Antimicrobial activity of SMAP-29 against the *Bacteroides fragilis* group and clostridia

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**Objectives:** The cathelicidin-derived peptide SMAP-29 exerts rapid and broad-spectrum antimicrobial activity against aerobic bacteria and fungi. In this study, the effects of the peptide against the *Bacteroides fragilis* group, including antibiotic-resistant isolates, *Clostridium perfringens* and *Clostridium difficile* reference and clinical isolates, were investigated.

**Methods:** The microbicidal activity of SMAP-29 against eight reference and 100 clinical anaerobic strains from a national collection was assessed using a microdilution susceptibility assay, and by determining the killing kinetics on selected strains. The killing mechanism was investigated further by means of a two-colour fluorescent permeabilization assay, and by scanning electron microscopy (SEM).

**Results:** The *Bacteroides fragilis* group, *Clostridium* reference strains and most clinical isolates were inhibited in vitro by 1–2 µM (3.2–6.4 mg/L) SMAP-29, and killed by 1.5- to 2-fold higher peptide concentrations. The anaerobic bacterial cells were 90%–100% permeabilized within 2 h of exposure to bactericidal concentrations of the peptide. The SEM images of bacteria exposed to SMAP-29 provide morphological evidence that the envelope is an important target of the bactericidal activity of this peptide. These results are consistent with earlier studies indicating that SMAP-29 kills aerobic bacteria with a membranolytic mechanism, and suggest that both aerobic and anaerobic bacteria share surface features that are targeted by this peptide.

**Conclusions:** These studies demonstrate that the spectrum of antibacterial activity of SMAP-29 includes the *B. fragilis* group and *Clostridium* species, and encourage further investigations of the therapeutic potential of this peptide.

Keywords: antimicrobial peptide, cathelicidin, anaerobic bacteria, membrane permeabilization

**Introduction**

The emergence of clinical bacterial strains exhibiting resistance to currently available antimicrobial agents is a worldwide threat. The evolution and rapid spread of strains carrying one or multiple antibiotic resistance mechanisms has affected the efficacy of traditional therapeutic approaches, and adds urgency to the search for antimicrobial agents with novel chemical structures and bacterial targets.¹

Several species of anaerobic bacteria are isolated frequently from human clinical specimens and may be responsible for serious infections. In particular, *Bacteroides fragilis* group strains among Gram-negative organisms, and *Clostridium perfringens*, *Clostridium difficile* and other *Clostridium* species among Gram-positive anaerobes may cause serious infections, i.e. mixed chronic/abscess processes, bacteraemia, post-operative wound infections and toxin-associated syndromes.² The rapid spread of antibiotic-resistant clinical isolates has impaired effective treatment of these anaerobic infections, by decreasing the efficacy of several classes of molecules currently used in therapy, such as β-lactams, tetracyclines, macrolides and clindamycin.³ Even metronidazole, which was long considered a fully active anti-anaerobe antibiotic, has become less effective following the emergence of less-susceptible *B. fragilis* isolates.⁴

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Several novel compounds are currently under investigation for their therapeutic potential. Among these are the cationic peptides of the innate immune system, which represent a wide natural host-defence mechanism. A large number of these molecules have been isolated from animals, plants and bacteria in the last two decades.\textsuperscript{5,6} Their \textit{in vitro} and \textit{in vivo} effects against several groups of pathogenic aerobic bacteria and fungi have been determined,\textsuperscript{6,7} revealing potent activity against these organisms. A limited number of investigations have revealed their effects on anaerobic bacteria.\textsuperscript{8–12}

In this study, the \textit{in vitro} activity of SMAP-29,\textsuperscript{13} a high-potency and broad-spectrum\textsuperscript{14} antimicrobial peptide belonging to the cathelicidin family,\textsuperscript{15} was evaluated against reference and clinical anaerobic bacteria belonging to the \textit{B. fragilis} group, \textit{C. difficile} and \textit{C. perfringens} species, including strains displaying antibiotic resistance mechanisms. The mechanism of antimicrobial activity was investigated using a two-colour fluorescence permeabilization assay and scanning electron microscopy (SEM).

