes to prevent aggregates. Subsequently, the lipid mixture 219 was vigorously whirlmixed every 10 minutes over the course of 1 hour and finally left at room 220 221 temperature for 1 hour, to allow the lipids to anneal. LUVs were prepared by extruding the lipid

(Eq. II)

mixture through, two double stacked 100 nm filters, a total of 10 times using a Nitrogen powered 222 223 extruder. The calcein containing liposomes were loaded on Sephadex G-50 columns to separate encapsulated calcein from free calcein and eluted with HEPES buffer. The size of the liposomes was 224 225 verified to be approximately 110 nm with a narrow size distribution by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern, Worcestershire, UK). Malvern DTS v. 5.10 software (Malvern, 226 Worcestershire, UK) was used for acquisition and analysis. By comparing the end volume with the 227 starting volume, the dilution of the initial liposome concentration was estimated and that lead to the 228 anticipation of a stock concentration of 971 µM of the liposomes. 229

230

231 Calcein release assay

232 Calcein release was done in a 96-well plate with shielded wells (MicroWell 96 optical bottom plate, NUNC, Roskilde, DK). 100 µL of peptide diluted in 10 mM HEPES buffer was added and immediately 233 before measurement of leakage, 80 µL of 45 µM liposome suspension was added for a final liposome 234 concentration of 20 µM. The liposome concentration was calculated from the starting concentrations of 235 lipids, taking into account the change in volume during preparation, and assuming a 100% yield from 236 the sephadex column. Measurements were performed in a FLUOstar OPTIMA plate reader at an 237 excitation wavelength of 485 nm and an emission wavelength of 520 nm over the course of 1 hour at 238 37°C. Maximum leakage of liposomes was acquired by lysis with 10% Triton and release following 239 peptide exposure, was calculated using equation (III), where F_{max} denotes fluorescence after addition of 240 10% Triton, F₀ represents background fluorescence of liposomes and F designates the fluorescence 241 242 intensity after peptide addition.

243 % leakage =
$$100 \bullet \left(\frac{F - F_0}{F_{\text{max}} - F_0} \right)$$
 (Eq. III)

245 **Results**

This study aimed at characterizing the bacterial inhibition and antibacterial mode of action of a set of 246 highly active antimicrobial peptides earlier identified by Fjell et al.¹². The GN peptide library was 247 screened for antibacterial activity against Gram-negative E. coli ATCC 25922 and P. aeruginosa 248 ATCC PAO1 and Gram-positive S. aureus ATCC 29213 bacteria. The minimal inhibitory 249 concentrations of the tested peptides demonstrated a significant spread in activity pattern from 1.5 to 250 100 µg/mL (Table 1). Potent broad spectrum activity against both Gram-negative and Gram-positive 251 252 bacteria was observed for GN-2, GN-4 and GN-6 peptides. For specific activity against E. coli, GN-14 exhibits as good antimicrobial activity as GN-2, GN-4 and GN-6 peptides. Similarly, a pronounced 253 antimicrobial activity against S. aureus was observed for GN-5 peptide (1.5 µg/mL), and GN-2 and 254 GN-6 peptides (3.1 µg/mL). However, in respect to a broad spectrum antimicrobial potential, peptide 255

256 GN-2 and GN-4 appear as best candidates.

In an attempt to explain the antibacterial peptide properties with classical chemical properties the 257 hydrophobic moment was measured for all the peptides. This quantitative measure of the peptides 258 259 amphipathicity indicates that antibacterial activity increases proportionally with the hydrophobic moment (Table 1). In order to further characterize the antimicrobial profiles of the GN peptides, their 260 ability to selectively target bacterial-versus mammalian cells was assessed using hemolytic assay. 261 Human red blood cells were exposed to different concentrations of GN peptides and the degree of lysis 262 was evaluated. All peptides resulted in a dose dependent release of hemoglobin, and toxicity is reported 263 264 as the highest concentration of peptides resulting in less than 10 % lysis compared to PBS treated control cells (Table 1). Comparing antibacterial and hemolytic potential, the peptide library clearly 265 separates into two sub-groups; GN-8, -9, -11 and -12 being hardly hemolytic with low antimicrobial 266 potential, while the remaining peptides demonstrate an overall higher antibacterial effect vet still only 267 modest hemolytic activity. Thus, for this set of peptides the tendency to lyse human red blood cells is 268 highly correlated with the ability to kill bacteria. In comparison, indolicidin, a small 13 amino acids 269 tryptophan and arginine rich antimicrobial peptide¹⁴, is used as an antibacterial reference peptide 270 indicating that most of the GN peptides have superior selectivity. To further complement the 271 272 assessment of the potential toxicity of the GN peptides, a MTT assay was performed on HeLa cells and 273 the inhibitory concentrations giving 50 % reduction (IC₅₀) of cellular metabolic activity were calculated (Table 1). The results are in agreement with the hemolytic activity, thus demonstrating that the

- 275 peptides with the highest antimicrobial activities are the peptides exhibiting the greatest metabolic
- inhibition in HeLa cells. Hence, peptides GN-2 and GN-4 stand out as most cytotoxic with IC_{50} values

277 of 47 and 37 μ g/mL, respectively.

To dissect the antimicrobial activity of GN peptides with a range of different assays, GN-2 was chosen as the most potent peptide with good selectivity index range of 16 to 32. Correspondingly, GN-6 which structurally differs from GN-2 peptide in the presence of phenylalanine and a patch of 4 consecutive tryptophan residues was also considered for characterization for the mechanism of bacterial inhibition.

