RESEARCH LETTER

Gomesin, a peptide produced by the spider *Acanthoscurria gomesiana*, is a potent anticryptococcal agent that acts in synergism with fluconazole

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gomesin; *Cryptococcus neoformans*; antimicrobial peptide.

Abstract

Gomesin is an 18-residue cysteine-rich antimicrobial peptide produced by hemocytes of the spider *Acanthoscurria gomesiana*. In the present study, the antifungal properties of gomesin against *Cryptococcus neoformans*, the etiologic agent of cryptococcosis, were evaluated. Gomesin bound to the cell surface of cryptococci, which resulted in cell death associated with membrane permeabilization. Antifungal concentrations of gomesin were not toxic for human brain cells. Supplementation of cryptococcal cultures with the peptide (1 μM) caused a decrease in capsule expression and rendered fungal cells more susceptible to killing by human brain phagocytes. The possible use of gomesin in combination with fluconazole, a standard antifungal drug, was also evaluated. In association with fluconazole, gomesin concentrations with low antimicrobial activity (0.1–1 μM) inhibited fungal growth and enhanced the antimicrobial activity of brain phagocytes. These results reveal the potential of gomesin to promote inhibition of cryptococcal growth directly or by enhancing the effectiveness of host defenses.

Introduction

Cryptococcus neoformans is an encapsulated yeast-like pathogen that causes meningoencephalitis in immunosuppressed individuals. Cryptococcal meningitis is one of the most common fungal infections developed by HIV-1 infected patients and is the third most frequent neurological complication in patients with AIDS (Del Valle & Piña-Oviedo, 2006). Options for the treatment of cryptococcal meningitis include amphotericin B during the first weeks, followed by fluconazole treatment until immune reconstitution (Bicanic & Harrison, 2004). The use of amphotericin B and fluconazole, however, can be accompanied by several undesired side effects, including toxicity for the host and fungal drug resistance (Dan & Levitz, 2006). In this context, it is clear that new alternatives for the treatment of cryptococcosis are required.

Antimicrobial peptides (AMPs) represent an abundant and diverse group of molecules that are produced by many tissues and cell types in a variety of microbial, plant and animal species (Brogden, 2005; Yount *et al.*, 2006). Their general properties such as amphipathicity and cationicity allow them to form pores in biological membranes, resulting in microbial death. AMPs also exert their antimicrobial activity by inhibiting cell-wall assembly, nucleic-acid and protein synthesis (Brogden, 2005), indicating that specific target molecules can be involved in these activities. Besides the direct action on microorganisms, AMPs can act as immune modulators (Brown & Hancock, 2006; Yount *et al.*, 2006).

Gomesin is a small-sized AMP produced and stored in hemocytes of the mygalomorph spider *Acanthoscurria gomesiana* (Silva *et al.*, 2000; Lorenzini *et al.*, 2003). The peptide, which contains 18 amino acids and two disulfide

bridges, adopts a β-hairpin-like structure, as determined by 2-D nuclear magnetic resonance and molecular dynamics studies (Mandard *et al.*, 2002). Gomesin presents potent antimicrobial activity against Gram-positive and -negative bacteria, protozoa and fungi (Silva *et al.*, 2000, Moreira *et al.* 2007). The putative mechanism involved in the antimicrobial action of gomesin includes membrane permeabilization (Mandard *et al.*, 2002, Fazio *et al*, 2006b), although the involvement of other targets had not been investigated.

The anticryptococcal effects of gomesin were evaluated in the present work. It is demonstrated that gomesin is a potent anticryptococcal agent that, alone or in combination with fluconazole, can interfere with *C. neoformans* growth and interaction with host cells.

Materials and methods

Microorganisms

Cryptococcus neoformans strains T_1-444 and HEC3393 (serotype A, clinical isolates) are, respectively, largely and poorly encapsulated isolates, as previously characterized (Rodrigues *et al.*, 1997; Barbosa *et al.*, 2006). Strain Cap67 is a well-characterized acapsular mutant of *C. neoformans* extensively used in previous studies. *Cryptococcus neoformans* was cultivated with shaking for 48 h, at 25 °C, in a chemically defined medium (Rosas *et al.*, 2001). Cell suspensions were quantified by counting the number of yeasts in a Neubauer chamber.

