Fungicidal activity of five cathelicidin peptides against clinically isolated yeasts

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Objectives: To investigate the in vitro antifungal activity of the structurally different cathelicidin peptides SMAP-29, BMAP-27, BMAP-28, protegrin-1 (PG-1) and indolicidin.

Methods: The in vitro antifungal and fungicidal activities of these antimicrobial peptides were respectively assessed via MIC determinations and killing kinetics assays. The effects of the peptides on membrane permeabilization and morphology were evaluated by flow cytometry, intracellular ATP release measurements and scanning electron microscopy.

Results: All five peptides showed a potent but differential antifungal activity against more than 70 clinical isolates belonging to over 20 different species of pathogenic fungi; some of which are resistant to amphotericin B and azoles. The MIC values of the peptides ranged between 0.5 and 32 μM, with PG-1 being the most effective and having the widest spectrum of activity. Filamentous fungi were instead found to be scarcely susceptible to the action of these cathelicidin peptides. All these cathelicidins rapidly killed Candida albicans and Cryptococcus neoformans cells in a dose- and time-dependent manner. The rapid uptake of propidium iodide into treated cells and morphological alterations apparent on their cellular surfaces suggest a killing mechanism based on membrane permeabilization and damage.

Conclusions: This study indicates that these five structurally varied host defence peptides are all endowed with the capacity to inactivate a number of fungal pathogens, irrespectively of their resistance to antifungal drugs, and suggests they might be potentially useful leads for the development of novel fungicidal agents.

Keywords: antifungals, host defence, cathelicidin, clinical isolates, pathogenic fungi, innate immunity, immunocompromised patients

Introduction

The incidence of fungal infections has increased significantly in the past decade due to the increase in immunocompromised subjects, including HIV/AIDS patients, oncology patients with chemotherapy-induced neutropenia and transplant recipients who are receiving immunosuppressive therapy.1,2 This phenomenon is aggravated by the rapid development of drug resistance against most of the currently used antifungal drugs, such as fluconazole, itraconazole and amphotericin B, which currently poses a major hurdle in antifungal therapy.3 The toxicity of some of the available antifungal agents, the intrinsic resistance to antifungal therapy observed in some genera and the development of resistance during treatment in others are becoming a major problem in the management of current antifungal therapies.3,4 The development of new antifungal agents with different mechanisms of action and acceptable toxicity is thus urgently needed.

In the past few years, a number of host defence peptides (HDPs) with antimicrobial activities have been isolated from a variety of natural sources. In mammals, a prominent role is played...
by peptides from three distinct gene families: the cysteine-rich α- and β-defensins⁵ and the cathelicids.⁶

The latter group includes a number of cationic and amphipathic peptides that display a variety of host defence functions with both a direct action against microbial cells and indirect actions that essentially modulate immune responses.⁶,⁷ All members of the cathelicidin family are distinguished by the presence of an N-terminal cathelin-like domain in their precursor, which explains the name, and a C-terminal region corresponding to the antimicrobially active peptides. These peptides show a remarkable diversity in amino acid sequence, size, charge and structure, including α-helical, elongated or β-hairpin conformations (Table 1). The number of different cathelicidins present in any organism varies between species, with a single congener found in (Table 1). The number of different cathelicidins present in any in vitro residues isolated from sheep and cow respectively. Their anti-bacterial activity has been well documented both in vitro and in vivo⁸–¹¹ and some data have been collected on their antifungal potential.¹²,¹³ These peptides are unstructured in solution, but can assume an amphipathic helical structure, covering part or all of the sequences, on contact with biological membranes, in which hydrophobic residues are relegated to one surface and insert into the lipid bilayer, while polar and cationic residues remain in contact with lipid head-groups. The mechanism of action of this type of peptide has been extensively studied and indeed has provided the paradigm for the mode of action of antimicrobial peptides (AMPs) in general.¹⁴,¹⁵ It involves the formation of transient pores or lesions in biological membranes as a principal step in their microbicidal activity.