282 First to differentiate between bactericidal or bacteriostatic mode of antibacterial activity, colony forming unit counts were monitored for *E. coli* cultures exposed to $1\times$, $2\times$, and $4\times$ MIC concentrations 283 284 of GN-2 and GN-6 peptide. This assay illustrated the ability of the different peptide concentrations to reduce the colony forming unit counts over a period of 180 minutes. The GN-2 peptide demonstrates 285 fast antimicrobial activity indicated by a decrease of exponentially growing bacteria within the first 40 286 minutes of exposure (Figure 1). The inhibition proceeds for 80 minutes after which there is a 287 288 reestablished bacterial growth observed. Similar pattern is observed for GN-6 peptide with a slightly 289 delayed inhibition. Bacterial inhibition is concentration dependent for both peptides, even though the standard error bars for GN-6 peptide at 4×MIC show high experimental variation. 290

To further elucidate the bacterial inhibition exploited by the peptides, bacterial mass was monitored by 291 optical density readings at 600 nm over a course of 5 hours. The control bacteria display exponential 292 growth until 120 minutes after which clear decrease in optical density is observed. There is a 293 294 reestablished growth after 180 minutes, indicating that the decrease after 2 hours is not due to limited nutrition and/or waste build-up (Figure 2). A number of antibiotics were analyzed in parallel for 295 growth inhibition patters so that the potential bacterial targets of GN-2 and GN-6 peptides could be 296 elucidated. In addition to the viability experiments of exponentially growing E. coli, the optical density 297 298 measurements revealed clear concentration dependent inhibition of the optical density readings when bacteria were exposed to 1×MIC concentration of both GN-2 and GN-6 peptide when compared to the 299 300 untreated bacteria. The inhibition is most pronounced at 2×MIC concentration for both peptides. With respect to 1×MIC concentration of both peptides, the inhibition pattern is different indicating distinct 301

ways of exerting antimicrobial activity halting bacterial growth. Both peptides at 2×MIC concentration
 demonstrate low OD measurements indicative of some degree of bacterial lysis.

304 Flow cytometry analyses were used to characterize the effect of GN-2 and GN-6 peptide on cell size 305 and DNA content. Exponentially growing E. coli were exposed to 1× and 4×MIC concentrations of GN-2 and GN-6 peptides and samples were analyzed every 20 minutes over a course of 3 hours for 306 DNA content per cell mass extrapolated by fluorescence/lightscatter measurements. Untreated E. coli 307 show fairly constant ratio of DNA per cell size indicative for a healthy growing bacterial population. 308 309 Exposure to 1× and 4×MIC concentration of GN-2 gives pronounced initial decrease of DNA content per cell size as compared with the control samples. After 40 minutes of exposure an increase of DNA 310 per cell size is observed for samples treated with 4×MIC concentration, referring to the reestablishing 311 of the DNA content at this point. At 120 minutes the effect of DNA content per cell size follows similar 312 ratio to that of the control, indicating a possible recovery (Figure 3A). Similar fashion of initial fast 313 and persistent decrease of DNA per cell size followed by an increase after 40 minutes is observed for 314 315 bacteria treated with 1×MIC concentration of GN-6 peptide.

316 The increase in cell size indicated by the lightscatter measurements observed in the flow cytometry 317 data (Figure S2) is supported by the microscopy images taken at time zero and 180 minutes for the control E. coli population. Bacteria treated with GN-2 peptide at 1× and 4×MIC concentration appear 318 slightly bigger in size than the control bacteria. Clear difference between cells treated with 1× and 319 320 4×MIC concentrations of GN-2 peptide is observed by the large areas of cell debris and bacteria 321 transparency indicating cell lysis in samples treated with 4×MIC (Figure 3C). Upon treatment with GN-6 peptide at 4×MIC concentrations, there is a clear morphological change presented by cells that 322 appear long and filamentous but not lysed (Figure 3C). This data is in agreement with the observations 323 from the flow cytometry analyzes on DNA, size and DNA /size. 324

Live/dead staining experiments with SYTO9 green-fluorescent nucleic acid stain and propidium iodide red-fluorescent stain, were used to analyze the membrane permeability capacity of GN-2. Under conditions where no membrane permeabilizing agent is present, bacteria with intact membranes will stain green (**Figure 4A**). The un-treated control cells are naturally predominantly staining green at all time-points, illustrating a healthy population of bacteria. *E. coli* exposed to GN-2 peptide at 1×MIC

concentration at the 10 and 20 minutes time-point, demonstrate about 90 % permeabilized membranes, 330 331 thus staining red. However, over time the surviving cells are replicating, resulting in an increasing percentage of green cells in the later time points (Figure 4B). Upon increasing the GN-2 peptide 332 333 concentration to 4×MIC, the membrane integrity stays disrupted throughout the assay of 120 minutes 334 (Figure 4C). To quantify the membrane permeability in a more un-biased fashion than microscopy, the same bacterial cultures were also analyzed using a multi-detection fluorescence plate reader. Plotting 335 the ratio of live and dead cells for cultures incubated with 1×, 2× and 4×MIC concentration of GN-2 336 337 peptide over time, clearly demonstrates how 2× and 4×MIC knocks down the viability to around 20% 338 and this level is kept for 120 minutes. Contrary, at 1×MIC of GN-2, the level of viable bacteria after 10 minutes is about the same as in the samples treated with 2× and 4×MIC of GN-2, but at 1×MIC the 339 culture re-growths with a doubling time of about 1 hour (Figure 4D). 340