Gomesin synthesis

Gomesin was manually synthesized on 4-methylbenzhydrylamine resin using the *t*-Boc strategy as described previously (Fazio *et al.*, 2006a).

Binding of gomesin to the surface of *C. neoformans*

A rabbit antibody to gomesin (Lorenzini *et al.*, 2003) was used as a probe in the analysis of cellular sites for the peptide in cryptococci. In these analyses, acapsular yeast were used since, although capsule expression seems not to regulate cryptococcal sensitivity to gomesin (Fig. 1), it has been described in the literature that the capsular polysaccharide negatively modulates the interaction of antibodies with the cell surface of *C. neoformans* (Rodrigues *et al.*, 2000). Yeast cells (10^6) were suspended in 100 μL of a 1 μM gomesin solution and incubated for 5 min at 37 °C. This concentration was chosen based on the results described in Fig. 1, as detailed in Results. The cell suspension was washed in phosphate-buffered saline (PBS) and then fixed in 4% paraformaldehyde. Yeast cells were then blocked and washed as described previously (Rodrigues *et al.*, 2000) and incubated with the rabbit antigomesin antibody (10 μg mL^{-1}) for 1 h at room temperature, followed by incubation with a rhodamine-labeled goat antirabbit IgG (Fc specific) antibody (Sigma). Yeast cells were finally observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired using a Color View SX digital camera and processed with the software system ANALYSIS (Soft Image System). Control preparations were developed as described above, except for the incubation with gomesin.

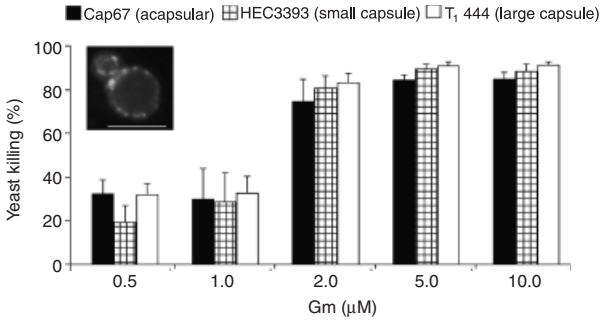

Fig. 1. Killing of *Cryptococcus neoformans* by gomesin (Gm). Immunofluorescence microscopy using strain Cap67 (inset) demonstrated that gomesin binds to the surface of cryptococci. Scale bar, 10 μm. Treatment of different *C. neoformans* strains with gomesin for five minutes followed by CFU determination revealed a dose-dependent profile of fungal killing by the peptide. Values of yeast killing were obtained by comparison of CFU counts of peptide-treated cells with untreated cells.

Antifungal effects of gomesin and fluconazole

Cryptococcus neoformans cells (strains HEC3393, T_1-444 and Cap67; 2×10^5 mL^{-1}) were treated with the peptide in concentrations varying from 0.5 to 10 μM (in PBS) for 5 min. Control systems were treated with PBS alone. Cell suspensions were then washed with sterile PBS and plated onto Sabouraud dextrose agar (SDA) plates for CFU counting. In some experiments, the anticryptococcal effects of fluconazole, alone or in combination with gomesin, were also evaluated, following the same protocol. All experiments were performed in triplicate sets and statistically analyzed using Student's *t*-test.