### Materials and methods

#### Organisms

A total of eight reference anaerobic strains were used in the assays: \textit{B. fragilis} NCTC 9343, \textit{Bacteroides distasonis} NCTC 11152, \textit{Bacteroides ovatus} NCTC 11153, \textit{Bacteroides thetaiotaomicron} NCTC 10582, \textit{Bacteroides eggerthii} NCTC 11155, \textit{Bacteroides vulgatus} NCTC 11154, \textit{C. difficile} ATCC 10463 and \textit{C. perfringens} ATCC 13124. In addition, 100 clinical isolates (70 from the \textit{B. fragilis} group and 30 \textit{Clostridium} spp.) isolated in the last 5 years by the Udine General Hospital and by the University Hospital were tested. \textit{Clostridium} spp. isolates included toxicogenic \textit{C. difficile} strains.\textsuperscript{16} All the isolates were subcultured from frozen stock cultures, stored at \textdegree C in 10% glycerol Brucella broth (Oxoid, Milan, Italy) vials, onto freshly made Brucella agar base (Oxoid) plates, supplemented with 5 mg/L of haemin (Sigma–Aldrich, Milan, Italy), 1 mg/L of vitamin K\textsubscript{1} (Sigma-Aldrich) and 5% defibrinated sheep blood (Oxoid). Plates were incubated in anaerobic jars (Anaerogen system, Oxoid) at \textdegree C for 48–72 h, and checked for purity. Colonies were picked up from plates and resuspended in Wilkins–Chalgren broth (Oxoid), supplemented with 5 mg/L haemin and 1 mg/L vitamin K\textsubscript{1}, for use in the antibiotic susceptibility assays.

#### Antimicrobial agents

SMAP-29 is a cathelicidin-derived peptide deduced from sheep myeloid mRNA, as previously reported.\textsuperscript{13} The peptide used in this study was synthesized chemically as a 28 residue peptide (RGLRLRLKRIAH-GVKKYGPTVLRIRIRIA), amidated at the C terminus, as indicated by the presence of C-terminal glycine, a common amidation signal in cathelicidin peptides.\textsuperscript{15} The correct peptide was obtained in >60% yield, and with a measured mass of 3198.0 \pm 0.3 compared with a calculated mass of 3197.99 Da, and was homogeneous after preparative purification, as confirmed by mass spectrometry and analytical RP-HPLC.

Standard laboratory powders of amoxicillin, cefotaxin, clindamycin and tetracycline (Sigma Aldrich) were reconstituted according to NCCLS indications.\textsuperscript{17}

#### Antimicrobial testing

Microdilution assays to establish MIC and MBC values of SMAP-29, as well as time–kill assays to determine the kinetics of inactivation of selected strains by the peptide, were performed as recommended by the NCCLS\textsuperscript{17} and by reference protocols.\textsuperscript{18} However, in order to prevent dehydration of samples and to optimize viable cell counting, the final broth volume of each well was increased to 300 \textmu L instead of 100 \textmu L. Supplemented Wilkins–Chalgren broth was implemented in the assays using \textit{B. fragilis} and \textit{Clostridium} strains. For each strain, experiments were performed in duplicate.

Aliquots of bacterial suspensions were dispensed into microtitre plate wells; different amounts of SMAP-29 were added to each well, in order to obtain a serial concentration range (0.8–32 mg/L) (0.25 \mu M to 10 \mu M), except for growth control wells, which contained an equal amount of bacterial suspension and PBS instead of SMAP-29. Negative controls contained SMAP-29 at the highest concentration used without bacterial suspension in Wilkins–Chalgren broth. Microtitre plates were incubated at 37\degree C in anaerobic jars (Anaerogen system, Oxoid) for 24–48 h. MICs were determined by visual reading of well turbidity, whereas MBCs were evaluated from the same test by viable counting assay: MBC was defined as the lowest concentration of peptide that killed 99.9\% of the test inoculum.\textsuperscript{18}

The killing kinetics of selected reference strains \textit{(B. fragilis} NCTC 9343, \textit{C. perfringens} ATCC 13124, \textit{C. difficile} ATCC 10463) were performed on the basis of standard protocols.\textsuperscript{18} The experiments were performed in duplicate for each strain. Bacterial suspensions were obtained during the late logarithmic phase from Wilkins–Chalgren broth cultures and adjusted to 10\textsuperscript{8}–10\textsuperscript{9} cfu/mL. Aliquots were dispensed into microtitre plate wells, and SMAP-29 was added to the final concentration: 0.5 \times MIC, 1 \times MIC, 2 \times MIC. At different time points (0, 2, 4, 6, 8 h), aliquots of samples were collected, briefly spun at 5200g three times (centrifuge mod.5810, Eppendorf, Milan, Italy), rinsed in PBS (to avoid antibiotic carry-over), serially diluted if needed to ensure accurate colony counting and cultured anaerobically on Brucella blood agar plates at 37\degree C for 24–48 h. Data were analysed and expressed as log\textsubscript{10} cfu/mL over time.