Indolicidin from cattle is a 13 residue Trp-rich peptide that assumes an extended, wedge-type conformation in the presence of biological membranes, with hydrophobic Trp residues in the wedge’s trough, flanked by positively charged regions.¹⁵ This peptide exerts a potent antibacterial activity and it has also been shown to display activity against the yeast Trichosporon beigeli, but it has also been reported to be cytotoxic towards host cells.¹⁶ It is thought to translocate across microbial membranes into the cytoplasm at concentrations close to the MIC value, and interact with internal targets, while it is membranolytic at higher concentrations.¹⁵

Pig protegrins, and in particular protegrin-1 (PG-1) and its variants, are possibly the most tested cathelicidins, having a rather rigid antiparallel β-sheet (β-hairpin) structure that is stabilized by two intramolecular disulphide bonds.¹⁷ This slightly convex hairpin clusters hydrophobic residues at the centre, which are flanked by positively charged regions on each side. Oligomerization on membrane insertion may play an important role in the mechanism of action.¹⁸ Protegrins exhibit potent and broad-spectrum activity against bacteria and some fungi,¹⁹,²⁰ and a synthetic derivative has been considered for the treatment of oral mucositis.²¹

All these different cathelicidins thus exert their antimicrobial activity by virtue of their cationic, amphipathic structures, although this consists of a flexible, winged arrangement of hydrophobic and charged residues in indolicidin, a rigid winged structure in the case of protegrin, and a rod-like longitudinally amphipathic structure in the case of SMAP-29 and the BMAPs. The mode of action somehow involves interaction with the microbial membrane and its subsequent permeabilization and/or disruption, although the mechanisms of interaction and modification are likely quite different.

The in vitro efficiency of cathelicidins as antibacterial agents has been extensively and systematically documented, also in view of commercial exploitation, and there is mounting in vivo evidence for such a role in innate defence.²²,²³ In contrast, the antifungal activity of these peptides, apart from protegrins, is less well defined, and has not yet been tested comparatively, although there is sufficient evidence that they should also be effective in this respect.

In this study we have compared the in vitro activity of BMAP-27, BMAP-28, SMAP-29, indolicidin and PG-1 against a number of nosocomial yeast strains, mainly Candida spp. and Cryptococcus neoformans strains under standardized conditions, and we have also investigated their mode of action. For all these peptides, killing is correlated to disruption of the cellular membrane, which may be a common essential step in their mechanism of action, making them effective also against drug-resistant strains.

### Materials and methods

**Peptide synthesis and purification**

SMAP-29, BMAP-27, BMAP-28, PG-1 and indolicidin were synthesized using the solid phase fluorenylmethoxy carbonyl chemistry, as reported previously.¹² Protected amino acids and PEG-PS resins were purchased from Advanced Biotech Italia (Milan, Italy) or Novabiotech (Merck, Darmstadt, Germany). All peptides were synthesized using a Pioneer™ Automated Synthesis System and purified by reversed-phase HPLC (AKTA Basic, Amersham Biotech, Uppsala, Sweden) on an X-Terra C18 column (19 × 300 mm; Waters, MA, USA) using appropriate acetonitrile-water 0–60% linear gradients in 0.05% trifluoroacetic acid. Disulphide bridges in PG-1 were formed by air-oxidation at a concentration of 0.1 mg/mL under gentle stirring.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Origin</th>
<th>Type</th>
<th>Sequence</th>
<th>Mol wt</th>
<th>q</th>
<th>[H]</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAP-27</td>
<td>cow</td>
<td>α-helical</td>
<td>GRFKRFRKKFKKLSPVPIPLHLL-am</td>
<td>3225.1</td>
<td>11</td>
<td>–0.4</td>
</tr>
<tr>
<td>BMAP-28</td>
<td>cow</td>
<td>α-helical</td>
<td>GGLRSLGRKLRAWKYGYPIPIRI-am</td>
<td>3073.9</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>SMAP-29</td>
<td>sheep</td>
<td>α-helical</td>
<td>RGLRLRGIHGVKGYPTVLRIRI-am</td>
<td>3198.0</td>
<td>10</td>
<td>–1.3</td>
</tr>
<tr>
<td>PG-1</td>
<td>pig</td>
<td>β-hairpin</td>
<td>RGLRLCYCRFFCVCYGR</td>
<td>2155.6</td>
<td>6</td>
<td>–2.0</td>
</tr>
<tr>
<td>Indolicidin</td>
<td>pig</td>
<td>linear</td>
<td>ILPWKWPWWPWR-am</td>
<td>1906.3</td>
<td>4</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*Charge (His considered neutral).  
bMean residue hydrophobicity, measured as described previously.²⁹
in 0.1 M Tris–HCl buffer, pH 7.5, for 24–36 h at room temperature. The molecular masses of all peptides were determined by an API-1 electrospay ionization mass spectrometer (PE SCIEX, Toronto, Canada). The purified peptides were lyophilized in 10 mM HCl, dissolved in double distilled water and stored at –20°C until use.

