In vitro and in vivo antimicrobial activity of granulysin-derived peptides against *Vibrio cholerae*

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**Objectives:** To determine the antibacterial activity of synthetic peptides derived from the cationic antimicrobial peptide granulysin against *Vibrio cholerae*.

**Methods:** The antibacterial activity of granulysin-derived peptides was assessed *in vitro* by microtitre and cfu assays. Toxicity against human peripheral blood mononuclear cells (PBMCs) was measured by propidium iodide uptake and haemolysis by measuring the levels of haemoglobin released after incubation of red blood cells (RBCs) with granulysin peptides. The ability of granulysin peptides to control bacterial growth *in vivo* was tested by the treatment of suckling mice infected with *V. cholerae* with granulysin peptides, administered by gavage 1 h after infection and determining the number of bacteria in the small and large intestines 24 h after infection.

**Results:** All peptides tested inhibited *V. cholerae* growth *in vitro*, and they were more effective against stationary phase cells. Two peptides, G12.21 and G14.15, effectively controlled bacterial growth *in vivo*. The peptides did not lyse RBCs and, with the exception of two peptides, exhibited very little toxicity against human PBMCs.

**Conclusions:** These results suggest that granulysin-derived peptides are candidates for the development of new agents for the treatment of *V. cholerae* infection.

Keywords: *V. cholerae*, antimicrobial peptides, cholera

**Introduction**

Cholera is a major life-threatening diarrhoeal disease associated with poor sanitation, particularly in developing countries. Humans apparently are the only natural host for *Vibrio cholerae*, a Gram-negative motile bacterium that causes cholera disease. Humans become infected with this bacterium following ingestion of contaminated food and water. After passage through the gastric barrier, *V. cholerae* colonizes the small intestine and secretes cholera toxin, which is responsible for the massive secretory diarrhoea that is a hallmark of the disease. Although fluid and electrolyte replacement either by oral hydration or intravenous fluid therapy is the treatment of choice for acute diarrhoea, the use of antibiotics for the treatment of severe cholera is recommended, as it significantly reduces the duration of diarrhoea, the volume of rehydration and the time of faecal excretion of *V. cholerae*, thereby decreasing transmission of the infection.

Tetracyclines and fluoroquinolones are the most commonly used antibiotics to treat cholera, and, in the past, most strains were susceptible to these drugs. However, a high incidence of *V. cholerae* strains resistant to commonly prescribed antibiotics has been reported in India and in other countries. The rise in antibiotic-resistant pathogens has led to the development of new therapeutic agents effective against these bacteria.

Antimicrobial peptides (AMPs) are an evolutionarily conserved component of the innate immune response and are found in all species. Cationic peptides are generally small (12–50 amino acids) with an overall positive charge (+2 to +9) due to the presence of excess basic residues. The overall charge, amphipathicity and interaction of a hydrophobic face with the membrane are the main characteristics that correlate with the microbicidal activity of AMPs.
effect of AMPs. Many of these peptides have broad-spectrum activity against bacteria, viruses and fungi, making them candidates for a new class of antibiotics. In vivo studies have also demonstrated the antibacterial activity of AMPs. In a mouse model, cathelicidins can control bacterial infection and prevent mortality when administered after bacterial challenge. Systemic administration of nisin, a lantionine-containing peptide from Lactococcus lactis, protects mice against infection by Streptococcus pneumoniae. Plectasin, a peptide that belongs to the family of defensins, has shown antibacterial activity against S. pneumoniae, and it is currently being examined in pre-clinical trials for the treatment of diseases like pneumonia. Despite all the efforts to develop new drugs for the treatment of infectious diseases, the number of multidrug-resistant strains of bacteria is still increasing, and the development of new drugs is imperative.