The antibiotic susceptibility of reference and clinical strains belonging to the \textit{B. fragilis} group to amoxicillin, cefotaxin, clindamycin and tetracycline was determined by a broth microdilution method.\textsuperscript{17} Breakpoints for susceptibility, intermediate susceptibility and resistance of \textit{B. fragilis} group isolates against antimicrobial agents other than SMAP-29 were based on NCCLS indications.\textsuperscript{17}

#### Membrane permeabilization assay

The ability of SMAP-29 to permeabilize anaerobic bacterial membranes was tested using a two-colour fluorescence assay of bacterial viability (LIVE/DEAD BacLight Bacterial Viability Kit, Molecular Probes, Eugene, OR, USA). The method utilizes mixtures of a green fluorescent nucleic acid stain (SYTO 9) and propidium iodide (PI), a red fluorescent nucleic acid stain. As indicated by the manufacturer, SYTO 9 labels all bacteria in a population with intact membranes. Conversely, propidium iodide only penetrates damaged bacterial membranes. The presence of both SYTO 9 and PI, as revealed by a decrease in the SYTO 9 fluorescence with respect to intact cells, denotes membrane damage. This method has been reported previously.\textsuperscript{19,20}

A 1:1 (v/v) mixture of the two dyes was used to monitor bacterial populations exposed to SMAP-29 during the killing-curve experiments. Aliquots of bacterial suspensions were taken, using the same peptide concentrations and interval time points as for the killing curves, washed three times with PBS and finally suspended in the same buffer. Final dilutions were performed to obtain an appropriate density for microscopic counts: 5.0 \times 10\textsuperscript{3}–6.5 \times 10\textsuperscript{8} cells/mL, which was estimated to be the ideal concentration range for counting a mean value of 100 cells/ optical field (total magnification: 650\times) (Axioskop, Zeiss, Germany).

Samples were exposed to a standard mixture of the two dyes, as indicated by the manufacturer’s protocol. A total amount of 10 \mu L of each sample was fixed on slides (fluorescence three-well slides, PBI, Milan, Italy) and examined under UV light (Axioskop, Zeiss). For each time point of observation, at least 25 different optical fields were considered, and a mean of 100 bacteria/optical field were counted. Values are expressed as

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Table 1. MIC and MBC values of SMAP-29 for anaerobic reference strains

<table>
<thead>
<tr>
<th>Reference strain</th>
<th>SMAP-29 MIC (mg/L)</th>
<th>SMAP-29 MBC (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. fragilis</em> NCTC 9343</td>
<td>3.2</td>
<td>6.4</td>
</tr>
<tr>
<td><em>B. distasonis</em> NCTC 11152</td>
<td>3.2</td>
<td>6.4</td>
</tr>
<tr>
<td><em>B. ovatus</em> NCTC 11153</td>
<td>6.4</td>
<td>12.8</td>
</tr>
<tr>
<td><em>B. thetaiotaomicron</em> NCTC 10582</td>
<td>6.4</td>
<td>12.8</td>
</tr>
<tr>
<td><em>B. eggerthii</em> NCTC 11155</td>
<td>3.2</td>
<td>4.8</td>
</tr>
<tr>
<td><em>B. vulgatus</em> NCTC 11154</td>
<td>3.2</td>
<td>4.8</td>
</tr>
<tr>
<td><em>C. difficile</em> ATCC 10463</td>
<td>2.4</td>
<td>4.8</td>
</tr>
<tr>
<td><em>C. perfringens</em> ATCC 13124</td>
<td>6.4</td>
<td>12.8</td>
</tr>
</tbody>
</table>

Table 2. Distribution of the MIC values of SMAP-29 towards anaerobic clinical isolates