**Fungal strains and growth conditions**

Over 70 clinical isolates including Candida spp., C. neoformans, Rhodotorula rubra, Pichia etchellsii and Pichia carsonii, Saccharomyces cerevisiae, Kloeckera apis, Aspergillus spp. and Penicilium spp. were collected from immunocompromised patients. The reference strains were C. neoformans, two capsulated strains ATCC 90112, ATCC 90113 and two acapsular mutants ATCC 52816, ATCC 52817, Candida albicans ATCC 90028 and ATCC 90029, Candida parapsilosis ATCC 90018, Candida glabrata ATCC 90030 and Aspergillus niger ATCC 16888.

Fungi were grown on Sabouraud agar plates at 30°C for 48 h. Inoculum suspensions were prepared by picking and suspending five colonies in 5 mL of sterile PBS. Filamentous fungi were grown on Sabouraud agar slants at 30°C for 7 days. The fungal colonies were then covered with 3 mL of PBS and gently scraped with a sterile pipette. The resulting suspensions were transferred to sterile tubes, and heavy particles were allowed to settle. The turbidity of the conidial spore suspensions was measured at 600 nm and was adjusted to obtain an appropriate inoculum.

**Antimicrobial assays**

The antifungal activity was evaluated by the broth microdilution susceptibility test following the guidelines of the National Committee for Clinical Laboratory Standards, to determine the MIC values.

Two-fold serial dilutions of each peptide were prepared in 96-well polystyrene microtitre plates (UNIFO, Treviso, Italy) in RPMI-1640 medium (Sigma-Aldrich) to a final volume of 50 μL. Each series included a well without peptide as a control. A total of 50 μL of the adjusted inoculum diluted in RPMI-1640 medium was added to each well to a final concentration of ~5 × 10⁴ cells/mL. Samples were then incubated at 30°C for 48 h. The MIC was taken as the lowest concentration of antimicrobial peptide resulting in the complete inhibition of visible growth after 48 h of incubation. Data are the mean of 4–6 independent determinations with values differing by one dilution at maximum.

Susceptibility of selected strains to amphotericin B, fluconazole and itraconazole was determined using the Etest (AB Biodisk, Solna, Sweden) according to the manufacturer’s instructions. Briefly, the inoculum suspension was adjusted to a turbidity equivalent to that of a 0.5 McFarland density standard and distributed over the entire surface of an RPMI-1640 agar plate. Inoculated plates were allowed to dry before Etest strips were applied to the medium. The plates were incubated at 35°C and results were read after 24 and 48 h. Interpretive breakpoints for fluconazole: susceptible, ≤ 8 mg/L; susceptible-dose dependent, 16–32 mg/L and resistant, ≥ 64 mg/L. Interpretive breakpoints for itraconazole: susceptible, ≤ 0.125 mg/L; susceptible-dose dependent, 0.25–0.5 mg/L and resistant, ≥ 1 mg/L. Amphotericin B susceptible, ≤ 1 mg/L.

**Killing kinetics assays**

Killing kinetics were determined using cultures of C. albicans and C. neoformans diluted in fresh RPMI-1640 medium to give ~5 × 10⁵ cells/mL. Peptides were added at different concentrations and the suspensions were then incubated in a shaking water bath at 30°C. At the indicated times, samples were removed, serially diluted with buffered saline solution (10 mM Na-phosphate, 145 mM NaCl, pH 7.4), plated in duplicate on Sabouraud agar and incubated for 36–48 h to allow colony counts. Data are the mean of at least four independent determinations with comparable results.

