Insights into the candidacidal mechanism of Ctn[15–34] – a carboxyl-terminal, crotalicidin-derived peptide related to cathelicidins

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Abstract

Purpose. Ctn[15–34], a carboxyl-terminal fragment of crotalicidin (a cathelicidin from the venom gland of a South American rattlesnake), has shown antifungal activity against clinical and standard strains of Candida species. The aim of the present work was to investigate the underlying mechanisms of the candidicidal activity of Ctn[15–34].

Methodology. The time-kill profile and drug synergism were evaluated by means of a microdilution assay and multi-parametric flow cytometry. The presumptive interaction of Ctn[15–34] with lipid membranes was estimated in vitro with a lipid-mimic compound, the chromogenic substance 4-nitro-3-(octanoyloxy)benzoic acid (4N3OBA).

Results/Key findings. The absorbance increment (at 425 nm) indicated a concentration- and time-dependent in-solution association between Ctn[15–34] and 4N3OBA. The interaction of Ctn[15–34] with Candida cells was confirmed by flow cytometric measurements with the 5(6)-carboxyfluorescein-labelled peptide (CF-Ctn[15–34]). Analysis of the killing time of Candida exposed to Ctn[15–34] and amphotericin B (AMB) showed that both the peptide and polyene drug reduce the number of c.f.u. but in mechanistically different ways. The Ctn[15–34] peptide alone caused yeast cell membrane disruption, which was confirmed by lactate dehydrogenase leakage and biomarkers of cell death mediated by necrosis.

Conclusion. Overall, Ctn[15–34] displays a synergistic antifungal activity with AMB, an effect that can be further developed into a multi-target therapeutic option with other antimycotics currently in use.

INTRODUCTION

Systemic fungal infections have drastically grown over the last three decades due to the increasing immunocompromised population, as a result of HIV infection and of diverse clinical interventions, like transplantation, cancer chemotherapy and steroid therapy [1]. The most disseminate fungal pathogens comprise species of Candida. For instance, Candida albicans is normally a commensal micro-organism in humans, but once the host is unable to trigger an adequate immune response, mucosal, cutaneous or invasive mycoses arise. The introduction of new systemic antifungal agents during the past decade has revolutionized the treatment of invasive mycoses. However, with these new therapies comes a need for an increased awareness of the limitations in their spectrum of activity, pharmacokinetics, and risk for drug interactions [2]. Amphotericin B (AMB) has been commonly used to treat serious fungal infections. Nevertheless, resistance to AMB has slowly been developing in selected Candida species and significant side effects associated with its use have
been observed, including nephrotoxicity [3]. To overcome these and other undesirable side effects, such as the occurrence of uncommon and resistant fungal strains or the limited spectrum of antifungal action, which polyene antifungals like AMB, thiazoles of new generations (fluconazole and analogues) and ectechinocandins (micafungin and caspofungin) are not totally able to circumvent, novel antifungal agents have been developed [4]. These antifungals target different cellular components and processes, like the fungal cell wall, or ergosterol and its biosynthesis. With a few exceptions, exemplified by arylamidine and thiepine derivatives, and lipopeptides and amphipathic β-peptides with helical fold, most novel antifungals are analogues of known chemical structures [4–8]. Therefore, despite these recent advances, the core critical problems with the antifungal arsenal, i.e. their effective applicability in clinic settings, limited by the usual toxicity to host cells and potential occurrence of microbial resistance to chemical structures that are similar, still persist. In this context, the discovery and development of alternative and more selective antifungal chemotherapeutics and therapeutic options are constantly required.