Granulysin, a member of the saposin-like protein (SAPLIP) family, is expressed by activated human natural killer cells and T lymphocytes. Recombinant granulysin exhibits activity against Gram-positive and Gram-negative bacteria, Mycobacterium tuberculosis, fungi and tumour cells. Granulysin comprises five α-helices connected by short-loop regions. Synthetic peptides corresponding to the central region of granulysin (helices 2–4) are cytotoxic against bacteria and mammalian tumour cells. Previously, we showed that mutation of cysteine or arginine residues or introduction of D-amino acids to disrupt the α-helix results in the loss of activity against mammalian cells with little or no effect on the antimicrobial activity. In this study, we examine the antimicrobial activity of a new panel of synthetic peptides based on helices 3 and 4 of granulysin against V. cholerae in vitro and in vivo.

Materials and methods

Bacterial strain and growth conditions

The V. cholerae O1 El Tor Inaba strain A1552, RifR, was used in this study. Bacteria were grown to stationary phase (18 h) and mid-log phase in standard Luria–Bertani (LB) broth medium at 37°C. Mid-log phase cultures were grown to an OD600 of 0.4 corresponding to 4.1 × 10⁹ organisms/mL (∼3 h).

Synthesis of granulysin peptides

Peptides were synthesized using 9-fluorenylethoxycarbonyl (Fmoc) chemistry. N°-Fmoc deprotection was achieved with 20% piperidine/N-methylpiperidolone (NMP) (3 × 5 min).

All standard Fmoc amino acid derivatives and resins were purchased from EMD Bioscience Inc. (San Diego, CA, USA). At the completion of synthesis, resins were washed with dichloromethane and dried (N₂ flush, 10 min). Peptides were cleaved from the resin and side chains were deprotected using modified reagent K [2,2,2-trifluoroethanol (TFE), 90% (v/v); phenol, 2.5% (v/v); water, 2.5% (v/v); and trisopropylsilane, 5%; 3 h]. The post-cleaving solutions were collected and the peptides precipitated by addition of ice-cold diethyl ether. The precipitated peptides were separated by centrifugation, washed using cold diethyl ether, spun-down again and dried in vacuo.

Peptides were purified by preparative reverse-phase high-performance liquid chromatography (RP-HPLC) to >95% homogeneity. The purity of peptides was evaluated by analytical RP-HPLC using a ProStar Dynamax® multisolvent delivery system connected to a photodiode array detector (monitoring at 220 nm), using a 4.6 × 150 mm Vydac C18 silica gel packed column (Hesperia, CA, 300 A˚ pore diameter, 5 µm particle size) in a linear gradient from 100% to 40% (v/v) solvent B [0.1% trifluoroacetic acid (TFA) in acetonitrile] in solvent A (0.1% TFA in water) over 40 min (flow rate: 1 mL/min). The integrity of each peptide was confirmed by amino acid analysis and matrix-assisted laser desorption ionization spectrometry (MALDI-MS) (The Beckman Center, Stanford, CA, USA). Peptides produced in this study were G11.24 (mol. wt 1419), G12.21 (mol. wt 2710), G12.34 (mol. wt 2776), G12.35 (mol. wt 2760), G12.37 (mol. wt 2892), G12.38 (mol. wt 3491), G12.39 (mol. wt 3804), G12.40 (mol. wt 4860) and G14.15 (mol. wt 2062). Their sequences and chemical properties are summarized in Table 1.

Circular dichroism (CD)

CD measurements for each peptide were taken with an Aviv spectropolarimeter (Aviv Instruments, Lakewood, NJ, USA) at room temperature, over the range from 190 to 260 nm. A pathlength of 0.1 cm was used. Peptides were assayed at 0.3 mM final concentration in 10 mM phosphate buffer, pH 7.4, or 50% TFE in 10 mM phosphate buffer. Results are expressed as percentage helicity.