Permeabilization of cryptococci after treatment with gomesin

Membrane permeabilization was evaluated using the method described by Mangoni *et al.* (2004), with minor modifications. The fluorochromes used were the double-stranded DNA-binding dye 4',6-diamidino-2-phenylindole (DAPI), to stain all fungal cells independently on viability and the green fluorescent probe fluorescein isothiocyanate (FITC), which is unable to traverse the cytoplasmic membrane of cells unless permeabilized. Based on this property, the percentage of permeabilization in a cryptococcal population was considered as the number of FITC-stained cells per 100

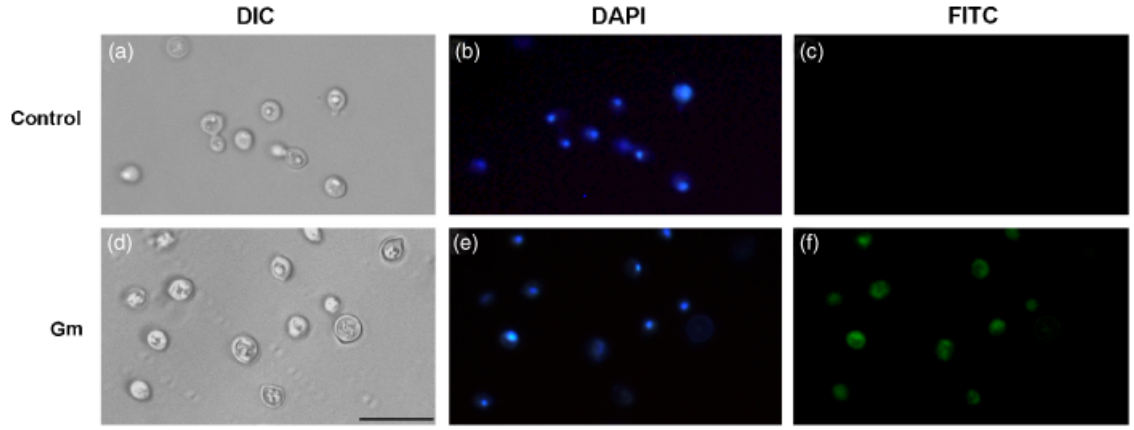

Fig. 2. Gomesin (Gm) induces permeabilization in encapsulated *Cryptococcus neoformans* cells (strain HEC3393). Control (a–c) or peptide-treated (d–f) yeast cells were sequentially incubated with DAPI and FITC. Microscopic analysis revealed that control and gomesin-treated cryptococci were stained with DAPI (b and e). Only the latter (f), however, were FITC-stained, while no green fluorescence was observed in control cells (c). Yeast cells observed under differential interferential contrast (DIC) are shown in a (control) and d (peptide-treated cells). Scale bar, 10 μm.

yeasts. Yeast cells (10^6) were suspended in 50 μL of a 2 μM gomesin solution for 5 min and further washed with PBS. This concentration was chosen based on the results described in Fig. 1, as detailed in 'Results'. Yeasts were then suspended in 50 μL of a DAPI solution (10 μg mL^{-1} in PBS). After 10 min at 30 °C, the DAPI solution was removed and the cells washed again with PBS. An FITC stock solution (10 mg mL^{-1} in dimethyl sulfoxide) was diluted to 6 μg mL^{-1} in PBS and used to resuspend the cells, followed by incubation at 30 °C for 30 min. After washing with PBS, yeast cells were transferred to glass slides and observed with an Axioplan 2 (Zeiss, Germany) as described above. Controls were run in the absence of the peptide. Cell viability was determined by CFU counting after peptide treatment, producing results essentially similar to those described in Fig. 2.