Due to previously established observation of membrane disruption as a primary mode of action of the 341 GN peptides, calcein leakage assay was performed. This assay allows characterization of peptides 342 based on the different potencies to provoke membrane leakage of calcein entrapped unilamellar 343 phospholipid vesicles (LUVs) made of POPC:POPG (3:7 molar ratio). Calcein is a self-quenching 344 molecule that at high concentration shows relatively low background fluorescence. As calcein 345 molecules are being released to the exterior of the vesicles, the fluorescence increases. The percentage 346 of calcein leakage can be plotted as a function of the concentration of the peptides. All GN peptides 347 were able to induce calcein leakage in a concentration dependent manner at endpoint reading of 60 min 348 349 (Figure 5A). But the release of calcein is much less significant that what is induced by melittin, a wellknown peptide with non-selective lytic properties, used as a positive control for giving distinct pattern 350 of liposome disruption ¹⁵. 351

As it was impossible to distinguish the lytic potential of the different GN peptides using an endpoint reading the release of calcein over time was monitored, enabling us to extract information about the capability of the peptides to induce calcein leakage. The GN-2 peptide demonstrates distinct kinetics of rapidly provoking calcein release from the liposomes, particularly at high concentrations (**Figure 5B**). Only a small concentration change from $3.5 \ \mu g/mL$ to $5.5 \ \mu g/mL$ of GN-2, changes the calcein release from about 30% to 100%. Conversely, the GN-6 peptide, which has slightly lower antimicrobial activity than GN-2, exerts a much more gradual release of calcein over time, which stabilizes after

about 10 minutes (Figure 5C). The gradual release is observed for a wider range of concentrations, 359 suggesting different mode of membrane activity or different modes of action for this peptide compared 360 to GN-2. This could potentially be reconfirmed and better characterized using the experimental design 361 of Russel et al. 2010¹⁶. A similar, but even more dramatic profile for gradual release is observed for 362 GN-14 where gradual increase in calcein leakage progressing over a course of 20 minutes is observed 363 (Figure 5D). The different patterns of calcein leakage attribute to the correlation of antimicrobial 364 peptide activity and their ability to disrupt bacterial membranes, where more active peptides cause 365 more instantaneous release of calcein from the liposome. 366

The secondary structure of GN peptides and melittin was inspected with circular dichroism (CD) 367 spectroscopy to see whether or not a confined structure is necessary for the antimicrobial, toxic or 368 membrane disrupting activities observed. Peptide secondary structures were measured in Tris buffer pH 369 7.4 and unilamellar phospholipid vesicles consisting of POPC:POPG:Cholesterol (5:2:3 molar ratio) 370 and POPC:POPG (3:7 molar ratio), the latter mimicking mammalian and bacterial membranes, 371 respectively (Figure S3). None of the peptides demonstrated any defined secondary structures in Tris 372 buffer, indicating random coils under these conditions. In presence of LUV that mimic mammalian 373 membranes, the control peptide melittin adopts a structural confirmation similar to that of an α -helix 374 with peak minima at 222 and 208 nm 17 . Using the DichroWeb K2D algorithm 18 , the α -helix content in 375 melittin was calculated to be 77 % which is in agreement with other publications ¹⁹. The CD spectra for 376 the GN peptides are similar for mammalian and bacterial membrane mimics and illustrate no well-377 defined secondary structure in either environment. Therefore, it is reasonable to assume that GN 378 peptides exert their antimicrobial, toxic and membrane disrupting activity without adapting any well-379 defined secondary structure. 380

381

383 Discussion

Due to the increased number of infectious agents that are resistant to many well-known classes of 384 antibiotics, there is a constant need of improved alternative drug classes and therapies. In this study we 385 present evaluation of the antimicrobial potencies of ten different peptides, GN-2 to GN-14, from the in 386 *silico* optimized peptide library previously reported on by Fiell *et al.*¹². These peptides are rich in 387 tryptophan residues along with lysine and arginine in various orders along the backbone (Table 1). The 388 contribution of the arginine, lysine and tryptophan residues to the antimicrobial activity is exerted by 389 390 primary interaction of the charged residues with the net negative charge distributed on the surface of the bacterial membranes followed by insertion of tryptophan residues in the interfacial region of the 391 phospholipid bilayer ²⁰. Beneficial features within the GN peptide library that exhibit somewhat higher 392 antimicrobial activity than others are the cationic and bulky three residues in the amino terminal with 393 specific annotation to the cationic residues which mediate interaction with the anionic lipids and lead to 394 membrane instability in Gram-negative bacteria models²¹. This observation is further supported with 395 the GN peptides -8, -9, -11 and -14 which have either leucine or isoleucine in addition to one cationic 396 (either lysine or arginine) and one tryptophan residues within their structure and exhibit lower 397 antimicrobial activities. 398