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>No. of isolates</th>
<th>1.6</th>
<th>3.2</th>
<th>4.8</th>
<th>6.4</th>
<th>9.6</th>
<th>12.8</th>
<th>19.2</th>
<th>50%</th>
<th>90%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram-negative anaerobes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. fragilis</em></td>
<td>10</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. distasonis</em></td>
<td>10</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. ovatus</em></td>
<td>10</td>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>B. thetaiotaomicron</em></td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>2</td>
<td>5</td>
<td></td>
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<td>3</td>
<td>1</td>
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<td></td>
<td></td>
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<tr>
<td><em>B. vulgatus</em></td>
<td>10</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td></td>
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<td>3</td>
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<tr>
<td>Bacteroides mordae</td>
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<td></td>
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<td></td>
<td>1</td>
<td>3</td>
<td>1</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>B. eggerthii</em></td>
<td>5</td>
<td></td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>0</td>
<td>6</td>
<td>13</td>
<td>28</td>
<td>19</td>
<td>4</td>
<td>0</td>
<td>6.4</td>
<td>9.6</td>
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<tr>
<td>Gram-positive anaerobes</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><em>C. difficile</em></td>
<td>10</td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>10</td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
<td>5</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clostridium butyricum</td>
<td>5</td>
<td></td>
<td></td>
<td>1</td>
<td>3</td>
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<td>Clostridium ramosum</td>
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<td></td>
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<tr>
<td>Total</td>
<td>30</td>
<td></td>
<td>2</td>
<td>10</td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>4.8</td>
<td>6.4</td>
<td>6.4</td>
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<tr>
<td>Grand total</td>
<td>100</td>
<td>1</td>
<td>8</td>
<td>23</td>
<td>38</td>
<td>25</td>
<td>4</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

mean percentage of red fluorescent bacteria versus mean percentage value of total amount of fluorescent cells, derived from multiple optical field counts and from at least duplicate experimental data sets.

Scanning electron microscopy

Late log phase cultures of *B. fragilis* NCTC 9343 and *C. perfringens* ATCC 13124 strains were resuspended in 1 × 10⁵ – 5 × 10⁶ cfu/mL in 0.1 M Ca²⁺- and Mg²⁺-free PBS (pH 7.4) and exposed to SMAP-29 at the MIC value. Control samples were run in the same microwell plate, in the presence of PBS only. Samples were incubated in anaerobic jars at 37 °C for 4 h; afterwards, bacterial suspensions were spun briefly at 5200 g at room temperature, washed in PBS and finally resuspended in 0.5 mL of the same buffer. Cells were fixed with an equal volume of 5% glutaraldehyde (Sigma) at 4 °C for 2 h, and filtered through Isopore filters (0.2 µm pore size; Millipore, Bedford, MA, USA). Filters were washed extensively with PBS, dehydrated with absolute ethanol, treated by gold coating and mounted on plates for SEM observation; samples were examined by a Leica Stereoscan (Deerfield, IL, USA) electron scanning microscope.

Results

Susceptibility of anaerobic reference strains to SMAP-29

The *in vitro* activity of the antimicrobial peptide SMAP-29 against selected anaerobic species was evaluated using eight reference strains. These included Gram-negative (*Bacteroides* spp.) and Gram-positive spore-forming species (*Clostridium* spp.). Both the inhibitory (MIC) and the bactericidal (MBC) concentrations were determined. As reported in Table 1, SMAP-29 displayed antimicrobial activity against all the reference strains, with no appreciable differences between Gram-negative and -positive species. The MIC values ranged between 2.4 and 6.4 mg/L (0.75–2.0 µM). The MBCs were 1.5 to 2 × MICs, consistent with a bactericidal mechanism of action for SMAP-29. It is also important to note that the values are similar.
or only slightly higher than those reported for a variety of aerobic bacteria, confirming the potency and broad spectrum of activity of SMAP-29.14

Activity of SMAP-29 against anaerobic clinical isolates

The MICs at which 50% and 90% of 100 clinical isolates were inhibited by SMAP-29 (MIC50, MIC90), and the distribution of MICs for these organisms, are summarized in Table 2. MICs of 3.2–12.8 mg/L (1–4 µM) were determined for all Gram-negative spp. The B. fragilis isolates tested had been screened previously for tetracycline and macrolide resistance genes carried by chromosomal conjugal elements.21 MICs of SMAP-29 against Clostridium spp. were in the range 1.6–9.6 mg/L (0.5–3 µM), except for one C. perfringens strain isolated from a blood culture that required higher peptide concentrations (MIC of 19.2 mg/L) (6 µM) (Table 2).

The peptide concentrations that inhibited the growth of 90% of all the isolates tested were in the range 6.4–9.6 mg/L (2.0 and 3.0 µM). These values are only slightly higher than those obtained for the reference strains shown in Table 1.

Of the 70 B. fragilis group isolates tested, 49 (70%) were found to be amoxicillin resistant, 28 (40%) clindamycin resistant, five (7%) cefoxitin resistant and 52 (74.3%) tetracycline resistant at breakpoint concentrations. The peptide efficiently inhibited both antibiotic-susceptible and -resistant B. fragilis group isolates (Table 3).