**Flow cytometric assay**

Flow cytometric assays were based on detection of increased permeability of fungal cells to propidium iodide (PI), a membrane impermeant DNA-intercalating dye, following treatment with cathelicidin peptides. Analyses were performed with a Cytoscan FC 500 (Beckman-Coulter, Inc., Fullerton, CA, USA) equipped with an argon-cooled argon laser (488 nm, 5 mW) and standard system configuration for orange-filtered light detection (620 nm). For these analyses, C. albicans or C. neoformans subcultured on Sabouraud agar were diluted in RPMI-1640 medium to give ~1 × 10⁵ cells/mL, and aliquots of the fungal suspension were then incubated with the peptides at 30°C for different times. A 0.2 μm-filtered solution of PI (Sigma-Aldrich) was then added to the peptide-treated fungi at a final concentration of 1 mg/L, and samples were acquired after 4 min of incubation at 30°C. To obtain a positive control for permeabilization, fungal suspensions were pelleted and resuspended in cold absolute ethanol for 30 min at –20°C. Ethanol was removed by aspiration following centrifugation at 1000 g for 10 min, and the pellet resuspended in RPMI-1640 medium. All experiments were conducted in triplicate. Data analysis was performed with the WinMDI software (Dr J. Trotter, Scripps Research Institute, La Jolla, CA, USA). Data are expressed as means ± SD.

**ATP release by peptide-treated fungi**

The amount of ATP released by peptide-treated fungi was determined by the ATP Determination Kit (Invitrogen) based on the luciferin/luciferase method.

The percentage of released ATP was estimated by using the equation [E–e/I] × 100, where E is the extracellular ATP content after treatment with the peptides, e is the extracellular ATP in untreated cells and I is the total ATP content of lysed cells (1 × 10⁵ cells/mL). Values were obtained from three independent experiments having comparable results.

**Scanning electron microscopy (SEM)**

Approximately 1 × 10⁵ cells/mL of C. neoformans and C. albicans were incubated at 30°C in RPMI-1640 for 60 or 120 min with different concentrations of SMAP-29 or PG-1. After incubation, cells were fixed on glass polylysine coverslips with an equal volume of 4% (v/v) glutaraldehyde in 0.2 M phosphate buffer, pH 7.4. After overnight fixation at 4°C, the samples were extensively washed with 0.2 M phosphate buffer, pH 7.0, then with doubly distilled water and subsequently dehydrated with a graded ethanol series. After lyophilization and gold coating, the samples were examined on a Leica Stereoscan 430i instrument (Leica Inc., Deerfield, IL, USA).

**Results and discussion**

**Antifungal activity of different cathelicidin peptides**

The antifungal activities of BMAP-27, BMAP-28, SMAP-29, PG-1 and indolicidin were evaluated towards 24 strains of C. albicans (22 clinical isolates and 2 ATCC reference strains) and 17 strains of C. neoformans (13 clinical isolates and 4 reference strains). Several of these strains were also assayed for resistance to commonly used antifungal drugs such as...
In vitro antifungal activity of cathelicidin peptides

amphotericin B, itraconazole and fluconazole. The distribution of the MIC values (Figure 1) indicates that all peptides displayed a potent activity against all \textit{C. neoformans} strains, with MIC values ranging from 0.25 to 4 \( \mu \)M. In particular, the bovine \( \alpha \)-helical BMAP-27 and BMAP-28 showed the highest anticryptococcal activity with MIC\(_{90}\) values of 1 and 2 \( \mu \)M respectively. No difference in susceptibility to the peptides was observed for the two acapsular strains of \textit{Cryptococcus} compared to the other capsulated strains (Figure 1). This aspect is interesting as the polysaccharide capsule of \textit{C. neoformans} has been associated with pathogenicity\(^{26}\) and was found to inhibit leucocyte phagocytosis.\(^{27}\)

All peptides were also active, at micromolar concentrations, against most \textit{C. albicans} strains (Figure 1), although their potency varied. Porcine PG-1 showed generally the most potent activity in this case, with MIC\(_{90}\) value of 4 \( \mu \)M, but the other four peptides also exhibited a good candidicidal activity (MIC values of 8–16 \( \mu \)M), with few exceptions. No difference in susceptibility

\[ \text{Figure 1. Distribution of the MIC values of the cathelicidin peptides against 24 strains of } \textit{C. albicans} \text{ (left-hand panel) and 17 strains of } \textit{C. neoformans} \text{ (right-hand panel). Bars representing the MIC values for the } \textit{C. neoformans} \text{ acapsular strains ATCC 52816 and ATCC 52817 are marked by the } ^* \text{ and } ^\# \text{ symbols respectively. Bars representing the MIC values of } \textit{C. albicans} \text{ L21 (resistant to amphotericin B, MIC = 32 mg/L) are marked by the } ^\$ \text{ symbol.} \]
was observed with the L21 isolate of *C. albicans*, which is resistant to amphotericin B (MIC = 32 mg/L) (Figure 1).