Effects of gomesin on capsule expression

To evaluate whether gomesin affects capsule expression in *C. neoformans*, 10^4 cells of the largely encapsulated strain T_1-444 were suspended in 100 μL of minimal media supplemented with 1 μM gomesin and cultivated for 48 h in 96-well plates. This concentration was chosen based on the results described in Fig. 1, as detailed in 'Results'. Control preparations were identical, except for the presence of gomesin. Yeast cells were collected and washed by centrifugation for further determinations of capsule expression. Capsule sizes, defined as the distances between the cell wall and the outer border of the capsule in India ink-stained yeast cells, were determined using IMAGEJ software (version 1.33), elaborated and provided by National Institutes of Health (NIH, http://rsb.info.nih.gov/ij/). Cell diameters were determined using the same software. Final measurements were presented as a ratio of capsule size/cell diameter. The ability of gomesin to regulate capsule synthesis and/or secretion was evaluated by cultivation of cryptococci in the presence of 1 μM gomesin. In this assay, *C. neoformans* cells that were cultivated in the presence of gomesin as described above were stained with Trypan blue and the number of viable (nonstained) yeast determined. The number of viable cryptococci (control cells or yeast obtained from gomesin-containing cultures) was then normalized to 10^4 and cells were again cultivated in the absence (control) or in the presence of 1 μM gomesin as described above. In 24 h intervals, 10 μL of the culture suspension was taken for CFU determination. The concentration of glucuronoxylomannan (GXM), the major capsular polysaccharide of *C. neoformans*, in the culture supernatants was determined in the same intervals, using capture ELISA as described previously (Casadevall *et al.*, 1992).

Yeast killing by human brain cells

Human brain phagocytes (U-87 MG, glioblastoma cells) were used in this study based on previous reports demonstrating the relevance of their interaction with *C. neoformans* (Lee *et al.*, 1994). They were provided by Dr Dumith Chequer Bou-Habib (Fiocruz, Rio de Janeiro, Brazil) and cultured in Dulbecco' modified Eagle's medium (Gibco-BRL, Gaithersburg, MD) supplemented with 2 mM L-glutamine and 10% complement-inactivated fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD). Viable fungal cells obtained from gomesin (1 μM)-containing cultures were washed in PBS and allowed to interact with brain phagocytes at a fungi : host cell ratio of 10 : 1 during 4 h at 37 °C. Nonassociated fungi were then removed by washing and host cells were lysed with sterile cold water. The resulting

suspension was plated onto SDA plates. After 48 h the number of CFU was determined. In some cases, after a 4 h incubation of *C. neoformans* with human cells, nonassociated fungi were removed by washing. Gomesin (0.1 μM) and/or fluconazole (1 μM) were diluted in fresh media and added to the systems. In this model, the cells were maintained at 37 °C for 24 h in the presence of the antifungal compounds and then lysed as described above for CFU counting. All experiments were performed in triplicate sets and statistically analyzed using Student's *t*-test.

Analysis of host cell damage by the lactate dehydrogenase (LDH) assay

To evaluate host cell injury induced by gomesin, human brain cells (U-87 MG) (10^5) were incubated with the peptide (0.1–10 μM, 100 μL) in fresh medium for periods of 24 h. Supernatants were then collected, centrifuged for removal of debris and assayed for the presence of released LDH as described previously (Barbosa *et al.*, 2006). Alternatively, human cells were incubated with the standard drug fluconazole (0.1–10 μM) in the same conditions as described above. Positive and negative controls consisted, respectively, of a U-87 MG Triton X-100 (10%) lysate and supernatants of nontreated human cells after 18 h of cultivation. All experiments were performed in triplicate sets and statistically analyzed using Student's *t*-test.

Results

Antifungal properties of gomesin

Treatment of different *C. neoformans* isolates with gomesin for 5 min resulted in a dose-dependent profile of fungal killing (Fig. 1). There was no evident correlation between capsule expression and susceptibility to gomesin. The peptide apparently uses the cell surface as a major binding site in *C. neoformans*, as inferred from the reactivity of gomesin with the cell wall of acapsular cells after 5 min incubation (Fig. 1, inset). Identical results were observed when gomesin was incubated with *C. neoformans* for 12 h (data not shown), suggesting that the peptide is not internalized.