To understand the overall activity of the GN peptides and their ability to selectively discriminate 399 between bacterial and mammalian membranes, and therefore obtain a selectivity index as a ranking tool 400 for the most potential peptide candidate, hemolytic and toxic effects were investigated. A strong 401 relationship between hydrophobicity and high antimicrobial activity and high toxicity has been reported 402 in the literature²². Such observation could not be observed in this study for the GN peptides. GN-2 and 403 GN-4 are the most active against both Gram-positive and Gram-negative bacteria and have both high 404 hemolytic profiles. They share the same charge distribution along their backbone differing only in 405 isoleucine (GN-2) and leucine (GN-4) at the C-terminal end and arginine (GN-2) and lysine (GN-4) at 406 the 4th position. The last feature suggests that the lysine residue in GN-4 peptide, which is previously 407 ascribed as a contributor to the higher lytic effects ²¹, exerts the same effect when compared to arginine 408 in GN-2 peptide (Table 1). 409

For a better differentiation of host cell toxicity, metabolic activities in HeLa cells were also monitored. 410 Comparing toxicity against HeLa cells with the hemolytic properties of the GN peptides, reveals a 411 disproportionally high toxicity towards the former (Table 1). This could be related to the peptides 412 exerting higher selectivity to the more anionic cancer cells, similar to previous observations²³. In 413 addition, peptides that successfully entered HeLa cells could target intracellular membranes that 414 resemble bacterial membrane such is the mitochondrial membrane and therefore inhibit metabolism 415 that could be lethal to eukaryotic cells²⁴. To further elucidate the observed toxicity of GN-2 and GN-4, 416 peptides toxicity measurements on a non-cancerous mammalian cells or animal studies should be 417

418 employed.

The current study demonstrate a fast decrease of exponentially growing E. coli when exposed to sub-419 MIC concentrations of both peptide GN-2 and GN-6 (Figure 1 and 2). The observed decrease is 420 probably due to metabolic disturbance that halts bacterial growth for about 80 minutes, after which the 421 growth is reestablished. This is also in agreement with the OD measurements, which revealed similar 422 decrease in the bacteria growth under the same conditions. Flow cytometry analysis show that over a 423 period of 80 minutes the cell size at MIC concentrations does not change significantly, thus supporting 424 the above stated evidence (Figure S2). In support of this, the microscopic images reveal no obvious 425 morphological difference except that the MIC treated samples appear slightly bigger in size. The 426 increase in cell size is a result of metabolic inhibition which delays the decrease in cell size which is 427 normally observed when bacterial suspension gets denser²⁵. In contrast to the bacteriostatic inhibition 428 at MIC concentration of both the GN-2 and GN-6 peptide, bactericidal inhibition is observed at 4×MIC 429 concentrations. Inhibition through bacterial lysis is ascribed to GN-2 peptide which causes decrease in 430 OD of *E. coli* over time, and such relationship has also been described in the literature 26 . The 431 mechanism behind the bactericidal killing for GN-6 demonstrates no signs of lysis rather than 432 filamentation which are reported as an increase in the lightscatter from the flow cytometry analysis ²⁷. 433 These results are further corroborated by the microscopy studies. Prior studies have shown that the SOS 434 response to ciprofloxacin and beta-lactams is ascribed to filamentation ²⁸ and the same can be induced 435 by multiple environmental stresses ²⁹. 436

437 Disturbed membrane integrity of *E. coli* is observed after 10-20 minutes of exposure of 1×MIC
438 concentration of GN-2 peptide, followed by gradual reestablishment or growth of cells that survived

the initial attack (Figure 4). The latter seems plausible as cells surviving exposure to the peptide would 439 440 be in a rough and rather hostile environment, thus explaining their very slow growth. The phenomena observed between 40 and 80 minutes, does also correlates with the observations of constant cell size 441 442 from flow cytometry analysis and a standstill in CFU. However, using live/dead staining analysis the reestablishment of membrane integrity has been ascribed to membrane reassembly which leads to 443 propidium iodide exclusion 30 or possible efflux pumps that drive propidium iodide out of the cell 31 . 444 The later mechanism would require presence of ATP for efflux pumps which could be in agreement 445 with membrane leakage and escape of ions upon peptides acting on membranes at low concentrations, 446 provoking ATP production ³². In summary, it may seem as GN-2 induce sufficient membrane 447 permeabilization for the entry of propidium iodine, but the process does not translate into full loss of 448 viability. In relation to this observation, prior studies have shown that Bac8c, a peptide with sticking 449 resemblance to the GN peptides, can causes membrane depolarization after 5 minutes of exposure 450 followed by membrane recovery after 90 minutes 33 . In case of 2× and 4×MIC concentrations of GN-2 451 peptide, the peptide consistently causes membrane leakage supported by the visual and quantitative 452 membrane leakage assays supported by CFU counts that show loss of bacterial viability (Figure 3 and 453 4). 454

The GN-2 peptide with a very narrow concentration range from 5.5 to $1.7 \,\mu\text{g/mL}$ induced instant 455 release of calcein from model membranes, corresponding to 100% and 5%, respectively (Figure 5). 456 This observation coincides with a proposed model of membrane active antimicrobial peptides binding 457 458 to the surface of the phospholipids parallel to the membrane (S-state) and upon reaching a threshold change their orientation into perpendicular position (I-state), thereby inducing a more profound 459 membrane disruption ³². The GN-14 peptide revealed a more gradual release of calcein content which 460 progresses over a period of 20 minutes. Such release has been ascribed to a mechanism of transient 461 pore formation ³⁴where liposomes re-stabilize resulting in plateau state of calcein release ³⁴⁻³⁵, leaving 462 partially depleted liposomes. Given that the GN peptides only are composed of 9 amino acids, the 463 toroidal pore model is the more plausible mechanism of membrane action due to the fact that the pores 464 in this model are stabilized by both lipids and peptides rather than only peptides as in the barrel-stave 465 model ³⁴. A membrane mechanism involving toroidal pores are further supported by the lack of well 466

defined secondary structures in the GN peptide library (Figure S3), and none helical peptides have also
 by others been demonstrated to work through this mechanism ³⁶.