Antifungal susceptibility testing was then extended to nine other species of *Candida* (including two multi-resistant isolates of *Candida famata*), to a panel of *Pichia*, *Rhodotorula*, *Kloeckera* and *Saccharomyces* strains, and a small panel of filamentous fungi including *Aspergillus* spp. and *Penicillium* spp. The MIC values are listed in Table 2, which include selected *C. albicans* and *C. neoformans* strains for comparison. Again, all the cathelicidin peptides were active against most of the yeast strains assayed, irrespective of resistance to other antimycotics, with MIC values ranging between 0.5 and 32 μM, while a generally lower activity was observed against the filamentous fungi species.

Porcine PG-1 globally displayed the most potent and broad-spectrum activity, showing MIC values of ≤8 μM towards most of the tested microbes, with the exception of two isolates of *Aspergillus* (MIC > 32 μM) (Table 2). The three α-helical peptides were even active in this respect. This is in line with previous observation of a lower efficacy for model helical antimicrobial peptides towards *A. niger* with respect to yeasts, which can however be improved with appropriate redesign.28,29 Interestingly, the two drug-resistant strains of *C. famata* did not show cross resistance to the AMPs (Table 2), suggesting that for this yeast the antifungal activity of cathelicidin peptides is likely directed against different targets compared with amphotericin B and azoles.

**Table 2. In vitro antifungal activity of the cathelicidin peptides towards collection and clinical isolates of filamentous and non-filamentous fungi**

<table>
<thead>
<tr>
<th>Species (no. of isolates)</th>
<th>BMAP-27</th>
<th>BMAP-28</th>
<th>SMAP-29</th>
<th>PG-1</th>
<th>indolicidin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. neoformans</em> ATCC 52816</td>
<td>0.25</td>
<td>0.5</td>
<td>0.5</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td><em>C. neoformans</em> ATCC 52817</td>
<td>1</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td><em>C. neoformans</em> L1</td>
<td>0.5</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td><em>C. albicans</em> C2</td>
<td>32–16</td>
<td>8</td>
<td>2</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td><em>C. albicans</em> L21</td>
<td>8</td>
<td>4</td>
<td>8</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td><em>C. famata</em> M100b</td>
<td>4–8</td>
<td>2–4</td>
<td>2</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><em>C. famata</em> SA550</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>16</td>
</tr>
<tr>
<td><em>C. parapsilosis</em> (4)</td>
<td>16–32</td>
<td>8</td>
<td>1–4</td>
<td>1–4</td>
<td>32–32</td>
</tr>
<tr>
<td><em>C. glabrata</em> (6)</td>
<td>32–32</td>
<td>8–16</td>
<td>16–32</td>
<td>2–8</td>
<td>32–32</td>
</tr>
<tr>
<td><em>Candida tropicalis</em> (2)</td>
<td>4–16</td>
<td>2–16</td>
<td>4–32</td>
<td>2–8</td>
<td>32–32</td>
</tr>
<tr>
<td><em>Candida guillermondii</em> (1)</td>
<td>4</td>
<td>2–8</td>
<td>0.5</td>
<td>2</td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>C. krasei</em> (2)</td>
<td>4</td>
<td>2–8</td>
<td>2–4</td>
<td>1–2</td>
<td>8</td>
</tr>
<tr>
<td><em>Candida humicola</em> (1)</td>
<td>8</td>
<td>8</td>
<td>16</td>
<td>2</td>
<td>32</td>
</tr>
<tr>
<td><em>Candida lusitaniae</em> (3)</td>
<td>16</td>
<td>4</td>
<td>4</td>
<td>2</td>
<td>8</td>
</tr>
<tr>
<td><em>Candida dubliniensis</em> (1)</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td><em>P. etchellsii</em> (2)</td>
<td>&gt;32</td>
<td>16–32</td>
<td>1–2</td>
<td>8</td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>P. carsonii</em> (1)</td>
<td>2</td>
<td>2</td>
<td>0.5</td>
<td>1</td>
<td>8</td>
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<tr>
<td><em>R. rubra</em> (3)</td>
<td>0.5–4</td>
<td>1–2</td>
<td>0.5–2</td>
<td>1–2</td>
<td>2–8</td>
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<tr>
<td><em>S. cerevisiae</em> (2)</td>
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<td>4–8</td>
<td>8–16</td>
<td>2–4</td>
<td>8–16</td>
</tr>
<tr>
<td><em>K. apis</em> (2)</td>
<td>16–32</td>
<td>4</td>
<td>4–8</td>
<td>2–4</td>
<td>8–32</td>
</tr>
<tr>
<td><em>Aspergillus</em> spp. (6)</td>
<td>&gt;32</td>
<td>8–32</td>
<td>&gt;32</td>
<td>8–32</td>
<td>&gt;32</td>
</tr>
<tr>
<td><em>Penicillium</em> spp. (2)</td>
<td>32–32</td>
<td>4–16</td>
<td>4–32</td>
<td>2–4</td>
<td>8–32</td>
</tr>
</tbody>
</table>