Lysis of human peripheral blood mononuclear cells (PBMCs)

Freshly isolated human PBMCs were resuspended in RPMI supplemented with 10% fetal calf serum (FCS) at a density of 1 × 10⁷ cells/mL. PBMCs (1 × 10⁶ cells in 100 µL) were incubated with 100 µL of peptide (50 or 10 µM) in 12 × 75 mm culture tubes at 37°C for 3 h. After this period, 50 µL of a 5 mg/L solution of propidium iodide (PI) was added to each tube, and the tubes were incubated in the dark for 4 min. PI incorporation of 10 000 cells was determined by flow cytometry (FACS Calibur, Becton & Dickinson, San Jose, CA, USA). The samples were analysed using Cell Quest software (BD Biosciences, San Jose, CA, USA). The PI-negative population in medium control samples was set as a reference to determine the percentage of PI-negative cells in all other samples. Peptide G8 was used as a positive control since it has been previously shown to induce cell death.

Haemolysis by granulysin-derived peptides

Red blood cells (RBCs) were separated from human peripheral blood by centrifugation at 1000 g for 5 min, washed three times with RPMI 1640 without phenol red and resuspended in the same medium supplemented with 20% FCS (RPMI-S). Granulysin peptides at 20 or 100 µM (100 µL/well in RPMI 1640) were added to 100 µL of a stock solution of RBCs (8% v/v) in RPMI-S to reach a final volume of 200 µL (final erythrocyte concentration 4% v/v and peptides 10 or 50 µM). The resulting suspension was incubated with agitation for 1 h at 37°C. After this, the samples were centrifuged at 800 g for 10 min. Haemoglobin release was monitored by measuring the absorbance of the supernatant at 540 nm in a microtitre plate reader (Molecular Devices, Sunnyvale, CA, USA). Controls for 0% haemolysis (blank) and 100% haemolysis consisted of RBCs in RPMI-S only and treated with 1% Triton X-100 (Sigma, St Louis, MO, USA), respectively. Melittin peptide was also used as a positive control for haemolysis. The results are expressed as the percentage of haemolysis obtained by the ratio of the difference in OD between the peptide-treated sample and the blank, and the difference in OD between the Triton X-100-treated sample and the blank.
Antimicrobial activity of granulysin-derived peptides against V. cholerae

Table 1. Amino acid sequence and chemical properties of granulysin-derived peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Amino acid sequence</th>
<th>No. of residues</th>
<th>Net positive charge</th>
<th>Hydrophobic residues</th>
<th>Glycine + proline</th>
<th>Percentage α-helix</th>
</tr>
</thead>
<tbody>
<tr>
<td>G11.24</td>
<td>rrvrrfmrr</td>
<td>10</td>
<td>6</td>
<td>3</td>
<td>0</td>
<td>RC</td>
</tr>
<tr>
<td>G12.21</td>
<td>rrvrrpmrryrssrprrlvr</td>
<td>20</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>RC</td>
</tr>
<tr>
<td>G12.34</td>
<td>rrvrrfmmrryrsrrrllv</td>
<td>20</td>
<td>11</td>
<td>6</td>
<td>0</td>
<td>18</td>
</tr>
<tr>
<td>G12.35</td>
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<td>11</td>
<td>5</td>
<td>1</td>
<td>18</td>
</tr>
<tr>
<td>G12.37</td>
<td>laurvl-rrvrrpmrryrsrrprrlv</td>
<td>20</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>RC</td>
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<tr>
<td>G12.38</td>
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<td>16</td>
<td>4</td>
<td>2</td>
<td>RC</td>
</tr>
<tr>
<td>G12.39</td>
<td>rrrrrrrrrrrrvrrpmrryrsrrprrlvr</td>
<td>27</td>
<td>18</td>
<td>4</td>
<td>2</td>
<td>RC</td>
</tr>
<tr>
<td>G12.40</td>
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<td>20</td>
<td>11</td>
<td>4</td>
<td>2</td>
<td>RC</td>
</tr>
<tr>
<td>G14.15</td>
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<td>15</td>
<td>8</td>
<td>4</td>
<td>1</td>
<td>32</td>
</tr>
</tbody>
</table>

PEG, polyethylene glycol; RC, random coil.

*One letter code has been used for amino acids. Upper case denotes L-isomer amino acids and lower case denotes D-isomer amino acids.