Antimicrobial peptides (AMPs) have emerged as potential antifungal agents, primarily because of their mechanisms of action, which impair the emergence of drug-resistant micro-organisms [9]. It has been well established that the majority of AMPs disturb membrane function and integrity through structural disruption and increased permeability, although this is not the sole mode of action and more subtle mechanisms determine the specificity and toxicity of the peptides [10]. In previous studies, catalicidcin precursors from the venom glands of South American pit viper snakes, named viperscids, were reported [11]. It was further demonstrated that Ctn[15–34], the carboxyl-terminal 20 amino acid-long fragment of crotalicidin (Ctn) (the viperscids from the rattlesnake *Crotalus durissus terrificus*), displays equivalent antibacterial and anticancer activities as the full sequence version Ctn, but with lower toxicity to healthy eukaryotic cells [12]. More recently, we demonstrated the good antifungal activity of Ctn[15–34] on different opportunistic yeasts and the reduced toxicity to human renal cells in comparison to the drug-of-choice AMB [13]. In the present study, we describe the killing kinetics and the main candidacidal mechanism of Ctn[15–34] involved in the antifungal activity against clinical and standard strains of *C. albicans*. Accordingly, the antifungal property of Ctn[15–34] can be further developed as a multi-target therapeutic option, in associations and formulations with antimycotics currently in use, to treat *Candida*-causing superinfection.

**METHODS**

**Peptides**

Ctn[15–34] (KKRLKKIFKKPMVIGVTIPF-amide) was prepared by solid phase synthesis, purified by HPLC and characterized by mass spectrometry analysis, as previously described [11, 12]. The fluorescent analogue, CF-Ctn[15–34], was similarly synthesized but, before deprotection of all radical groups, a 1.1 equivalent of 5(6)-carboxyfluorescein was added to the resin and left overnight for the completion of dye coupling. For all experiments, 1 mM peptide stock solutions were prepared in deionized water and stored in aliquots at 4 °C for up to 6 weeks.

**Fungi**

The *C. albicans* strains used in this study were from the American Type Culture Collection (ATCC90028) and clinical isolates (LABMIC107 and 108) from the Santa Casa de Misericordia Hospital at Sobral (Ceará, Brazil). Their identification, when applicable, was confirmed with CHROMagar-Candida (CHROMagar Company, Paris, France) in a VITEK 2 automated system (BioMerieux, Marcy-l’Étoile, France) with YST card. These strains 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 (phosphate 35 mM, NaCl 150 mM, pH 7.4). 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.

**Antifungal assays**

MIC of Ctn[15–34] against the fungal strains were determined by the broth microdilution method, based essentially on the document M27-A3 from the Clinical and Laboratory Standards Institute (CLSI) [14]. Determination of all MICs for all *C. albicans* strains was performed in 96-well microtitre plates (E&K Scientific, Santa Clara, CA, USA). The peptide stock solutions were used to make twofold serial dilutions with Rosewell Park Memorial Institute 1640 medium (RPMI) to a final volume of 100 µl well−1. Next, 100 µl of fungal suspension, containing 2×10⁶ c.f.u. ml−1 (equivalent to 0.5 in McFarland’s scale), in RPMI were added to each well. Final peptide concentrations ranged from 0.0195 to 40 µM. AMB (Sigma Chemical, St. Louis, MO, USA) was used as a standard antimycotic (positive control) against yeasts. MIC was, like usual, defined as the lowest peptide concentration at which no fungal growth was visually observed after 48 h at 30 °C.

**Time-kill assays**

*C. albicans* cells (~1×10⁶ c.f.u. ml−1) were inoculated in RPMI containing Ctn[15–34] (10 µM) or AMB (2 µM). The tubes were incubated (30 °C; 200 r.p.m.), and 100 µl aliquots were removed at predetermined time points (0, 4, 8, 12 and 24 h). The aliquots were serially diluted (10-fold) in PBS and plated on yeast extract/potato dextrose agar plates. The numbers of colonies were counted after incubating the plates at 30 °C for 48 h.

**Synergism detection assays as evaluated by flow cytometry**

The antifungal activity of Ctn[15–34] when combined with AMB was determined by the checkerboard technique, a method used to estimate drug interaction by calculating the
fractional inhibitory concentration (FIC) index. In 96-well microtitre plates, 50 µl of fungal suspensions (with 2×10⁶ c.f.u. ml⁻¹ = 0.5 in McFarland’s scale) in RPMI were added to wells containing the peptide and AMB in the same final concentration ranges (0.0195–40 µM peptide, 0.0312–16 µM AMB), as previously established for the MIC determination. 