The MIC values were determined in RPMI-1640 medium according to the guidelines of the NCCLS.

*C. famata* M100 was resistant to amphotericin B (MIC = 12 mg/L) and to itraconazole (MIC = 6 mg/L) 

*C. famata* SA550 was resistant to amphotericin B (MIC = 32 mg/L) and susceptible-dose dependent to itraconazole and fluconazole (MIC = 0.5 and 16 mg/L respectively).

MICs >32 μM were observed with *A. niger* ATCC 16888 and *A. flavus* 495G.

**Killing kinetics of cathelicidin peptides**

The antifungal activity of BMAP-27, BMAP-28, SMAP-29 and PG-1 was further investigated by analysing their killing kinetics against two representative isolates: *C. neoformans* L1 and *C. albicans* C2. Results indicated that all the cathelicidins displayed a rapid, concentration-dependent, fungicidal action towards both yeast species. In particular, at a concentration equal or slightly higher than the MIC, they caused at least a 99% viability reduction of *C. neoformans* cells within 10–20 min of incubation (Figure 2), while the killing of *C. albicans* cells appeared somewhat less rapid (Figure 3). The antifungal efficiency of the peptides showed some differences: porcine PG-1 and ovine SMAP-29 caused a total killing of the fungal cells at 5–10 μM, while bovine BMAP-27 and BMAP-28 at comparable concentrations considerably reduced the number of viable cells without a complete killing of the fungi (Figures 2 and 3).
drugs. For example amphotericin B, when tested against several species of Candida, reached the fungicidal endpoint (99.9% killing) only at four times its MIC value and after 2–24 h incubation, depending on the strain assayed, while fluconazole and itraconazole only have a fungistatic activity against clinical isolates of C. neoformans.

Effects of the peptides on the membrane permeability and cell morphology

To investigate the mechanism by which cathelicidin peptides kill C. neoformans and C. albicans, their effect on membrane integrity was evaluated by flow cytometry. Non-viable, permeabilized cells were marked using the fluorescent probe PI that is incorporated and stains the nucleic acids only in cells with damaged membranes. Treatment of C. albicans with each of the peptides at 5 µM concentration caused the appearance of PI-permeable yeast cells after only 10 min of incubation, consistent with the killing kinetics. The percentage of PI-positive cells increased up to 100 min of incubation and PG-1 was the most effective peptide with 95% of PI-positive cells at this time point (Figure 4). An even more rapid and marked effect was observed when C. neoformans was incubated with each peptide at 1 µM. In this case, after 60 min of treatment, most of the cells became permeabilized (Figure 4). By comparison, within this time period, 10 µM amphotericin B caused significantly less permeabilization of either yeast strain (7% PI-positive cells after 100 min), in accordance with its slower kinetics of antifungal action. Altogether these results indicate that cathelicidin peptides, despite having their different structural features, affect yeast cells by injuring their membranes, and thus increasing permeability. This observation was also confirmed by measuring ATP released from peptide-treated cells. Incubation of C. neoformans or C. albicans for 10 min with 5 µM SMAP-29 or 5 µM PG-1 caused a 95% decrease in intracellular ATP content (Figure 5). Such a rapid action on cell viability is usually associated with a cell membrane permeabilization effect, as shown for other antimicrobial peptides.