*Helical content (%) calculated from CD measurement in 50% TFE.

*Lauric acid: CH₃(CH₂)₁₀COOH (mol. wt = 2000).

*Peptide G14.15 sequence includes the original loop 2 of granulysin.

Assay for antibacterial activity

Determination of MIC was performed using microtiter 96-well plates (E & K Scientific, Santa Clara, CA, USA). Melittin from honey bee venom (Sigma), polymyxin B sulphate (Sigma) and granulysin-derived peptides were diluted to 40 μM in 10 mM phosphate buffer, pH 7.4. Two-fold serial dilutions of this solution ranging from 0.039 to 40 μM were prepared in 10 mM phosphate buffer, pH 7.4. LB broth well, and the plates were incubated overnight with shaking at 37°C. The MIC endpoint was defined as the lowest peptide concentration at which no bacterial growth was measurable after overnight incubation.

Infection and treatment of infant mice

BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were bred at the Research Animal Facility at Stanford University Medical Center. Studies reported in this work were performed using protocols approved by the Institutional Review Board. Suckling BALB/c mice (Charles River Laboratories, Wilmington, MA, USA) were bred at the Research Animal Facility at Stanford University. Studies reported in this work were performed using protocols approved by the Institutional Review Board. Suckling mice were used for oral infection with V. cholerae. Briefly, 4- to 5-day-old suckling mice were separated from their mothers 1 h prior to inoculation with V. cholerae. Stationary phase cultures of V. cholerae were washed twice (3000 g, 5 min) in 10 mM phosphate buffer, pH 7.4 + 0.03% LB and the final concentration adjusted to 2 × 10⁹ cfu/mL. Eight microlitres of blue food colouring dye (McCormick & Co., Hunt Valley, MD, USA) was added per millilitre of bacterial suspension to visualize the inoculum in the animals. Each mouse was inoculated with 5 μL of bacterial suspension (containing 1 × 10⁶ cells) delivered into their mouth by pipette. One hour later, 40 μL of peptide (8 mg/kg mouse) diluted in 10 mM phosphate buffer, pH 7.4 + 0.03% LB containing the blue dye was administered directly into the stomach by gavage with polyethylene tubing (PE10; Clay Adams, Parsippany, NJ, USA) connected to a 30 gauge needle on a 1 mL syringe (Becton & Dickson, Franklin Lakes, NJ, USA). Proper inoculation of peptides and bacteria into the stomach was verified by visualizing the blue dye in the stomach. The control group received 40 μL of 10 mM phosphate buffer, pH 7.4 + 0.03% LB. Mice were then kept in a 30°C humidified incubator for 18–20 h and sacrificed by CO₂ inhalation. The large and small intestines were washed twice (3000 g, 5 min) in 10 mM phosphate buffer, pH 7.4 + 0.03% LB. Serial dilutions of the homogenates were plated onto LB agar supplemented with 100 mg/L rifampicin. The plates were incubated overnight at 37°C, and bacterial colonies were enumerated the following day by automatic colony counter (aCOLyte colony counter, UK).

Statistical analyses

Differences between peptide-treated group and control group were evaluated using the unpaired Student’s t-test with Welch’s correction. The one-sided P values were calculated, and statistical significance was accepted within 95% confidence limits.

Results

Amino acid sequence and properties of granulysin-derived peptides

For this study, we selected a panel of synthetic peptides originally based on the primary sequence of helix 3 and helix 4 of granulysin. All L-isomer amino acids in the original sequence of granulysin were replaced by D-isomer amino acids, as others and we, have shown that the latter are much more resistant to proteases. The amino acid sequences and other properties of these peptides are shown in Table 1. G11.24 has two arginine substitutions at positions 2 and 6, and the cysteine at position 4 has been replaced with serine. Peptide G12.34 contains the G11.24 sequence plus a modification of helix 4, with four more arginine residues at positions 12, 15, 17 and 18. Peptide G12.21 includes two proline residues at positions 7 and 16 to interrupt any helical structure. G12.35 is identical to G12.37, with one proline substitution at position 16. G12.39, G12.37, G12.15 and G12.40 are identical to G12.21 with
In vitro antimicrobial activity of granulysin-derived peptides against *V. cholerae*