Cells treated with either peptide or AMB alone, at their respective MIC values, along with untreated fungal suspensions were used as controls. After 48 h incubation at 30 °C, results were visually observed and the FIC index (FICI) was calculated as FICI=FIC(A)+FIC(P), where ‘A’ represents peptide (Ctn[15–34]) and ‘P’ AMB. FIC(P), in turn, was calculated as the MIC(P) (combined)/MIC(P) (alone) ratio, while FIC(A) was calculated as the MIC(A) (combined)/MIC(A) (alone) ratio. Drug interaction was classified as synergism if FICI ≤ 0.5; no interaction when 0.5 < FICI ≤ 4.0; and antagonism if FICI > 4.0 (13). The synergistic effect of Ctn[15–34] with AMB was further demonstrated with C. albicans cells by flow cytometry. Fungal suspensions containing 2×10⁶ c.f.u. ml⁻¹ (~0.5 in McFarland’s scale) in RPMI were treated with Ctn[15–34] (0.31 µM) and AMB (0.5 µM), either alone or combined, for 24 h. After these incubation periods, cells were washed and propidium iodide (PI) at a final concentration of 1.49 µM was added to the yeasts as a marker of cell viability/membrane disruption. C. albicans cells were then run in a FACScalibur flow cytometer (Becton–Dickinson, San Jose, CA, USA) and 10,000 live events were analysed with the Cell Quest software (Becton–Dickinson). Cells without treatment were used as controls and experiments were carried out in triplicate.

**In-solution interaction assay of Ctn[15–34] with synthetic chromogenic lipid**

Ctn[15–34] at 5 or 10 µM was initially mixed with reaction buffer (final concentration 10 mM Tris-HCl, 10 mM CaCl₂, 100 mM NaCl, pH 8.0) containing 2 mM of the lipid 4-nitro-3-(octanoyloxy)benzoic acid (4N3OBA), as the molecular indicator. In the assay buffer (pH 8.0), the ionized form of 4N3OBA increases in relation to the reciprocal lipophilic non-ionized species, which is predominant in low pH (e.g. LogD=1.93 at pH 7.4 and 2.71 at pH 5.5) (CSID:16788382, www.chemspider.com/Chemical-Structure.16788382.html, accessed 20 July 2017). Thus, the interaction of the Ctn[15–34] peptide with the lipid-mimic was measured by monitoring the increase of 4N3OBA chromogenicity through absorbance readings at 425 nm, after 10, 20, 30 and 40 min of incubation, at constant temperature (37 °C). The assay buffer without the peptide was used as the control and all measurements were carried out in triplicate.

**Cell interaction assay of carboxyfluorescein (CF)-Ctn[15–34] and C. albicans cells**

C. albicans (~1×10⁶) cells were first harvested at the logarithmic phase and suspended in RPMI. Next, these yeasts were incubated at 30 °C with the carboxyfluorescein-labelled, CF-Ctn[15–34], at its MIC and 0.5xMIC, for 0.5, 1, 2 and 4 h. After each incubation period, cells were washed and PI at a final concentration of 1.49 µM was added to the pathogenic yeasts. These treated yeast cells were then analysed by flow cytometry as mentioned previously. Fungal cells without treatment were used as controls and experiments were carried out in triplicate.

**Enzymatic assessment of membrane lysis induced by Ctn[15–34]**

Candida suspensions (~0.5 in McFarland’s scale) were exposed to Ctn[15–34] (from 0.25xMIC to 8xMIC) for 4 and 8 h, in RPMI with and without protease inhibitor cocktail (PIC) (protease inhibitor mix HP Plus, SERVA Electrophoresis GmbH, Heidelberg, Germany). The leaked amount of lactate dehydrogenase (LDH) from fungal cells with a peptide-disrupted membrane was fluorometrically measured with the homogeneous membrane integrity assay kit CytoTox-ONE (Promega, Madison, WI, USA). The fluorescent signal, which is proportional to cells with disrupted membranes (short incubation time, in RPMI with PIC) or to viable cells (long incubation time, in RPMI without PIC), was measured (λ_exc=560 nm/λ_em=590 nm) in the microplate reader BioTek Synergy HT (BioTek Instruments, Winooski, VT, USA).

