Article

Giving a Hand: Synthetic Peptides Boost the Antifungal Activity of Itraconazole against *Cryptococcus Neoformans*

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Abstract: *Cryptococcus neoformans* is a multidrug-resistant human pathogenic yeast responsible for infections in immunocompromised patients. Here, Itraconazole (ITR), a commercial antifungal drug with low effectiveness against *C. neoformans*, was combined with different synthetic peptides *Mo*-CBP3-PepII, *Rc*Alb-PepII, *Rc*Alb-PepIII, PepGAT, and PepKAA. The mechanisms of action responsible for the synergistic effect were evaluated for the best combinations by Fluorescence Microscopy (FM). The synthetic peptides enhanced the activity of ITR by 10-fold against *C. neoformans*. Our results demonstrated that the combinations could induce pore formation in the membrane and overaccumulation of ROS on *C. neoformans* cells. Our findings indicate that our peptides successfully potentialize the activity of ITR by reducing it by 10-fold to reach antifungal activity against *C. neoformans*. Therefore, synthetic peptides are potential molecules to act as co-adjuvant agents in treating Cryptococcal infections.

Keywords: azole drugs; cryptococcal meningitis; resistance; synergism; synthetic antifungal peptides

1. Introduction

Fungal diseases are a threat to human health. From mild mycosis to severe lung infections, fungi affect over 300 million people worldwide, causing 1.6 million deaths annually [1]. In addition to the low number of new drugs available, in the past 40 years, it was observed an increasing resistance of these pathogens to traditional antifungal medications and treatments [2,3]. Thus, there is a need for new treatments for fungal diseases, which could be developed from new molecules with antifungal activity.

Among the deadliest fungal pathogens is the human-pathogenic yeast *C. neoformans*, a highly virulent yeast that can cause pneumonia and meningitis. *C. neoformans* is the leading cause of mortality among immunocompromised individuals, such as organ transplant patients and cancer patients undergoing chemotherapy [4]. The *C. neoformans* virulence is a combination of some unique traits such as a polysaccharide capsule, which protects from phagocytosis, thermotolerance to 37 °C, presence of melanin protecting from UV light, and a variety of extracellular enzymes that act as defense mechanisms [1]. All these features, plus drug misuse over the years, led to a rapid increase in drug resistance among the various strains [2], turning *C. neoformans* into a severe problem for human health.

The solution may lie in discovering drugs with different mechanisms of action than conventional drugs. Recently, synthetic antimicrobial peptides have emerged as potential candidates because they are based on natural antimicrobial peptides but have high activity and low toxicity levels [5–7]. In addition to being employed alone, synthetic peptides could be applied synergistically with commercial drugs to improve their action [8–11]. For

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example, Souza et al. [11] revealed that synthetic peptides improved the activity of Griseofulvin against dermatophytes. By studying the mechanism of action, authors revealed that synthetic peptides induced pore formation in the fungus membrane and increased the intracellular concentration of Griseofulvin, which has a cytoplasmatic target [11].

Recently, our research group reported five synthetic peptides (*Mo*-CBP3-PepII, *Rc*Alb-PepII, *Rc*Alb-PepIII, PepGAT, and PepKAA), with higher activity against *C. neoformans* that can induce pore formation of its membrane [7]. This study aimed to evaluate the ability of those peptides to improve the activity of a commercial drug ITR against *C. neoformans*. Additionally, the mechanisms of action were assayed to understand somehow this synergic effect happened.

2. Materials and methods

2.1. Biological material and chemicals

C. neoformans (ATCC 32045) was obtained by the Department of Biochemistry and Molecular Biology of the Federal University of Ceará (UFC), Fortaleza, Brazil. All the chemicals used in the experiments were obtained from Sigma Aldrich (São Paulo, SP, Brazil).

2.2. Synthetic peptides

The synthetic peptides *Mo*-CBP3-PepII, *Rc*Alb-PepII, *Rc*Alb-PepIII, PepGAT, and PepKAA, were chemically synthesized by ChemPeptide (Shanghai, China) and were analyzed for purity and quality (≥95%) by reverse-phase high-performance liquid chromatography and mass spectrometry.