The antibacterial activity of granulysin peptides against *V. cholerae* was tested in vitro by microtitre plate assay. The MIC of each peptide is shown in Table 2. All of the peptides tested had similar MICs in stationary phase cells range between 1.25 to 2.5 \( \mu M \) and 10 to 20 \( \mu M \) in log phase cells (Table 2); the MIC of melittin in stationary phase cells was 2.5 and 40 \( \mu M \) in log phase cells; the MIC of polymyxin B in stationary phase cells was 1.25 and 20 \( \mu M \) in log phase cells. Results were confirmed by cfu assay (data not shown). These results indicate that log phase *V. cholerae* are more resistant to granulysin-derived peptides, melittin and polymyxin than are stationary phase cells (Table 2).

Lytic activity of granulysin peptides against human PBMCs

The toxicity of granulysin peptides against human PBMCs was tested by incubating PBMCs with peptides (10 or 50 \( \mu M \)) for 3 h and then measuring the PI uptake (Table 3). The granulysin derivative peptide G8 was used as a positive control for PI uptake. Peptides G11.24 and G12.21 did not exhibit toxicity against PBMCs. G12.34, G12.35, G12.38, G12.39 and G14.15 exhibited toxicity against human PBMCs only at 50 \( \mu M \). Peptides G12.37 and G12.40 were toxic even at 10 \( \mu M \) (Table 3). Toxicity against human PBMCs seems to correlate with the size of the peptide and with the ability to form \( \alpha \)-helix upon association with membranes (Tables 1 and 3). Attachment of lauric acid and PEG to the amino terminus of the peptide G12.21 greatly increased peptide toxicity (G12.37 and G12.40; Table 3) and did not improve their antimicrobial activity against *V. cholerae in vivo* (Table 2) and in vivo (data not shown).

### Table 2. Antibacterial activity of granulysin-derived peptides against *V. cholerae*

<table>
<thead>
<tr>
<th>Peptide</th>
<th>MICa (( \mu M ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>stationary phase cells</td>
</tr>
<tr>
<td>G11.24</td>
<td>1.25</td>
</tr>
<tr>
<td>G12.21</td>
<td>1.25</td>
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<tr>
<td>G12.34</td>
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<td>G12.37</td>
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<tr>
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<td>2.5</td>
</tr>
<tr>
<td>G12.39</td>
<td>2.5</td>
</tr>
<tr>
<td>G12.40</td>
<td>2.5</td>
</tr>
<tr>
<td>G14.15</td>
<td>1.25</td>
</tr>
<tr>
<td>Melittin</td>
<td>2.5</td>
</tr>
<tr>
<td>Polymyxin B</td>
<td>1.25</td>
</tr>
</tbody>
</table>

*aAntimicrobial activity was determined in three to four independent microtitre assays with a discrepancy of not more than one dilution.

bNM, not measured.

### Table 3. Propidium iodine (PI) uptake by and haemolysis of PBMCs incubated with granulysin-derived peptides

<table>
<thead>
<tr>
<th>Peptide</th>
<th>PI uptake (%)</th>
<th>Haemolysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 ( \mu Ma )</td>
<td>50 ( \mu Ma )</td>
</tr>
<tr>
<td>G8</td>
<td>NMb</td>
<td>55</td>
</tr>
<tr>
<td>G11.24</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>G12.21</td>
<td>0.0</td>
<td>1.0</td>
</tr>
<tr>
<td>G12.34</td>
<td>3.0</td>
<td>27.0</td>
</tr>
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<td>G12.35</td>
<td>0.0</td>
<td>19.0</td>
</tr>
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<td>G12.37</td>
<td>10.0</td>
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<td>G12.38</td>
<td>2.0</td>
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<tr>
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<td>G12.40</td>
<td>23.0</td>
<td>59.0</td>
</tr>
<tr>
<td>G14.15</td>
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<td>19.0</td>
</tr>
<tr>
<td>Melittin</td>
<td>NM</td>
<td>NM</td>
</tr>
</tbody>
</table>

*aPeptide concentration.

bNM, not measured.