**Biochemical analysis of necrotic and apoptotic markers**

C. albicans cells were exposed to treated Ctn[15–34] or AMB, at their respective MIC and 0.5xMIC, for 12 and 24 h at 30 °C. After each treatment period, these yeasts were incubated with 7-actinomycin D (7-AAD) and Annexin V (AX), by using the PE Annexin V Apoptosis Detection Kit I (BD Pharmingen), to assess the cellular integrity and the externalization of phosphatidylserine (PS), respectively. The labelled yeast cells were then analysed by flow cytometry using the same instrumentation and software as described above. Yeasts not exposed to either peptide or the AMB were used as controls and experiments were in triplicate. The results were scored as follow: AX-/7AAD-, viable cells; AX+/7AAD-, indicative of early apoptosis; AX-/7AAD+, necrosis; AX+/7AAD-, late apoptosis/necrosis.

**Statistical analyses**

All experiments were carried out in triplicate. The obtained means±SEM were compared through one-way ANOVA in the synergy detection by flow cytometry and Ctn[15–34] C. albicans interaction assays, and Bonferroni post hoc tests using the software SPSS version 16.0 (IBM Corporation, Somers, NY, USA). Differences were considered statistically significant if *P<0.05.

**RESULTS**

Ctn[15–34] and carboxyfluorescein-labelled Ctn [15–34]

The Ctn[15–34] peptide and its fluorescein-labelled analogue, CF-Ctn[15–34], were prepared as carboxyl-terminal amide forms by solid-phase syntheses with high purity (>95 % by HPLC) and with the respective molecular weights.
of 2371.10 and 2729.41, as obtained by liquid chromatography coupled to a mass spectrometer. The peptide stock solutions at 1 mM with deionized water did not show signs of degradation for at least 6 weeks of storage at 4°C.

**Antifungal activity of Ctn[15–34] and killing kinetics**

Initially, the MICs of Ctn[15–34] and AMB against standard and clinical isolates of *C. albicans* were determined (Table 1) and confirmed not only our previous findings [13] but also established the experimental parameters for subsequent mechanistic studies. Based on the MICs of Ctn[15–34] and AMB for both strains described here, the time-kill (killing kinetics) assays were next performed to evaluate the growth inhibition profile of *C. albicans* when exposed to these drugs. Results displayed in Fig. 1 showed that both drugs are able to effectively inhibit the growth of *C. albicans* in agar plates after 8 h of treatment at their MICs. However, while Ctn[15–34] quickly reduced the number of c.f.u. between 0 and 4 h and slowly between 4 and 8 h, the AMB profile was the opposite (slow reduction during the first 4 h and a fast decrease between 4 and 8 h).

**Synergism of Ctn[15–34] against *C. albicans* strains as assessed by flow cytometry**

Drug interaction studies between Ctn[15–34] and AMB were confirmed *in vitro* by the checkerboard dilution assay against the above *C. albicans* strains. According to the MIC results (Table 1), concentrations of AMB at sub-inhibitory values together with Ctn[15–34] at well-belw (more than a log) their MICs could inhibit the growth of all *C. albicans* strains. This drug interaction, which is classified as a synergism, could be further observed by flow cytometry using a clinical *C. albicans* strain (LABMIC0107) and cell-non-permeant PI as a viability marker (PI-staining increases as a result of membrane disruption and the higher number of dead cells) (Fig. 2). The Ctn[15–34] alone at 0.31 µM was not enough to allow PI-staining of *C. albicans* cells (green line, Fig. S1a, available with the online version of this article) and results were similar to untreated cells (black line). When 0.5 µM AMB was used instead, 20 % (±2.6) of 10 000 remaining live cells (red line, Fig. S1b) showed an increased fluorescent signal related to PI-staining (arrow) compared to untreated cells. Moreover, when Ctn[15–34] and AMB are associated at these later concentrations, a significant 10 % increase in fluorescent signal (30±1.0%) was observed in these PI-stained yeasts (blue line, Fig. S1c), compared to cells treated with only the polycene drug.