The ability of gomesin to induce permeabilization in *C. neoformans* cells was evaluated by a fluorescence-based method. Levels of fungal killing after treatment of cryptococci with the peptide concentrations of 2, 5 and 10 μM were similar (Fig. 1); hence the 2 μM concentration was selected as the lowest gomesin dose presenting effective fungicidal effects. After treatment of *C. neoformans* (strain HEC3393) with PBS (control) or the peptide for 5 min, cryptococci were sequentially stained with DAPI and FITC. Analysis of these cells by fluorescence microscopy (Fig. 2) revealed nuclear staining of both control and peptide-treated yeasts after incubation with DAPI. FITC staining, however, was only observed in the cytoplasmic compartments of peptide-treated yeasts, indicating that gomesin causes permeabilization in *C. neoformans*. The percentage of permeabilized cells corresponded to 79%, which is in full agreement with the results demonstrated in Fig. 1. Both encapsulated cells and acapsular mutants were evaluated, producing similar patterns of fluorescence after gomesin treatment (data not shown).

Gomesin-treated cryptococci present an impaired ability to express capsular polysaccharides and reduced resistance against brain phagocytes

The suggestion that gomesin interacts with the cryptococcal cell surface (Fig. 1) led to the evaluation on whether cultivation of *C. neoformans* in the presence of gomesin also affects capsule expression. Based on the results presented in Fig. 1, the concentration of 1 μM was chosen, since it was the higher gomesin concentration at which inhibitory effects were relatively low. *Cryptococcus neoformans* cultivated in the presence of gomesin exhibited an average increase in cell diameter (not including capsule size) of around 38%, compared with control cells ($P > 0.05$). Cells obtained from the gomesin-containing medium were significantly less effective than control cells ($P < 0.0001$) in their ability to express capsular polysaccharides (Fig. 3a) as concluded from the increased ratio of capsule size per cell diameter. Microscopic observations revealed that virtually all cells had smaller capsules, although the level of capsule inhibition varied considerably in the cryptococcal population. Defective capsule expression or assembly was apparently related to an impaired ability of synthesis and/or secretion of GXM, since the concentration of soluble polysaccharide in culture fluids was significantly smaller ($P < 0.01$) in the presence of the peptide (Fig. 3b). This effect was apparently not related to smaller cell number in the culture or other metabolic faults, since the growth rates of control or peptide-treated cryptococci were very similar. The fact that viable cells proliferated normally in the presence of gomesin may indicate that gomesin-resistant yeasts could be selected after cultivation in the presence of the peptide, although it has not been experimentally proved.

The susceptibility of gomesin-treated cells to the antimicrobial action of brain phagocytes was also evaluated. The results shown in Fig. 3c demonstrate that peptide-treated cells are significantly more susceptible to killing by human phagocytes than control yeasts ($P < 0.0001$). The recovery of viable yeasts when gomesin-treated cells were used in phagocytosis assays represented only 5% of the values obtained after incubation of control yeasts with phagocytes. The enhanced susceptibility to macrophages was apparently not related