The morphology of the peptide-treated yeast cells was also examined by SEM. C. neoformans cells, after 60 min of incubation with SMAP-29, assumed a smoother aspect with the disappearance of the polysaccharide capsule (Figure 6). Similar morphological alterations have been reported with C. neoformans treated with fluconazole and amphotericin B. The antifungal effects of these two antymycotic drugs are considered to be respectively mediated by binding to cell membrane sterols and by alteration of sterol synthesis. These effects compromise the membrane and its barrier functions. Treatment of C. albicans cells with PG-1 for 1 h instead produced remarkable effects on the cellular surface, with extensive roughening and blebbing (Figure 6).

Overall, these observations are consistent with a mechanism in which the cell membrane is a principal, and possibly the most important target for these peptides, although the diverse types

Figure 2. Killing kinetics of the cathelicidin peptides against C. neoformans L1. Fungal cells were incubated with the indicated concentrations of the peptides in RPMI-1640 medium. At the indicated times, survivors were diluted in buffered saline and plated to allow colony counts. Filled circles indicate control runs in the absence of peptides. Results are mean values of at least four independent determinations.
of morphological alteration may indicate different consequences of membrane damage. Furthermore, the nature and effects of the peptide interaction with the fungal membrane must be different to those of amphotericin B and fluconazole, as they are active against strains resistant to these drugs (see Figure 1 and Table 2). This aspect is further confirmed by the susceptibility of Candida krusei to the peptides’ action (Table 2), whereas this species is known for its intrinsic resistance to fluconazole.36
In vitro antifungal activity of cathelicidin peptides

For most AMPs, antimicrobial potency tends to correlate somewhat with increased cytotoxicity, and the cathelicidins are no exception. This is probably due to the membranolytic nature of their antimicrobial action, although they do show a certain selectivity for bacterial cells, due to their stronger electrostatic interaction with their anionic envelope components, so that lethal concentrations are generally significantly lower for bacteria than host cells. For a qualitative comparison, the cytotoxic concentrations against nucleated mammalian cells derived from a number of studies are reported in Table 3. In this respect, one could expect these AMPs to be active towards yeast cells, which are eukaryotic, at higher concentrations than for bacteria, with a consequent increased cytotoxicity towards host cells. In fact, we report MIC values against most yeast cells which are of the same order as the MIC values reported against bacteria, albeit under somewhat different assay conditions, and these are significantly lower than those reported to cause lysis of erythrocytes. It is as yet unclear why yeast cells are more susceptible than host cells to the action of these peptides, but it may relate to the quite different cell envelope composition.

In conclusion, this study reports the comparative activities of five different cathelicidin peptides against clinically relevant yeasts and fungi, some of which were also tested with the antifungal drugs amphotericin B and fluconazole. Despite belonging to three distinct structural classes (linear, α-helical and β-hairpin), all of the cathelicidin peptides showed a potent and rapid, concentration-dependent, antifungal activity, with a wide spectrum of activity even if this was not completely imposable. The small, β-hairpin protegrin was overall the most active towards Candida species, while the helical BMAP and SMAP peptides were the most active towards Rhodotorula, Pichia and Cryptococcus. A common feature of the antifungal activity of all the peptides is that it correlates with the capacity to rapidly permeabilize the cell envelope. This mechanism seems to differ from those of the clinically used antifungal drugs, and thus results in a lack of cross resistance.