2.3. Synergism assay

The synergism assay was performed as described by [11]. The combinations with SAMPs and Itraconazole were constituted of peptides MIC_{50} [7] + Itraconazole (ITR) 500 (1x) μg mL^{-1}, peptides [5xD] + ITR [1xD], peptides [10xD] + ITR [5xD], peptides [10xD] + ITR [10xD] (Supplementary Table 1). The cells were grown on YPD agar for approximately 15 days, and next, cryptococcal cells were resuspended in YPD medium and standardized at 10^6 cells mL^{-1}. The assay was performed in 96 well-plates, incubated for 24 h at 30 °C. Then, the absorbance was measured at 600 nm using an automated microplate reader (Epoch, Biotek, Santa Clara, CA, USA). The best combinations were used to study the mechanisms of action.

Table 1. Combined antifungal activity between synthetic peptides and ITR against *C. neoformans*

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Treatments	% of inhibition of *C. neoformans* growth
DMSO-NaCl	0
ITR 1x	45.3 ± 0.021
ITR 5xD	12.2 ± 0.003
ITR 10xD	2.4 ± 0.004
Mo-CBP$_3$-PepII 1x (MIC$_{50}$)	50.0 ± 0.004
Mo-CBP$_3$-PepII 5xD	12.4 ± 0.001
Mo-CBP$_3$-PepII 10xD	2.1 ± 0.009
*Rc*Alb-PepII 1x (MIC$_{50}$)	50.0 ± 0.001
*Rc*Alb-PepII 5xD	2.6 ± 0.005
*Rc*Alb-PepII 10xD	1.5 + 0.007
*Rc*Alb-PepIII 1x (MIC$_{50}$)	50.0 ± 0.004
*Rc*Alb-PepIII 5xD	10.6 ± 0.003
*Rc*Alb-PepIII 10xD	5.5 + 0.002
PepGAT 1x (MIC$_{50}$)	50.0 ± 0.009
PepGAT 5xD	17.1 ± 0.003
PepGAT 10xD	7.8 + 0.01
PepGAT 1x (MIC$_{50}$)	50.0 ± 0.005
PepGAT 5xD	20.4 ± 0.009
PepGAT 10xD	1.8 ± 0.002
Mo-CBP$_3$-PepII 1x + ITR 1x	78.8 ± 0.004
Mo-CBP$_3$-PepII 5xD + ITR 1x	74.5 ± 0.008
Mo-CBP$_3$-PepII 10xD + ITR 1x	73.8 ± 0.009
Mo-CBP$_3$-PepII 5xD + ITR 5xD	84.1 ± 0.001
Mo-CBP$_3$-PepII 10xD + ITR 5xD	87.2 ± 0.002
Mo-CBP$_3$-PepII 5xD + ITR 10xD	71.7 ± 0.005
Mo-CBP$_3$-PepII 10xD + ITR 10xD	70.8 ± 0.006
Mo-CBP$_3$-PepII 1x + ITR 1x	78.8 ± 0.002
*Rc*Alb-PepII 1x + ITR 1x	83.9 ± 0.001
*Rc*Alb-PepII 5xD + ITR 1x	74.8 ± 0.006
*Rc*Alb-PepII 10xD + ITR 1x	65.6 ± 0.007
*Rc*Alb-PepII 5xD + ITR 5xD	82.3 ± 0.002
*Rc*Alb-PepII10xD + ITR 5xD	63.5 ± 0.005
*Rc*Alb-PepII 5xD + ITR 10xD	71.7 ± 0.004

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*Rc*Alb-PepII 10xD + ITR 10xD	70.8 ± 0.003
RcAlb-PepIII 1x + ITR 1x	73.8 ± 0.004
RcAlb-PepIII 5xD + ITR 1x	74.5 ± 0.001
RcAlb-PepIII 10xD + ITR 1x	69.8 ± 0.01
RcAlb-PepIII 5xD + ITR 5xD	84.1 ± 0.02
RcAlb-PepIII 10xD + ITR 5xD	73.9 ± 0.009
RcAlb-PepIII 5xD + ITR 10xD	66.3 ± 0.001
RcAlb-PepIII 10xD + ITR 10xD	49.0 ± 0.008
PepGAT 1x + ITR 1x	73.4 ± 0.02
PepGAT 5xD + ITR 1x	69.4 ± 0.005
PepGAT 10xD + ITR 1x	66.3 ± 0.011
PepGAT 5xD + ITR 5xD	59.0 ± 0.02
PepGAT 10xD + ITR 5xD	54.2 ± 0.001

2.4. Cell Membrane Integrity Assay

The cell membrane integrity of *C. neoformans* was performed as described by [7]. After the incubation for 24 h, as described above, the samples were washed three times with sterile 0.15 M NaCl and centrifuged (5000× *g* 5 min at 4 °C) to remove the YPD medium. Then, the cells were incubated with Propidium Iodide (PI) for 30 min in the dark at room temperature (22 °C ± 2). Next, they were washed and centrifuged two times at the same conditions as mentioned. Finally, the cryptococcal cells were observed with a fluorescence microscope (Olympus System Bx 60, Tokyo, Japan) in a 535 nm excitation and 617 nm wavelength.