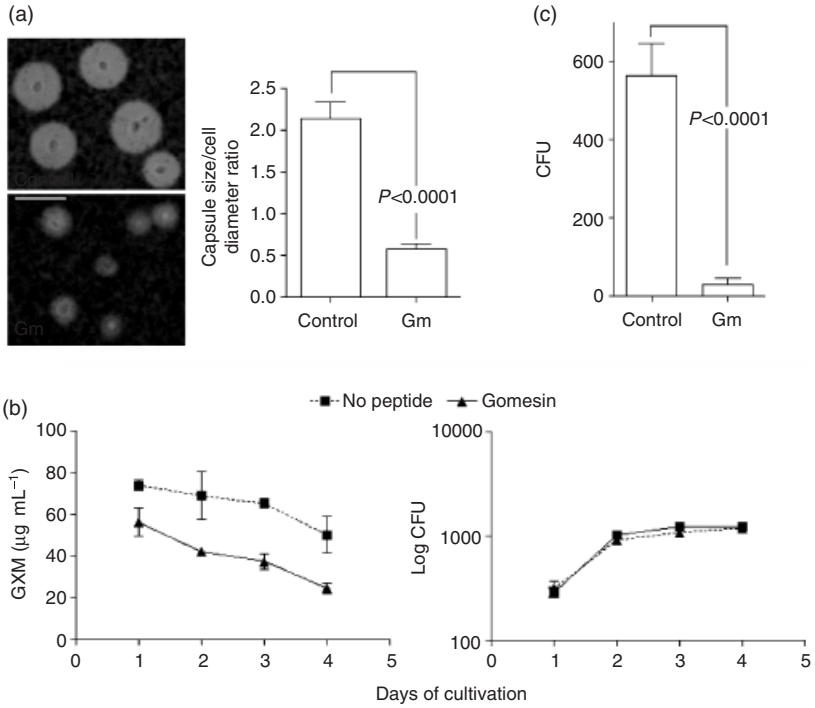

Fig. 3. Growth of *Cryptococcus neoformans* (strain T_1-444) in the presence of gomesin (Gm; 1 μM) results in inhibition of capsule expression (a, b) and increased susceptibility to killing by brain cells (c). (a) Microscopic observations of India ink preparations (left panels) and quantitative determinations of capsule size in control or Gm. Scale bar, 10 μm. (b) *Cryptococcus neoformans* cells were grown in control conditions or in the presence of 1 μM Gm for determination of GXM concentration (left) in culture supernatants or yeast viability (right). At different periods of cultivation, GXM concentrations were reduced in the presence of gomesin. (c) Control fungi or yeast cells grown in the presence of Gm were allowed to interact with brain cells for further CFU determination, which demonstrated that fungal killing is higher in peptide-containing cultures.

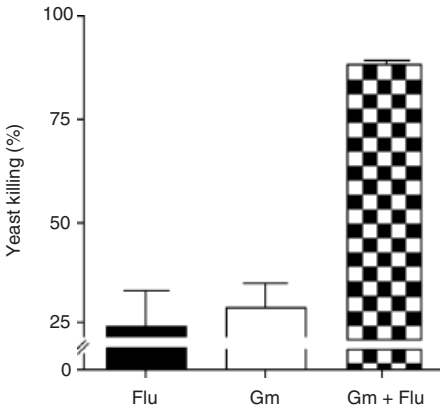

Fig. 4. Fluconazole (Flu) and gomesin (Gm) act synergistically. Values of yeast killing were obtained by comparison of CFU counts of drug/peptide-treated cells with untreated yeasts in three different experiments. Treatment of yeast cells with gomesin (1 μM) or fluconazole (1 μM) alone results in partial inhibition of fungal growth, while a combination of both compounds induces high levels of fungal killing.

to the antimicrobial effects of gomesin alone, since the indices of fungal killing by the 1 μM dose of the peptide were considerably lower than those observed after incubation with macrophages (for comparison, Figs 1, 3 and 4).

Synergistic effects of gomesin and fluconazole

The possible use of gomesin at concentrations below the range of effective antimicrobial activity in combination with the standard drug fluconazole to kill cryptococci was also evaluated. Dose–response curves of the anticryptococcal activity of fluconazole (data not shown) indicated that, in the presence of 1 μM fluconazole, only partial fungal killing (25%) was obtained under the conditions used in this study. At the same concentration, similar levels of antifungal activity were obtained for gomesin (Fig. 4). When both antimicrobial components were combined, however, fungal killing was close to 90%. These results indicate that gomesin and fluconazole act synergistically to kill *C. neoformans*. Therefore, it was tested whether gomesin and fluconazole could produce similar results in infected phagocytes.

The toxicity of gomesin and fluconazole to human brain phagocytes by the release of LDH, a classical marker of cell injury, was evaluated. Figure 5a shows the LDH activity of U-87 MG cells treated with each drug. Gomesin and fluconazole were significantly toxic only at 10 μM, using positive (Triton lysate) and negative (no treatment) controls as references. A partially inhibitory dose of fluconazole (1 μM) and the lowest dose of gomesin (0.1 μM) were then selected for further experiments. Fluconazole (1 μM) and gomesin (0.1 μM) were combined and their concomitant ability to cause injury in brain cells was tested. LDH activity from these culture supernatants was similar to that found in culture fluids on untreated cells, suggesting that, at low concentrations, a combination of fluconazole and gomesin is not toxic to brain cells. The results presented in Fig. 5b demonstrate that brain phagocytes, in the presence of fluconazole and gomesin alone, produced only partial inhibitory activity, as concluded from the comparison of