The most commonly used animal model to study *V. cholerae* intestinal colonization is the suckling mouse. The neonatal mouse model has been successfully used to demonstrate passive protection by immunoglobulin A and virstatin against intestinal colonization by *V. cholerae*. Based on this, we used a modified version of the neonatal mouse model to test the ability of granulysin peptides to control *V. cholerae infection in vivo*. Cohorts of mice (eight animals per group) were infected by pipetting 5 \( \mu L \) of the stationary phase bacteria (10\(^6\) cfu/mouse) directly into their mouths, which better mimics the natural infection in humans. One hour after bacterial inoculation, 40 \( \mu L \) of 10 mM phosphate buffer, pH 7.4 + 0.03% LB or 40 \( \mu L \) of peptide (8 mg/kg/mouse) in 10 mM phosphate buffer, pH 7.4 + 0.03% LB was administered by gavage. After 24 h, the small and large intestines were removed for bacterial quantification. Following inoculation of 10\(^6\) cfu/mouse, 1–4 × 10\(^5\) cfu/mouse (mean: 1.74 × 10\(^6\) ± 2.79 × 10\(^5\)) were recovered 24 h later.
Antimicrobial activity of granulysin-derived peptides against *V. cholerae*

![Graph showing antimicrobial activity of granulysin-derived peptides against V. cholerae](image)

**Figure 1.** Granulysin-derived peptides kill *V. cholerae in vivo*. BALB/c mice were infected with $1 \times 10^6$ *V. cholerae* El Tor strain A1552. After 1 h, granulysin peptides (8 mg/kg/mouse) were delivered into the stomach by gavage. Animals in the control group were given an equal volume of 10 mM phosphate buffer + 0.03% LB. Results depict the number of cfu/mouse in each group and are representative of at least four experiments. (a) Peptide G12.21. (b) Peptide G14.15.

With the exception of G11.24, all peptides shown in Table 2 were tested *in vivo*. As shown in Figure 1, peptides G12.21 and G14.15 significantly controlled bacterial growth *in vivo*; the total number of cfu recovered in mice treated with G12.21 or G14.15 was 20% to 30% of that in the control group (Figure 1). Peptides G12.34, G12.35, G12.37, G12.38, G12.39 and G12.40 did not reduce or increase the number of *V. cholerae* recovered (data not shown). Although a single dose of G12.21 or G14.15 results in a significant decrease in the number of bacteria recovered, it did not clear the infection.

**Discussion**

Outbreaks of cholera have been reported in developing countries in which *V. cholerae* strains resistant to the currently used antibiotics are appearing.\(^2\)\(^4\)\(^5\) Thus, development of new antibiotics against these resistant strains is critical. In the present study, we investigated the activity of a panel of synthetic peptides derived from granulysin against *V. cholerae*. The peptides tested were based on helix 3 and helix 4 of granulysin. All peptides tested exhibited very similar antimicrobial activity against *V. cholerae in vitro* (Table 2), independent of their length, net positive charge and helical content.