**Interaction of Ctn[15–34] with the synthetic lipid 4-nitro-3-(octanoyloxy)benzoic acid**

Since PI is a non-permeant nucleic acid fluorescent dye which can only enter cells with damaged membranes and disruption of cytoplasmic membranes is the main mechanism of action of most AMPs, our next step was to evaluate whether Ctn[15–34] could interact with lipids. The synthetic lipidic compound 4N3OBA was incubated with the peptide Ctn[15–34] at 10 and 5 µM, which is respectively equivalent to the MIC and 0.5xMIC against *C. albicans*, and the result was plotted as shown in Fig. 3. As can be observed, the proportional increment of 43NOBA absorbance (λmax=425 nm) was a consequence of the concentration- and time-dependent interaction between the the Ctn[15–34] peptide and the chromogenic lipid in solution.

**The membrane lytic effect of Ctn[15–34] measured by lactate dehydrogenase leakage**

To corroborate the data of the increased population of PI-stained *Candida* cells exposed to the peptide and the *in vitro* interaction of Ctn[15–34] with the chromogenic 43NOBA, the fluorometric measure of LDH leakage confirmed the membrane damage induced by increasing the Ctn[15–34] concentration (Fig. 4). Importantly, by adjusting the time and composition of the assay solution for *Candida* cells, membrane damage or cell viability could be determined with the LDH/NADH/diaphorase/rezasurin-coupled reactions from the commercial assay system.

**Cell membrane disruption of *C. albicans* by CF-Ctn [15–34]**

These results prompted us to investigate membrane disruption in clinical *C. albicans* (LABMIC0107) by Ctn[15–34] using flow cytometry and a fluorescent-labelled version of

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**Table 1. MICs of Ctn[15–34] and AMB either alone or combined against clinically isolated strains of C. albicans**

<table>
<thead>
<tr>
<th>Crotalicidin/AMB</th>
<th>C. albicans LABMIC0108</th>
<th>C. albicans LABMIC0107</th>
<th>C. albicans ATCC90028</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CIM (µM)</td>
<td>CIM (µM)</td>
<td>FICI*</td>
</tr>
<tr>
<td>Ctn[15–14]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>0.007</td>
<td>0.25</td>
</tr>
<tr>
<td>AMB</td>
<td>2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Ctn[15–34]</td>
<td>20</td>
<td>0.31</td>
<td>0.26</td>
</tr>
<tr>
<td>AMB</td>
<td>2</td>
<td>0.5</td>
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<tr>
<td>Ctn</td>
<td>20</td>
<td>0.31</td>
<td>0.26</td>
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<tr>
<td>AMB</td>
<td>2</td>
<td>0.5</td>
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*FICI, fractional inhibitory concentration index; S, synergy.*
the peptide (Fig. 5). Thus, carboxyfluorescein-labelled Ctn [15–34] at 10 µM, Fig. 5b shows that up to the first 30 min of incubation, the peptide interacted with C. albicans cells (green ‘FL1’ fluorescence) compared to the control (Fig. 5a), which can be observed by the shift of cell-associated fluorescence from the lower left to the lower right (carboxyfluorescein, CF+/PI-) quadrants. Up to that period of treatment, however, the peptide did not cause much membrane damage, like indicated by low PI-staining (red ‘FL2’ fluorescence). Therefore, the fluorescence ratio between FL1 and FL2 had comparatively higher mean scores (Fig. 5d). However, after 1 h incubation (Fig. 5c), the interaction of the CF-Ctn[15–34] peptide with C. albicans caused a more noticeable cell membrane damage in the yeast, as an increase of PI fluorescent signal was observed (shift towards the upper right quadrant, the CF+/PI+) and, thus, the ratio between FL1 and FL2 decreased (Fig. 5d).