Previous studies have shown that melittin is a pore forming membrane active peptide that acts in an all 469 or none manner ^{19c, 37}, resulting in these immediate release profiles of calcein. Since the current study 470 showed similar kinetics in mediating calcein release for both melittin and GN-2, it is reasonable to 471 assume that the GN-2 peptide also acts by pore formation mechanism. However the calcein release for 472 mellitin is much more pronounced at lower concentrations when compared to the release for peptide 473 GN-2 Fig.5A. Having said that it is important to stress out that melittin and GN-2 act with different 474 potencies of 16.3 (3.5 µg/mL) lipid/peptide molar ratio for melittin and 5.4 (5.5 µg/mL) for the GN-2 475 peptide. Furthermore, we would argue that GN-6 which results in a much more gradual release of 476 calcein, also could work through pore formation. The difference in kinetics of these peptides (GN-2 477 and GN-6) might be related to the lifetime of the pores ^{35, 38}, the size of the pores and the number of 478 pores ³⁸, all of which reflects on the release of the entrapped molecules from the LUVs. Even though 479 pore formation may seem the best mechanism of action of tested GN peptides, the possibility of 480 membrane disruption exploited by a mixture of different mechanism should not be excluded ³⁹. The 481 release of calcein for both peptides, GN-2 and GN-6, levels off over time (Figure 5B-C) and this type 482 of behavior has been reported to indicate transient pore formation during the initial insertion step into 483 the lipid bilayer ³⁴. 484

In conclusion, based on the experimental data obtained in the current study the primary antibacterial mechanism of action of GN-2 and GN-6 is associated with membrane permeabilization at high peptide concentrations with different kinetics and potencies. At more moderate peptide concentrations it appears that the peptides affect on metabolic disturbance, and further work is required to strengthen these observations.

490

491 Acknowledgements

This work was in part funded by The Danish Council for Independent Research (grant # 10-085287).
We also acknowledge Professor Lars Holm Øgendal (Niels Bohr Institute, Copenhagen University) for

- 494 dynamic light scattering measurements of our liposomes, and Jacob Krake (Roskilde University) for
- 495 LC-MS analysis.

- 497 **References**
- 498 1. Nathan, C., Antibiotics at the crossroads. *Nature* **2004**, *431* (7011), 899-902.
- von Nussbaum, F.; Brands, M.; Hinzen, B.; Weigand, S.; Habich, D., Antibacterial natural products in medicinal chemistry--exodus or revival? *Angew Chem Int Ed Engl* **2006**, *45* (31), 5072-129.
- Klevens, R. M.; Morrison, M. A.; Nadle, J.; Petit, S.; Gershman, K.; Ray, S.; Harrison, L. H.; Lynfield, R.;
 Dumyati, G.; Townes, J. M.; Craig, A. S.; Zell, E. R.; Fosheim, G. E.; McDougal, L. K.; Carey, R. B.; Fridkin, S. K.,
 Invasive methicillin-resistant Staphylococcus aureus infections in the United States. *JAMA* 2007, *298* (15),
 1763-71.
- 505 4. Fischbach, M. A.; Walsh, C. T., Antibiotics for emerging pathogens. *Science* **2009**, *325* (5944), 1089-93.
- 506 5. Jenssen, H.; Hamill, P.; Hancock, R. E., Peptide antimicrobial agents. *Clin Microbiol Rev* **2006**, *19* (3), 491-507 511.
- Hamill, P.; Brown, K.; Jenssen, H.; Hancock, R. E., Novel anti-infectives: is host defence the answer? *Curr Opin Biotechnol* 2008, *19* (6), 628-36.
- Find The State of Content of Co
- 512 8. Jenssen, H.; Hancock, R. E., Antimicrobial properties of lactoferrin. *Biochimie* **2009**, *91* (1), 19-29.
- Wang, G.; Li, X.; Wang, Z., APD2: the updated antimicrobial peptide database and its application in peptide
 design. *Nucleic Acids Res* 2009, *37* (Database issue), D933-7.
- Fjell, C. D.; Jenssen, H.; Hilpert, K.; Cheung, W. A.; Pante, N.; Hancock, R. E.; Cherkasov, A., Identification of
 novel antibacterial peptides by chemoinformatics and machine learning. *J Med Chem* 2009, *52* (7), 2006-15.
- 517 11. Godballe, T.; Nilsson, L. L.; Petersen, P. D.; Jenssen, H., Antimicrobial beta-peptides and alpha-peptoids.
 518 *Chem Biol Drug Des* 2011, 77 (2), 107-16.
- 519 12. Fjell, C. D.; Jenssen, H.; Cheung, W. A.; Hancock, R. E.; Cherkasov, A., Optimization of antibacterial peptides
 520 by genetic algorithms and cheminformatics. *Chem Biol Drug Des* **2011**, *77* (1), 48-56.
- 13. Wiegand, I.; Hilpert, K.; Hancock, R. E., Agar and broth dilution methods to determine the minimal
 inhibitory concentration (MIC) of antimicrobial substances. *Nat Protoc* 2008, *3* (2), 163-75.
- 14. (a) Falla, T. J.; Karunaratne, D. N.; Hancock, R. E. W., Mode of action of the antimicrobial peptide
 indolicidin. *Journal of Biological Chemistry* **1996**, *271* (32), 19298-19303; (b) Selsted, M. E.; Novotny, M. J.;
 Morris, W. L.; Tang, Y. Q.; Smith, W.; Cullor, J. S., Indolicidin, a novel bactericidal tridecapeptide amide from
 neutrophils. *The Journal of biological chemistry* **1992**, *267* (7), 4292-5.
- 527 15. Shai, Y., Mode of action of membrane active antimicrobial peptides. *Biopolymers* **2002**, *66* (4), 236-48.
- 16. Russell, A. L.; Kennedy, A. M.; Spuches, A. M.; Venugopal, D.; Bhonsle, J. B.; Hicks, R. P., Spectroscopic and
 thermodynamic evidence for antimicrobial peptide membrane selectivity. *Chem Phys Lipids* 2010, *163* (6),
 488-97.
- 531 17. Greenfield, N. J., Using circular dichroism spectra to estimate protein secondary structure. *Nat Protoc* 2006,
 532 1 (6), 2876-90.
- 18. Whitmore, L.; Wallace, B. A., Protein secondary structure analyses from circular dichroism spectroscopy:
 methods and reference databases. *Biopolymers* 2008, *89* (5), 392-400.