Antibacterial activity of many drugs can be dependent on the bacterial growth phase, and log phase cells are usually more susceptible to antibiotics than stationary phase cells.\(^2\)\(^4\)\(^5\) However, we observed that log phase *V. cholerae* are more resistant to granulysin derivatives than stationary phase cells, suggesting that sensitization to granulysin peptides does not occur during cell division. Bacterial resistance/susceptibility to a particular drug depends on the mechanism of action of the drug. Log phase cells are more susceptible than stationary phase cells because most drugs target enzymes that are highly expressed during cell division, including those involved in cell-wall synthesis, DNA synthesis, metabolism and protein synthesis.\(^2\)\(^2\) It is likely that the main target of many cationic AMPs is the lipid bilayer itself, since these agents do not exhibit any stereospecific interactions with chiral receptors or enzymes.\(^2\)\(^3\) Log phase *V. cholerae* were also more resistant to melittin and polymyxin B sulphate (Table 2). Melittin and polymyxin B are AMPs with distinct mechanisms of action. Melittin disrupts the lipid bilayer by pore formation, reorganization of lipid assembly that includes vesiculization of multilayers, fusion of small lipid vesicles, fragmentation into discs and micellar aggregation (reviewed in Raghuraman and Chattopadhyay\(^1\)\(^5\)). Polymyxin B, on the other hand, does not appear to induce membrane pore formation. Rather, the bactericidal effect of polymyxin B seems to involve the formation of molecular contacts between the inner and outer membranes of the bacteria and the induction of lipid exchange, thereby resulting in loss of compositional specificity of the membrane and osmotic instability.\(^2\)\(^4\) By comparison, less is known about the mechanism of action of granulysin-derived peptides. A recent study of the biophysical interaction of a panel of granulysin peptides (G12.21, G12.34 and G12.35) with lipopolysaccharide (LPS) demonstrates that granulysin peptides bind to LPS and convert LPS into a multilamellar form with no change of acyl chain fluidity\(^2\) as opposed to polymyxin B that induces fluidity of LPS acyl chain.\(^2\)\(^6\) It is possible that the mechanism of action of the granulysin peptides is similar to that of melittin, but a detailed study of the biophysical and biochemical interaction of granulysin peptides with bacterial membranes is necessary.

The differences in the effect of granulysin peptides between log phase and stationary phase cells may also reflect differences in gene expression during the growth phase. For example, it has been shown that ToxR, a transcription factor that regulates the expression of a set of nearly 60 genes involved in *V. cholerae* pathogenicity,\(^2\)\(^7\) mediates resistance to P2 [a bactericidal/ permeability-increasing (BPI) derived peptide] in log phase cells by activating the expression of the outer membrane protein gene U (OmpU). In stationary phase cells, both OmpU and OmpT genes induce resistance of *V. cholerae* to P2 peptide.\(^2\)\(^8\) Levels of OmpU and OmpT are regulated by the *V. cholerae* growth phase. In mid-log phase cultures, ompU is expressed and ompT is repressed; in stationary phase cells, both ompU and ompT are expressed.\(^2\)\(^9\) Absence of OmpU also increases the susceptibility of mid-log phase cells to polymyxin B sulphate by nearly 1000-fold relative to wild-type cells.\(^2\)\(^8\) It is possible that OmpU and other genes may confer resistance of mid-log phase *V. cholerae* cells to our peptides.

Modifications were made at the amino terminus of G12.21 to increase antimicrobial activity and resistance to proteases (Table 1). Previous studies showed that the attachment of fatty acids to cationic peptides could increase their antibacterial activity.\(^2\)\(^9\) Fatty acids are considered non-toxic and have broad-spectrum activity against Gram-positive and Gram-negative bacteria.\(^3\)\(^0\)\(^3\)\(^1\) Formulations of lauric acid and mupirocin are effective against *Staphylococcus aureus* in a murine nasal model.\(^3\)\(^0\) We found that acylation of G12.21 with lauric acid (G12.37) did not improve its activity against *V. cholerae in vitro* (Table 2) or *in vivo* (data not shown). Moreover, addition of the fatty acid rendered this peptide more toxic against human PBMCs (Table 3). Not surprisingly, the addition of lauric acid did not change the secondary structure of G12.21; the two proline residues at positions 7 and 16 prevent formation of any helical structure. The addition of fatty acids to peptides can be an important factor in controlling peptide self-assembly and in determining its oligomeric state, and specific secondary structure may not play a central role in controlling the biological activity of lipopeptides.\(^3\)\(^2\) We did not investigate oligomerization and self-assembly of peptide G12.21 and its derivatives, however, no correlation between antimicrobial effect of these peptides and helical content was observed (Table 2).
Attaching a PEG moiety to peptides and protein drugs has been used to improve in vitro efficacies of these drugs, since PEGylation prolongs the circulating half-life of drugs and reduces immunogenicity (reviewed in Harris and Chess33). PEGylation of G12.21 did not improve its antimicrobial activity in vitro (Table 2) or in vivo (data not shown), and no change of the α-helical content was observed (Table 1). Imura et al. also observed that addition of PEG did not alter secondary structures of tachypleisin I in solution or in membranes. PEGylation reduced the antimicrobial activity of nisin and tachypleisin I peptides in vitro.43,45 In agreement with these findings, we observed a 2-fold increase in the MIC of peptide G12.40 (the PEGylated version of G12.21) and this peptide was highly toxic against PBMCs in vitro (Table 3). PEGylation can increase the solubility and improve the pharmacokinetic profile of some drugs, but it can also decrease specific activity leading to undesired effects.36