**Evaluation of biochemical markers for necrosis and apoptosis**

Finally, we verified the main pathway by which Ctn[15–34] causes cell death in C. albicans and compared it with the death pathway triggered by AMB after treatment with sub-inhibitory and inhibitory concentrations of both compounds for 12 and 24 h (Fig. 6). As depicted in Fig. 6(a), Ctn [15–34] showed that there were more cells undergoing apoptosis than necrosis at both sub-inhibitory and inhibitory concentrations of peptide, within 12 h of treatment. However, by 24 h of incubation (Fig. 6b), the percentage of cells going through necrosis increased in detriment to cells undergoing apoptosis, with a considerable increase of C. albicans yeasts committed to late apoptosis/necrosis after Ctn[15–34] treatment, remarkably at 10 µM (17.87 % – upper left quadrant, Fig. S2). In contrast, AMB mainly induced an initial cell death in Candida (5.4 % – lower right quadrant, Fig. S2) by early apoptosis, which can be observed especially at lower concentrations (the 0.5xMIC, Fig. 6d), while after treatment with a concentration of 2 µM (the MIC) for 24 h (14.8 % of the remaining live cells – upper left quadrant, Fig. S2), AMB also induced late apoptosis/necrosis (Fig. 6d). These killing patterns coincide with the profile of candidacidal activity observed in Fig. 1, being initially and mechanistically different between the peptide and the AMB.

![Fig. 1. Killing-time curves of C. albicans by Ctn[15–34] or AMB at their respective MICs. Assays were performed in triplicate in RPMI with 2 % MOPS at 30 °C for 24 h. Samples were withdrawn at the indicated times and evaluated for c.f.u.](image1)

![Fig. 2. Candidacidal synergism between Ctn[15–34] and AMB measured by flow cytometry. The combined sub-inhibitory concentrations of Ctn [15–34] (0.31 µM) and AMB (0.5 µM) results in an improved antifungal effect. Bars correspond to numeric values in the representative histograms of flow cytometric experiments reported in Fig. S1.](image2)
DISCUSSION

The search and development of new antifungal agents are constantly demanding due to the emergence and spread of drug-resistant microbial strains, like potential pathogenic species of *Candida*, the causative agent of invasive candidiasis (the most prevalent systemic mycoses worldwide). Previously, we reported the *in vitro* antifungal activity of Ctn [15–34] against standard and clinical isolates of *Candida* species [13]. In that work, Ctn[15–34] was shown to display a selective activity against several species and strains of *Candida* and a much lower toxicity to healthy eukaryotic cells (human kidney-2 cell line and human red blood cells), even lower than AMB. In the present study, by combined methodologies, we assessed the main underlying mechanism by which Ctn[15–34] can kill *in vitro* clinical isolates of *C. albicans*. The MICs from both standard and clinical *C. albicans* strains were initially confirmed (Table 1); the time-kill assays were further performed to compare over time the candidacidal or fungistatic capacities of Ctn[15–34] and AMB against clinical isolates of *C. albicans*. Although both the peptide and drug were able

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**Fig. 3.** In-solution interaction of Ctn[15–34] with anionic lipid. The anionic lipid 4N3OBA was incubated with an increase of peptide Ctn [15–34] concentration, i.e. 0 (NT), 5 (MIC/2) and 10 (MIC) µM, and the absorbance at 425 nm read at different time points. (Absorbance arbitrary units, mean±SEM with n=3.)

**Fig. 4.** Cell viability and the membrane lytic effect in *C. albicans* exposed to the Ctn[15–34] peptide. Fungal cells were incubated with increasing peptide concentrations, above and below the MIC for Ctn[15–34] (10 µM), for 4 h and 8 h, at 30°C. The membrane damage (LDH leakage) and the cell viability were measured with the reagent CytoTox-ONE. Short exposure time (4 h) of *C. albicans* with peptides, in RPMI supplement with PIC, reveals membrane damage and LDH activity in *C. albicans* (ATCC90028) (●). Long treatment time (8 h), in RPMI without PIC, shows the cell viability (~▲~). Measurements were in triplicates.
to inhibit yeast growth after 8 h of treatment, they reduced the number of c.f.u. by different mechanistic patterns (Fig. 1). This fact suggested that Ctn[15–34] and AMB might exert their effect by distinct mode of action against C. albicans. With this premise, the checkerboard assays were re-tested to confirm our earlier findings, i.e. the antifungal effect of the peptide and the polyene drug alone and the potentiation of antifungal activity mediated by the combination of the peptide and AMB, administered together [13]. Our previous and present results (Table 1) demonstrated that Ctn[15–34] acted synergistically (FICI ≤0.5) with AMB at lower concentrations than their respective MICs. This synergism effect of Ctn[15–34] and AMP corroborates other studies in which natural or synthetic AMPs associated with peptide or non-peptide antibiotics can effectively act synergistically [15].