19. (a) Bechinger, B., Structure and functions of channel-forming peptides: magainins, cecropins, melittin and
alamethicin. *The Journal of membrane biology* **1997**, *156* (3), 197-211; (b) Brogden, K. A., Antimicrobial
peptides: pore formers or metabolic inhibitors in bacteria? *Nature reviews. Microbiology* **2005**, *3* (3), 238-

- 538 50; (c) Wessman, P.; Stromstedt, A. A.; Malmsten, M.; Edwards, K., Melittin-lipid bilayer interactions and 539 the role of cholesterol. *Biophysical journal* **2008**, *95* (9), 4324-36.
- 20. (a) Chan, D. I.; Prenner, E. J.; Vogel, H. J., Tryptophan- and arginine-rich antimicrobial peptides: structures
 and mechanisms of action. *Biochimica et biophysica acta* 2006, *1758* (9), 1184-202; (b) Schibli, D. J.; Epand,
 R. F.; Vogel, H. J.; Epand, R. M., Tryptophan-rich antimicrobial peptides: comparative properties and
 membrane interactions. *Biochem Cell Biol* 2002, *80* (5), 667-77.
- 544 21. Wadhwani, P.; Epand, R. F.; Heidenreich, N.; Burck, J.; Ulrich, A. S.; Epand, R. M., Membrane-active 545 peptides and the clustering of anionic lipids. *Biophysical journal* **2012**, *103* (2), 265-74.
- 546 22. Chen, Y.; Guarnieri, M. T.; Vasil, A. I.; Vasil, M. L.; Mant, C. T.; Hodges, R. S., Role of peptide hydrophobicity
 547 in the mechanism of action of alpha-helical antimicrobial peptides. *Antimicrobial agents and chemotherapy* 548 2007, 51 (4), 1398-406.
- Slaninova, J.; Mlsova, V.; Kroupova, H.; Alan, L.; Tumova, T.; Monincova, L.; Borovickova, L.; Fucik, V.;
 Cerovsky, V., Toxicity study of antimicrobial peptides from wild bee venom and their analogs toward
 mammalian normal and cancer cells. Peptides 2012, 33 (1), 18-26.
- 552 24. Eliassen, L. T.; Berge, G.; Leknessund, A.; Wikman, M.; Lindin, I.; Lokke, C.; Ponthan, F.; Johnsen, J. I.;
 553 Sveinbjornsson, B.; Kogner, P.; Flaegstad, T.; Rekdal, O., The antimicrobial peptide, lactoferricin B, is
 554 cytotoxic to neuroblastoma cells in vitro and inhibits xenograft growth in vivo. International journal of
 555 cancer. Journal international du cancer 2006, 119 (3), 493-500.
- Steen, H. B., Flow cytometry of bacteria: glimpses from the past with a view to the future. J Microbiol
 Methods 2000, 42 (1), 65-74.
- 26. Pasotti, L.; Zucca, S.; Lupotto, M.; Cusella De Angelis, M. G.; Magni, P., Characterization of a synthetic
 bacterial self-destruction device for programmed cell death and for recombinant proteins release. Journal
 of biological engineering 2011, 5, 8.
- 27. Walberg, M.; Gaustad, P.; Steen, H. B., Rapid assessment of ceftazidime, ciprofloxacin, and gentamicin
 susceptibility in exponentially-growing E. coli cells by means of flow cytometry. Cytometry 1997, 27 (2),
 169-78.
- Kohanski, M. A.; Dwyer, D. J.; Collins, J. J., How antibiotics kill bacteria: from targets to networks. Nature
 methods in the second seco
- 30. Moussa, M.; Perrier-Cornet, J. M.; Gervais, P., Damage in Escherichia coli cells treated with a combination
 of high hydrostatic pressure and subzero temperature. Appl Environ Microbiol 2007, 73 (20), 6508-18.
- 570 31. Stocks, S. M., Mechanism and use of the commercially available viability stain, BacLight. Cytometry A 2004,
 571 61 (2), 189-95.
- 572 32. Huang, H. W., Molecular mechanism of antimicrobial peptides: the origin of cooperativity. Biochimica et
 573 biophysica acta 2006, 1758 (9), 1292-302.
- 574 33. Spindler, E. C.; Hale, J. D.; Giddings, T. H., Jr.; Hancock, R. E.; Gill, R. T., Deciphering the mode of action of 575 the synthetic antimicrobial peptide Bac8c. Antimicrobial agents and chemotherapy 2011, 55 (4), 1706-16.
- 576 34. Hugonin, L.; Vukojevic, V.; Bakalkin, G.; Graslund, A., Membrane leakage induced by dynorphins. FEBS
 577 letters 2006, 580 (13), 3201-5.
- 35. Arbuzova, A.; Schwarz, G., Pore-forming action of mastoparan peptides on liposomes: a quantitative
 analysis. Biochimica et biophysica acta 1999, 1420 (1-2), 139-52.