Kinetics of the bactericidal activity

Figure 1 shows the killing kinetics for SMAP-29 against three reference strains: B. fragilis NCTC 9343, C. perfringens ATCC 13124 and C. difficile ATCC 10463 (Figure 1). Bacterial viability was determined as cfu counts. In all cases, SMAP-29 was found to inhibit the bacterial growth at 0.5 × MIC, and was bactericidal at higher concentrations (≥ 1 × MIC) in a dose- and time-dependent manner. Particularly, at 2 × MIC, ~corresponding to the MBC for all the species analysed, the peptide caused one and two log decreases of B. fragilis and C. difficile after 2 h incubation, respectively, with >10⁸ organisms/mL killed at 8 h for C. difficile (Figure 1). Slower killing kinetics were observed for C. perfringens ATCC 13124, with a maximum colony decrease of about 3.5 log after 8 h incubation with SMAP-29 at 2 × MIC.

Membrane permeabilization

To investigate the killing mechanism, we next assessed the ability of SMAP-29 to permeabilize bacterial membranes at the MBC, the MIC, and 0.5 × MIC peptide concentrations. The results of this analysis are shown in Figures 2 and 3. The staining of the isolates shifted progressively from green to red after the addition of SMAP-29, indicating an increase in permeability (Figure 2). The number of permeabilized cells increased in a peptide concentration- and time-dependent manner (Figure 3). In particular, a rapid and efficient permeabilization was observed after treating B. fragilis, C. perfringens and C. difficile reference strains with SMAP-29 at peptide concentrations corresponding to the MIC and at twice the MIC (roughly equivalent to MBC), with 80%–100% of the cells permeabilized within the first 2 h of incubation (Figure 3). A relatively slow change in permeability was observed when the same strains were treated with SMAP-29 at 0.5 × MIC (Figure 3). In this case, ~40% of the cells were permeabilized 2 h after the addition of the peptide. The percentage of permeabilized cells steadily increased over time at 0.5 × MIC, apart from C. perfringens. A decrease in the rate of permeabilization was observed when this strain was incubated with the peptide at 8 h.
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compared with 6 h (Figure 3), and was accompanied by a corresponding decrease in the killing activity (Figure 1). This behaviour may be explained by the ability of \textit{C. perfringens} to recover slightly over time in the presence of 0.5 \( \times \) MIC of the peptide (Figure 1). A clear temporal correlation is observed between the killing (Figure 1) and the permeabilization (Figure 3) kinetics, and comparable peptide concentrations are required for both events. These observations thus support a killing mechanism mediated by perturbation of bacterial membranes.\textsuperscript{22}

**Morphological changes induced by SMAP-29 on target bacteria**

To visualize the effects of SMAP-29 on anaerobes, scanning electrographs (at 8000–20 000× magnification) were obtained from the Gram-negative \textit{B. fragilis} NCTC 9343 strain and the Gram-positive spore-forming \textit{C. perfringens} ATCC 13124 strain, after treatment with the peptide (Figure 4). With both strains, control cells incubated in broth in the absence of the peptide exhibited a regular, smooth surface (Figures 4a, c), whereas cells incubated with SMAP-29 at concentrations corresponding to the MIC value revealed severe membrane damage consistent with disruption of the membrane integrity. In fact, \textit{B. fragilis} cells exposed to 1 \( \times \) MIC (6.4 mg/L) of SMAP-29 for 4 h, revealed large globular surface protrusions (Figure 4b), and \textit{C. perfringens} cells exposed to the peptide for the same time length appeared wrinkled, with numerous small clefts regularly distributed on the bacterial cell surface (Figure 4d).

**Discussion**

Several published studies indicate that the \( \alpha \)-helical peptide SMAP-29 kills a variety of aerobic bacteria and several fungal species.\textsuperscript{1,14} However, limited information exists on the effects of this peptide against anaerobic species. In this study, we extended the investigations of the activity of SMAP-29 to \textit{Clostridium} and \textit{Bacteroides} spp. and found that the peptide effectively inhibits the \textit{in vitro} growth of both Gram-negative and Gram-positive spore-forming anaerobic isolates. Interestingly, this molecule appears to be equally active against the \textit{Bacteroides fragilis} group isolates, which are resistant to traditional anti-anaerobe antibiotics. The small differences between MIC and MBC values, and the results of the killing kinetics, indicate that the effects of SMAP-29 are bactericidal, and both a PI-based permeabilization assay and SEM images of SMAP-29-treated bacteria suggest that the bacterial membranes are an important target of this molecule. These results are in accordance with the ability of SMAP-29 to permeabilize the bacterial membranes of aerobic strains, as previously observed using \textit{Escherichia coli} ML35.\textsuperscript{14} Thus, the effects of SMAP-29