Importantly now, results from such checkerboard tests were reiterated and confirmed by flow cytometry analysis in addition to the microdilution method. Indeed, after 24 h of peptide and drug treatment, the remaining live Candida cells were analysed with PI as a non-permeant viability marker and as a fluorescent dye to detect cells that were killed as a result of membrane disruption (Fig. 2). PI-positive cells indicated that the dye stained more yeast cells that were treated with Ctn[15–34] and AMB together rather than with only the polyene drug. Similar synergistic effects against an ATCC C. albicans strain were previously observed with AMB, at sub-inhibitory concentrations, when it was incubated with the glycoside acteoside, as determined by analytical flow cytometric measurements, [16].

Although flow cytometry methods for antimicrobial/antifungal susceptibility tests have not yet been completely standardized, they have been in use for more than 20 years and their quantifiable results are equivalent to those data obtained with standard CLSI methods [17]. Moreover, flow cytometry can provide reliable and faster results comparable to the traditional protocols (e.g. 2–6 h compared to 24–72 h in the case of microdilution methods), which can have a tremendous impact on the analytical, time cost and clinical outcome for patients [18]. Herein, analytical flow cytometry was applied to quantitatively verify the effect of the crotalidin-derived peptide, Ctn[15–34], against patient-isolated C. albicans strains, highlighting the clinical importance of
disclosing new antifungal agents or their combined therapeutic associations.

Concomitantly to flow cytometry evaluation, we turned our attentions back to the interaction of Ctn[15–34] with anionic lipids, which is essential to understand the structural activity relationship. Since most AMPs, including cathelicidins, interact with negatively charged bacterial and fungal phospholipids and induce subsequent membrane disruption [19], a simple biochemical test was initially and preliminarily carried out to verify whether Ctn[15–34] alone could interact with a given lipid. For this assessment, an assay based on the chromogenic lipid 4N3OBA was chosen. The molecule of 4N3OBA contains an eight-carbon lipid tail connected to a chromophore (3-hydroxy-4-nitrobenzoic acid) that is covalently linked and is colourless except these two functional chemical groups are separated by enzymatic cleavage [20]. Despite the most usual enzymatically catalysed-based readout of the chromogenic 4N3OBA, we based our analysis, like devised here, on the interaction of the peptide and lipid, which resulted in alteration of the electronic transition state and in the light-absorbing properties of the chromogenic 4N3OBA moiety upon peptide binding. Thus, the cationic Ctn[15–34] peptide (net positive charge=+8) appeared to interact to ionized 4N3OBA, modify its physico-physical properties and induce the increase of light absorbance in a concentration- and time-dependent manner (OD$^{425\text{nm}}$=0.03 at 5 µM and OD$^{425\text{nm}}$=0.18 at 10 µM). To confirm that the membrane is the main target of Ctn[15–34] activity, as seen by the increased number of PI-stained Candida cells and the in vitro interaction of Ctn[15–34] with the lipid-mimic 43NOBA, the membrane integrity was assayed based on the measure of LDH leakage. In this case, when Candida cells are exposed to peptide concentrations below the MIC (<10 µM), for 4 h at 30°C, a low level of LDH activity is detected in the medium, while with concentrations above the MIC (up to 80 µM) a relatively high level of LDH is observed (Fig. 4). Obviously, such a pattern contrasts with the profile of cell viability, in which Candida cells show a relatively high vitality at concentrations very inferior to the MIC, when exposed for 8 h at 30°C, while clearly they were efficiently killed with quantities of Ctn[15–34] over 10 µM (Fig. 4).