- 36. (a) Irudayam, S. J.; Berkowitz, M. L., Influence of the arrangement and secondary structure of melittin
 peptides on the formation and stability of toroidal pores. Biochimica et biophysica acta 2011, 1808 (9),
 2258-66; (b) Sengupta, D.; Leontiadou, H.; Mark, A. E.; Marrink, S. J., Toroidal pores formed by
- antimicrobial peptides show significant disorder. Biochim Biophys Acta 2008, 1778 (10), 2308-17.
 37. Allende, D.; Simon, S. A.; McIntosh, T. J., Melittin-induced bilayer leakage depends on lipid material
 properties: evidence for toroidal pores. Biophysical journal 2005, 88 (3), 1828-37.
- 38. Rex, S.; Schwarz, G., Quantitative studies on the melittin-induced leakage mechanism of lipid vesicles.
 Biochemistry 1998, 37 (8), 2336-45.
- 39. (a) Pistolesi, S.; Pogni, R.; Feix, J. B., Membrane insertion and bilayer perturbation by antimicrobial peptide
 CM15. Biophys J 2007, 93 (5), 1651-60; (b) Teixeira, V.; Feio, M. J.; Bastos, M., Role of lipids in the
- 590 interaction of antimicrobial peptides with membranes. Progress in lipid research 2012, 51 (2), 149-77.
- 591 592



В



Figure 1. Viability of exponentially growing E. coli ATCC 25922 exposed to 1x, 2x, and 4x MIC concentrations of (A) GN-2 peptide and (B) GN-6 peptide over a course of 3 hours. Data represent mean and SEM of 3 independent experiments. 97x148mm (600 x 600 DPI)



Figure 2. Optical density measurements of exponentially growing E. coli ATCC 25922 over 5 hours. Data presents mean of 3 independent experiments based. Bacterial growth at 37°C in a 96-well plates from a starting inoculum of OD600 of 0.1 has been measured with 8 minutes intervals. (A) Bacteria exposed to antibiotics at MIC concentrations, Ampicillin (4 μg/ml), Tobramycin (0.78 μg/ml), Tetracycline (1 μg/ml) and Ciprofloxacin (0.05 μg/ml). (B) Bacteria exposed to GN-2 peptide at 1x MIC (6.2 μg/ml) and 2x MIC (12.5 μg/ml) and GN-6 peptide at 1x MIC (12.5 μg/ml) and 2x MIC (25 μg/ml) concentrations. 42x12mm (600 x 600 DPI)



Figure 3. Peptide effect on E. coli morphology. (A,B) Flow cytometry data of GN-2 and GN-6 peptide influence on the DNA content per cell mass represented by fluorescence/lightscatter ratio, respectively. Data collected over a course of 3 hours in a microtitter plate format with an inoculum of OD600 of 0.1 and incubated at 37°C between extractions. (C) Microscope images of ethanol stained flow cytometry samples of E. coli treated with GN-2 and GN-6 peptide at 1x and 4xMIC concentration for 3 hours. Bacteria without cellular content appear transparent. Pictures taken at same magnification with a Leica DM5000 B microscope. 87x113mm (600 x 600 DPI)



-●- GN-2 6.25 μg/ml -■- GN-2 12.5 μg/ml -▲- GN-2 25 μg/ml

Figure 4. Visualization and quantification of membrane permeabilization of GN-2 peptide on E. coli ATCC 25922 over a period of 120 minutes. (A) Control cells. (B) Bacteria exposed to $1 \times MIC$ GN-2 peptide. (C) Bacteria exposed to $4 \times MIC$ GN-2 peptide. (D) Percentage of live bacteria after treatment with $1 \times$, $2 \times$ and $4 \times MIC$ concentration of peptide GN-2. Pictures taken with Leica DM5000B microscope using mercury lamp. $118 \times 138 \text{mm}$ (600 x 600 DPI)



Figure 5. Calcein release from POPG:POPC vesicles. (A) Calcein leakage measurements from 20 μM POPG:POPC (7:3) vesicles at 37°C at various peptide concentrations in a 96-well format assay at 60 min. All readings are baseline corrected by subtracting values from wells containing only liposomes. 100% leakage is estimated as a total calcein release from liposomes exposed to 10 % Triton X-100. Represented results are mean values of three independent experiments with good reproducibility. Standard error bars are omitted for clarity. Calcein leakage measurements from 20 μM POPG:POPC (7:3) vesicles at 37°C at various concentrations of (B) GN-2, (C) GN-6 and (D) GN-14, in a 96-well format assay over a period of 1 hour. 76x74mm (600 x 600 DPI)