Previous studies have shown that arginine oligomers can cross cell membranes, facilitating the transport of drugs, peptides and proteins into mammalian cells.7 Arginine oligomers can also transport inhibitory compounds into different evolutive forms of Toxoplasma gondii in vitro and in vivo.38 Synthetic l-arginine polymers enhance LPS responses in vitro, increasing production of cytokines by human blood cells, and this effect correlates with the ability of the l-arginine polymers to bind to LPS.39 Two peptides in our panel, G12.38 and G12.39, have extensions of 5 and 7 arginine residues, respectively (Table 1). The in vitro antibacterial effect of both peptides was similar or less than that of the parent peptide, G12.21 (Table 2). Moreover, administration of G12.38 and G12.39 in vivo did not reduce the number of V. cholerae recovered in comparison with the control group (data not shown).

We used two different assays to evaluate peptide toxicity in vitro. Haemolysis is widely used to investigate toxicity of drugs, and none of the granulysin peptides tested lysed RBCs. We also investigated whether these peptides exhibit toxicity against PBMCs (Table 3). Peptides G11.24 and G12.21 were not toxic in vitro against PBMCs, and peptides G12.38 and G12.39 showed minimal toxicity (<10%) at 50 μM. The remaining peptides exhibited some degree of toxicity at 50 μM (G12.34, G12.35, G12.37, G12.40 and G14.15) or at 10 μM (G12.37 and G12.40). Toxicity against PBMCs correlated to some extent with peptide length and helical content, properties identified as important by others.40

V. cholerae is an extremely hardy organism that can survive the low pH of the stomach, ultimately reaching the small bowel where it colonizes. To survive and multiply in the small bowel, V. cholerae must resist many AMPs present in the human intestine, including β-defensins, cathelicidin and BPI.23,41 An increase in the expression of V. cholerae outer membrane protein, OmpU, by ToxR is responsible for the resistance to P2 and to polymyxin B.28 It may be possible that the genes that confer resistance of V. cholerae to the natural AMPs present in the gut may also play a role in conferring resistance of this bacterium to granulysin peptides.

The neonatal mouse model suffers from some limitations, including the small volume that can be administered and the difficulty in delivering of multiple doses of peptides in vivo. Nonetheless, we demonstrate that peptides G12.21 and G14.15 could overcome the low pH and proteases present in the gut to effectively control V. cholerae growth in vivo (Figure 1). The granulysin peptides also exhibited bactericidal activity against V. cholerae cells in vitro and it is possible that G12.21 and G14.15 are not only inhibiting the bacterial growth but also killing V. cholerae in vivo; however, a more detailed histopathological investigation of the intestinal cells is necessary. Finally, it is also important to note that none of the mice treated with granulysin peptides died or showed any macroscopic signs of toxicity. These findings suggest that these or related peptides provide new clues for the development of new agents for cholera therapy.

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Transparency declarations
None to declare.

References
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