![Figure 1. Killing kinetics of bacteria exposed to SMAP-29: (a) \textit{B. fragilis} NCTC 9343; (b) \textit{C. perfringens} ATCC 13124; (c) \textit{C. difficile} ATCC 10463. Strains were grown in the absence of the peptide (solid circles) or in the presence of SMAP-29 at 0.5 \( \times \) MIC (solid triangles); 1 \( \times \) MIC (solid squares); 2 \( \times \) MIC (solid diamonds), respectively. Vertical axis: cfu/mL (log_{10}). Horizontal axis: observation times (h).](image1)

![Figure 2. Two-colour fluorescence assay of bacterial viability (representative images): (a) \textit{B. fragilis} NCTC 9343, (b) \textit{C. perfringens} ATCC 13124. From top to bottom: control cells; samples exposed to the MIC of SMAP-29 for 2 and 6 h, respectively. Bacterial cells with intact membrane appear green-stained, whereas red-stained cells indicate a damaged membrane. Bar = 1 µm.](image2)
on both aerobic and anaerobic bacteria appear to be based similarly on membrane lysis, despite the existence of several differences in the composition of the membrane lipid between aerobic (E. coli) and anaerobic (B. fragilis) Gram-negative bacteria, based on biochemical studies of lipid A.23

It is important to note that prior studies of the in vivo efficacy of SMAP-29 have shown that this peptide is well tolerated and efficient when inoculated in sheep lung in a model of acute pneumonia against the respiratory pathogen Mannheimia haemolytica.24 Furthermore, SMAP-29 has been shown to provide full protection to mice.
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injected intraperitoneally with lethal concentrations of *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* and encapsulated *E. coli*. In this respect, it should be noted that *B. fragilis* group species are primarily involved in intra-abdominal abscess formation and in peritonitis. However, a low therapeutic index has been observed when the peptide was administered intravenously, and further structure–activity relationship studies are required to identify synthetic SMAP-29 analogues with reduced systemic toxicity.

Detailed studies of anti-anerobe potential are only reported for a minority of other antimicrobial peptides. Pexiganan, a 22 amino acid analogue of the magainin peptides isolated from the skin of the African clawed frog, is one of the most extensively investigated. Pexiganan proved to be effective against a variety of Gram-negative and -positive anaerobic species at concentrations of 64 mg/L or less, and was most active (MIC<sub>90</sub> ≤ 8 mg/L) against *Porphyromonas gingivalis*. Cecropin-melittin analogues (CAMEL), containing portions of the amino acid sequences of the two peptides, were found to be active against anaerobic microorganisms, with an MIC<sub>90</sub> of 4 mg/L against *B. fragilis*, fusobacteria, propionibacteria and peptostreptococci. In a structure–activity relationship study, 16 CAMEL analogues exhibited MIC<sub>90</sub> in the range 2–32 mg/L against 60 clinical anaerobic strains. Interestingly, in the study by Oh et al., the activity of the hybrid peptides was compared to the bactericidal activity of commonly used antibiotics, and found to be equal or superior to those of metronidazole, cefotaxin, ciprofloxacin and chloramphenicol, although inferior to those of imipenem, clindamycin and piperacillin. Among the cathelicidin peptides, the porcine protegrin PG-1 was reported to kill within 30–60 min some reference strains of Gram-negative anaerobic periodontal pathogens, such as *Fusobacterium nucleatum*, *Porphyromonas gingivalis* and *Prevotella intermedia*, at concentrations in the range 5–50 mg/L. In a recent study, SMAP-29 has been shown to be active against an *F. nucleatum* reference strain, with an MBC value of 10 mg/L, but not against a *P. gingivalis* reference strain (MBC > 100 mg/L).

Overall, these data support the therapeutic usefulness of SMAP-29 in topical applications, and encourage further investigations aimed to evaluate the suitability of SMAP-29, or of synthetic analogues of this peptide, for application in mixed and anaerobic human infections. The synergistic effects of natural antimicrobial peptides and β-lactams, as shown by Darveau et al., suggest the use of SMAP-29 in association with traditional antibiotic treatments.

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References