Peptide	Sequence	HPLC retention-	Hydrophobic moment	Minimal Inhibitory Concentration (µg/ml)			< 10 % _ Hemolysis	IC50
				<i>L. con</i>	1 . ucr uginosu	S. uureus		
GN-2	RWKRWWRWI-CONH ₂	6.33	5.4	6.2	3.1	3.1	100	47
GN-3	KWWRWRRWW-CONH ₂	6.62	3.3	12.5	25	6.2	100	106
GN-4	RWKKWWRWL-CONH ₂	6.54	5.5	6.2	3.1	6.2	100	37
GN-5	KKRWWWWR-CONH ₂	7.10	2.8	12.5	6.2	1.5	100	131
GN-6	RKRWWWWFR-CONH ₂	7.08	2.8	12.5	6.2	3.1	100	97
GN-8	IWKRWWWKR-CONH ₂	4.54	2.2	25	100	50	> 400	199
GN-9	RIWKIWWKR-CONH ₂	4.63	4.8	25	25	> 50	> 400	> 200
GN-11	IKWKRWWWR-CONH ₂	5.88	3.4	12.5	50	25	>400	> 200
GN-12	KWWKIWRWR-CONH ₂	6.13	2.9	25	12.5	12.5	>400	173
GN-14	RLWKRWWIR-CONH ₂	4.43	4.4	6.2	100	25	100	> 200
Indolicidin	ILPWKWPWWPWRR-CONH2	2 ND	1.2	25	100	12.5	100	> 200

Table 1. Peptide characteristics, antimicrobial activity and toxicity

Table 1: Hydrophobic moment is a quantitative measure of amphipathicity calculated with HydroMCalc using the CCS scale. Median MIC values of GN peptides in μ g/ml against *E. coli* ATCC 25922, *P. aureginosa* PA01 and *S. aureus* ATCC 29213 obtained by 3 repeated experiments. Hemolysis values are the mean concentration in μ g/ml recorded over 3 experiments that induced less than 10% hemolysis. IC50 values in μ g/ml is a toxicity measurement resulting from the MTT assays performed on HeLa cells, and calculated from the equation Y=Bottom + (Top-Bottom)/(1+10^((LogIC50-X)*HillSlope)).

Figure 1. Viability of exponentially growing *E. coli* ATCC 25922 exposed to 1x, 2x, and 4x MIC concentrations of (**A**) GN-2 peptide and (**B**) GN-6 peptide over a course of 3 hours. Data represent mean and SEM of 3 independent experiments.

Figure 2. Optical density measurements of exponentially growing *E. coli* ATCC 25922 over 5 hours. Data presents mean of 3 independent experiments based. Bacterial growth at 37°C in a 96-well plates from a starting inoculum of OD_{600} of 0.1 has been measured with 8 minutes intervals. (**A**) Bacteria exposed to antibiotics at MIC concentrations, Ampicillin (4 µg/ml), Tobramycin (0.78 µg/ml), Tetracycline (1 µg/ml) and Ciprofloxacin (0.05 µg/ml). (**B**) Bacteria exposed to GN-2 peptide at 1x MIC (6.2 µg/ml) and 2x MIC (12.5 µg/ml) and GN-6 peptide at 1x MIC (12.5 µg/ml) and 2x MIC (25 µg/ml) concentrations.

Figure 3. Peptide effect on *E. coli* morphology. (**A**,**B**) Flow cytometry data of GN-2 and GN-6 peptide influence on the DNA content per cell mass represented by fluorescence/lightscatter ratio, respectively. Data collected over a course of 3 hours in a microtitter plate format with an inoculum of OD_{600} of 0.1 and incubated at 37°C between extractions. (**C**) Microscope images of ethanol stained flow cytometry samples of *E. coli* treated with GN-2 and GN-6 peptide at 1x and 4xMIC concentration for 3 hours. Bacteria without cellular content appear transparent. Pictures taken at same magnification with a Leica DM5000 B microscope.

Figure 4. Visualization and quantification of membrane permeabilization of GN-2 peptide on *E. coli* ATCC 25922 over a period of 120 minutes. (**A**) Control cells. (**B**) Bacteria exposed to 1xMIC GN-2 peptide. (**C**) Bacteria exposed to 4xMIC GN-2 peptide. (**D**) Percentage of live bacteria after treatment with 1x, 2x and 4xMIC concentration of peptide GN-2. Pictures taken with Leica DM5000B microscope using mercury lamp.

Figure 5. Calcein release from POPG:POPC vesicles. (A) Calcein leakage measurements from 20 μ M POPG:POPC (7:3) vesicles at 37°C at various peptide concentrations in a 96-well format assay at 60 min. All readings are baseline corrected by subtracting values from wells containing only liposomes. 100% leakage is estimated as a total calcein release from liposomes exposed to 10 % Triton X-100. Represented results are mean values of three independent experiments with good reproducibility. Standard error bars are omitted for clarity. Calcein leakage measurements from 20

μM POPG:POPC (7:3) vesicles at 37°C at various concentrations of (**B**) GN-2, (**C**) GN-6 and (**D**) GN-14, in a 96-well format assay over a period of 1 hour.