Our in vitro results suggest that this family of innate immune peptides may play a role in antifungal host defence. Mammals such as the artiodactyles, from which the studied peptides derive, simultaneously produce several different cathelicidin peptides, belonging to all three structural classes. Some antifungal protection, especially at epithelial and mucosal surfaces, has been reported for the single human and mouse cathelicidins, which belong to the helical group.37

Our results also suggest that cathelicidin AMPs may have potential for development as novel antifungal agents. In favour of this are the rapid action and broad spectrum, the fact that they are active against species that are multiple resistant to currently used antymycotics, and that they are amenable to production by both chemical and recombinant methods.38,39 The fact that cathelicidin AMPs can also display signalling, chemotactic and other activities that modulate both defensive and healing processes may provide

![Figure 6. Scanning electron microscopy of 10^7 cfu/mL yeast cells treated with peptides. C. neoformans L1 untreated (a) and treated for 1 h with 20 μM SMAP-29 (b). C. albicans C2 untreated (c) and treated for 1 h with 40 μM PG-1 (d).](image)

Table 3. Cytotoxic activities of cathelicidin peptides against mammalian nucleated cells

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Cell lines</th>
<th>Assay</th>
<th>Conditions</th>
<th>Effects</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMAP-27, BMAP-28</td>
<td>neutrophils</td>
<td>PI uptake</td>
<td>1–6 μM, 30 min</td>
<td>PI+ &lt; 10%</td>
<td>13</td>
</tr>
<tr>
<td>BMAP-27, BMAP-28</td>
<td>neutrophils</td>
<td>PI uptake</td>
<td>30 μM, 30 min</td>
<td>PI+ = 94%</td>
<td>13</td>
</tr>
<tr>
<td>BMAP-27, BMAP-28</td>
<td>resting lymphocytes</td>
<td>PI uptake</td>
<td>6 μM, 30 min</td>
<td>PI+ &lt; 10%</td>
<td>41</td>
</tr>
<tr>
<td>BMAP-27, BMAP-28</td>
<td>myeloid tumour cells</td>
<td>PI uptake</td>
<td>6 μM, 30 min</td>
<td>PI+ = 60–90%</td>
<td>41</td>
</tr>
<tr>
<td>BMAP-27, BMAP-28</td>
<td>lymphoid tumour cells</td>
<td>PI uptake</td>
<td>6 μM, 30 min</td>
<td>PI+ = 40%</td>
<td>41</td>
</tr>
<tr>
<td>SMAP-29</td>
<td>epithelial cells</td>
<td>dye uptake</td>
<td>2 μM, 24 h</td>
<td>10% cell death (EC50 = 7–8 μM)</td>
<td>42</td>
</tr>
<tr>
<td>Indolicidin</td>
<td>monocytes, epithelial cells</td>
<td>MTT assay</td>
<td>10–300 μg/mL, 24 h</td>
<td>&lt;100 μg/mL (52 μM) not cytotoxic</td>
<td>43</td>
</tr>
<tr>
<td>Protegrin-1</td>
<td>epithelial cells</td>
<td>MTT assay</td>
<td>1–75 μg/mL, 20 h</td>
<td>EC50 = 22.4 μg/mL (10.3 μM)</td>
<td>44</td>
</tr>
</tbody>
</table>

# aCell lines: U937, HL60, K562.
# bCell lines: Jurkat, CEM-CCR, CEM-VLB.
# cCell lines: MDCK.
# dCell lines: THP-1 (monocytes), HBE (epithelial cells).
# eCell lines: HTB-33, CCL-185.
# fFlow cytometric determination of PI (propidium iodide).
# gFluorescence determination of Alamar Blue (Biosource).
# hAbsorbance determination after MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction.
an added value to their development as multifunctional anti-
infective agents. This process will however present some
difficulties. Firstly, due to the membranolytic nature of their
antimicrobial activity, these peptides can be quite toxic to host
cells. Secondly, their activity can be decreased by serum
components, and this use presents the problems faced by
peptides in general as systemic agents. For this reason, the most
likely uses are as topical agents. In this respect, a first attempt to
peptides in general as systemic agents. For this reason, the most
components, and their use presents the problems faced by
undergoing chemotherapy, was not successful. We feel that the
a debilitating polymicrobial condition, characterized by the pre-
sence of numerous bacterial and fungal species, affecting patients
undergoing chemotherapy, was not successful. We feel that the
lessons learned from these pioneering studies, combined with the
application of methods for reducing toxicity, will considerably
improve chances of successful applications in the future,
especially in view of the worsening fungal resistance problem.

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Transparency declarations

We declare that in the period of research leading to this manu-
script, we have not accepted fees or grants from any organization
or have had connections with any company (including con-
sultancy contracts, ownership of stocks, etc.), which could affect
the conclusions therein.

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