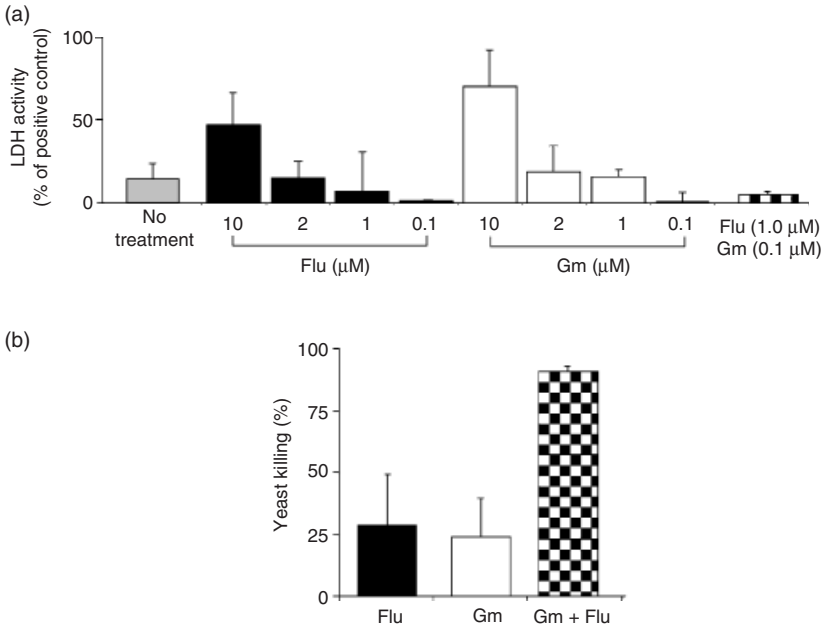

Fig. 5. Anticryptococcal effects of nontoxic doses of fluconazole during interaction of fungal cells with human brain phagocytes. (a) Cell damage induced by fluconazole and gomesin. The basal level of enzymatic activity (no treatment) was considered as the oxidation of NADH by culture supernatants of human brain cells kept in culture for 24 h, in the absence of drugs. The positive control of enzyme activity, taken as 100%, consists of a cell lysate obtained by extraction with Triton. The 1 μM dose of fluconazole and the lowest gomesin concentration tested (0.1 μM) were selected for treatment of infected phagocytes (b). CFU counts were determined after lysis of infected human cells. Values of yeast killing were obtained by comparison of CFU counts of drug/peptide-treated cells with those found in untreated, infected phagocytes.

CFU counts from control or drug/peptide-treated phagocytes. In the presence of a combination of both antimicrobial compounds, however, an impressive increase in antifungal activity was observed.

Discussion

Gomesin was the first peptide isolated from spider exhibiting antimicrobial activities (Silva *et al.*, 2000), including several pathogens. The mechanisms by which gomesin exert its antimicrobial effects are not well known but, since the peptide is cationic and present both hydrophobic and hydrophilic domains, plasma membrane insertion followed by its permeabilization might be at least one of its action mechanism, as discussed previously (Mandard *et al.*, 2002, Fazio *et al.*, 2006b). Gomesin does not act *via* a stereospecific receptor for several bacterial and fungal strains as demonstrated by Silva *et al.*, 2000.