Since not only the maximal in vitro interaction between peptide and lipid was effective with 10 µM of the peptide, but also the membrane damage in fungal cells was becoming evident, this concentration was maintained to evaluate by flow cytometry the interaction of a fluorescein-labelled peptide analogue, CF-Ctn[15–34], with C. albicans cells. In this analytical procedure, PI was again used to monitor the membrane disruption in the pathogenic yeast cell that is caused by the peptide (Fig. 5). While the peptide interacted with cells in the first 30 min of exposure, which was noticed

Fig. 6. Detection of the cell death pathway in C. albicans cells exposed to Ctn[15–34] or AMB. C. albicans cells were exposed to sub-inhibitory and inhibitory concentrations of Ctn[15–34] (a, b) or AMB for 12 and 24 h (c, d). Markers for apoptosis and necrosis were respectively, AX and 7-AAD. Scores were: AX-/7-AAD-, viable cells; AX+/7-AAD-, early apoptosis; AX-/7-AAD+, necrosis; AX+/7-AAD-, late apoptosis/necrosis. Data are from representative flow cytometry scattering graphs and mean percentage values were from three independent experiments, each with 10 000 live events, Fig. S2.
by increased green fluorescence associated to the Candida cells; it took 1 h of treatment to observe the pronounced C. albicans membrane damage (increased cell-associated red fluorescence). This set of experiments was based on a previously published work by Freire and colleagues [21], in which they used time-resolved flow cytometry assay (TR-FCA) to examine bacterial cell membrane permeabilization mediated by pepR – a designed AMP. In fact, by using TR-FCA combined with surface plasmon resonance and atomic force microscopy, some of us demonstrated that Ctn[15–34] permeabilizes the cytoplasmic membrane of Gram-negative bacteria immediately upon its addition to the microbial cell cultures, killing more than 90 % of cells within less than 1 h. Moreover, Ctn[15–34] causes preferential disruption of model vesicles that mimic microbial and tumour cell membranes rather than compositions that mimic eukaryotic healthy cell membranes [22].

Last but not least, the C. albicans cell death pathway was investigated by flow cytometry using biochemical markers for apoptosis and necrosis (Figs 6 and S2). Although both AMB and Ctn[15–34] induces late apoptosis and necrosis as the definitive mode of killing C. albicans, at their respective MICs, after 24 h of treatment, the onset of action to induce necrotic cell death is, at sub-inhibitory concentrations, distinctly pronounced for the peptide in comparison to the polyene AMB drug. These findings are in concordance with other studies that demonstrated that the entry of AMPs in Candida cells resulted in accumulation of reactive oxygen species and led to late cell necrosis [23, 24]. Thus, in contrast to AMB, we observed that Ctn[15–34] annihilates C. albicans in two phases, an immediate one: an initial concentration- and time-dependent interaction to and disruption of the cell membrane, in which apoptosis is barely detected, followed by a second phase that intensifies the membrane disruption and necrotic response. Therefore, there is a potentiating necrotic killing effect of Candida cells and faster reduction of pathogen burden, induced by Ctn[15–34] compared to the polyene drug, in which this firstly triggers an apoptotic response, mainly at sub-inhibitory concentration, as experimentally observed (Fig. 6). Despite this mechanistic contrastive effect, an important fact is that these antifungal agents kill pathogenic Candida by apoptosis and necrosis, as also observed for caspofungin [25]. However, the Ctn[15–34] peptide appears more efficacious in triggering the necrotic pathway of cell death, which would be an interesting intrinsic property for the control of an eventual disseminate fungal infection that needs a rapid intervention.

In conclusion, the present work sheds some light on the anti-Candida activity of Ctn[15–34] by means of flow cytometric analysis. Time-kill assays showed that Ctn[15–34] could interrupt the growth of C. albicans after the same incubation period as AMB at their respective MICs. Importantly, when the peptide and polyene drug were administered together, synergism was observed, which is beneficial for the potential combinatorial therapy of candidiasis. In addition, Ctn[15–34] alone promoted earlier cell membrane disruption, as the first phase of killing, and induced cell death in C. albicans mainly by necrosis, as confirmed with biochemical markers after Ctn[15–34] treatment. Further studies will be addressed to verify whether Ctn[15–34] is capable of interacting with intracellular target components of Candida cells, contributing to the collapse of their cellular processes and the modulation of the immune response of host cells.

**References**


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