The anticryptococcal activity of gomesin was previously suggested (Silva *et al.*, 2000), but key information such as cellular targets, toxicity for brain cells, effects on capsule expression and host susceptibility were not explored. Due to the antimicrobial potential of the peptide and clear problems with cryptococcosis treatment, the current study aimed at the evaluation of the anticryptococcal activity of gomesin. Data from this study indicate that a short period of incubation of different *C. neoformans* strains with the peptide results in fungal death. The cryptococcal sensitivity to gomesin is apparently not related to capsule expression, since isolates differently expressing capsular polysaccharides and an acapsular mutant were similarly affected by the peptide. The ability of gomesin to cross the capsular barrier and kill cryptococci is of potential relevance, since *C. neoformans* can express very large capsules during infection (Rodrigues *et al.*, 1999).

Gomesin also affects capsule expression in *C. neoformans*. A reduced capsule was associated with a decreased concentration of soluble GXM, which is regularly secreted in culture supernatants and used for distal capsule growth (Zaragoza *et al.*, 2006). GXM is also the major capsular polysaccharide and virulence factor of *C. neoformans* (Rodrigues *et al.*, 1999; Vecchiarelli, 2000; Perfect & Casadevall, 2002). Gomesin-treated cells had an increased diameter, as described for *C. neoformans* cells with defective mechanisms of GXM secretion (García-Rivera *et al.*, 2004). The mechanisms by which gomesin could inhibit capsule secretion and/or expression are still unclear but, since gomesin is apparently not internalized by cryptococci, it can be speculated that low concentrations of the peptide (1 μM) could affect signaling pathways related to capsule expression. In higher concentrations, cell death pathways could be activated. It is also demonstrated that yeast cells treated with gomesin were more efficiently killed by brain cells than by control yeast. In this context, this study indicates that the peptide could act in the control of cryptococcosis by killing *C. neoformans* and also by down modulating the expression of its major virulence factor.

Low doses of fluconazole and gomesin alone presented no antimicrobial activity but, in combination, a strong inhibition of fungal growth was observed, with no damage to human cells. The mechanisms by which gomesin and fluconazole could act synergistically are still unknown. It can be speculated that, since gomesin can induce cell

permeabilization in *C. neoformans*, the amount of fluconazole necessary to effectively reach the fungal cytoplasm and inhibit ergosterol synthesis could be decreased in the presence of the peptide. Combination antifungal therapy is in fact an alternative for the treatment of cryptococcosis. For instance, recent clinical data confirm that amphotericin B, which also induces membrane permeabilization, and the inhibitor of DNA synthesis flucytosine represent the most active combination for patients with cryptococcal meningitis although they both present significant toxicity (Baddley & Pappas, 2005).

It has been recently suggested that *C. neoformans* is a facultative intracellular pathogen (Feldmesser *et al*., 2001; Tucker & Casadevall, 2002). It is therefore believed that the intracellular environment works as a protective niche for *C. neoformans* during infection, avoiding interference of the host immune response and drug-mediated killing. In our model, combined low doses of fluconazole and gomesin resulted in enhanced indices of killing of *C. neoformans* by brain phagocytes. Supposedly, gomesin could enhance the permeability of host cells, allowing fluconazole to reach the intracellular environment and inhibit fungal growth. In this context, it has been described that several cationic and amphipathic peptides have the potential to be used as effective enhancers of the intracellular delivery of macromolecules (Kueltzo & Middaugh, 2003), genes (Kichler *et al*., 2006) and drugs (Trehin & Merkle, 2004). In the presence of gomesin, therefore, conventional antifungal therapeutics could be used in smaller and, consequently, less-toxic concentrations. Another possibility is that, even in lower doses of gomesin (0.1 μM), capsule expression in *C. neoformans* could be affected, rendering fungal cells more susceptible to the antimicrobial action of macrophages.

The current data indicate that gomesin, individually or in combination with fluconazole, could be of considerable application as a new anticryptococcal agent. In this regard, the potential to control cryptococcosis in animal models are the focus of future studies. In addition, the mechanisms by which the peptide binds to the cell surface of cryptococci inducing permeabilization and killing remain not understood. Purification of putative gomesin-binding molecules from *C. neoformans* cells followed by structural elucidation will represent crucial analyses on this subject.

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Authors' contribution

L.N. and M.L.R. share senior authorship on this manuscript.

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