2.4. Overproduction of Reactive Oxygen Species (ROS)

The ROS overproduction was evaluated according to the method Dias et al. [12]. The analysis was conducted as described by PI assay. Next, 9 µL of 2′,7′ dichlorohfluorescein diacetate - DCFH-DA (Sigma, St. Louis, MI, USA) was added and incubated in the dark for 30 min at room temperature (22 °C ± 2). Next, they were washed and centrifuged two times at the same conditions mentioned above. Then, the cryptococcal cells were observed under a fluorescence microscope (Olympus System BX 41, Tokyo, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

2.5.3/7. caspase activity

The caspase activity was measured after cell incubation for 24 h, in the presence and absence of synthetic peptides and ITR, according to the methodology described by Qorri and Harless [13]. The samples were washed and centrifuged as described above, and next, the cells were incubated with CellEvent ® de 3 µL (ThermoFisher, São Paulo, SP, Brasil) for 30 min in the dark at room temperature (22 °C ± 2). Then, the samples were washed and centrifuged again. Finally, the cryptococcal cells were observed under a fluorescence microscope (Olympus System BX60) with an excitation wavelength of 342 nm and emission wavelength of 441 nm.

2.6. Statistical Analysis

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Experiments were performed in triplicates. The statistical analyses were performed using GraphPad Prism (version 5.01) for Microsoft Windows. All data obtained in the assays were compared using the one-way analysis of variance (ANOVA), followed by the Tukey test ($p < 0.05$).

3. Results

3.1. Combined anticryptococcal activity of synthetic peptides combined with ITR

The method used to perform the activity between synthetic peptides and ITR was that developed by Souza et al. [11]. All the concentrations without dilution [1x] used to produce the combination were defined by Aguiar et al. [7], and 5-fold dilutions [5xD] and 10-fold dilutions [10xD] combinations were done following Souza et al. [11] and all presented in supplementary table 1. In total, were performed 36 combinations between all synthetic peptides and ITR with three dilutions (Table 1).

For all experiments, a solution made of 5% DMSO in 0.15 M NaCl (DMSO-NaCl) was used as a control (Table 1). At the concentration of 500 μg mL^{-1} [1x], ITR inhibited only 45.3% of *C. neoformans* growth (Table 1). At concentrations of 100 [5xD] and 50 [10xD] μg mL^{-1} inhibitory activity of ITR against *C. neoformans* dropped to 12.2 and 4.5%, respectively (Table 1). All synthetic peptides presented the MIC_{50} at 25, 0.04, 0.04, 0.04, and 0.04, respectively, for Mo-CBP3-PepII, RcAlb-PepII, RcAlb-PepIII, PepGAT, and PepKAA (Table 1). As expected, all synthetic peptides have their inhibitory activity against *C. neoformans* affected at concentrations of 5xD and 10xD dilutions (Table 1).

Regarding the combinations made by synthetic peptides and ITR, it a threshold was established to choose the best combinations. Only the combinations with an inhibitory activity ≥ 80% were considered (Table 1). Based on that, the best combinations were Mo-CBP3-PepII [5xD] + ITR [5xD], Mo-CBP3-PepII [10xD] + ITR [5xD], RcAlb-PepII [1x] + ITR [1x], RcAlb-PepII [5xD] + ITR [5xD], and RcAlb-PepIII [5xD] + ITR [5xD] by inhibiting, respectively, 84.1, 87.2, 83.9, 82.3, and 84% the growth of *C. neoformans* (Table 1). All combinations presented great results; however, the best combination is made by Mo-CBP3-PepII [10xD] + ITR [5xD], which inhibits the growth of *C. neoformans* by 87.2% (Table 1). Alone, Mo-CBP3-PepII [10xD] and ITR [5xD] presented an inhibition, respectively, of 2.1 and 12.2% of *C. neoformans* growth (Table 1). All the best combinations were further used to understand the mechanism of action of a combined activity.

3.2. Membrane pore formation on C. neoformans cells

The first mechanism analyzed was the ability to induce pore formation on *C. neoformans* cells by the propidium iodide (PI) uptake assay. The PI uptake is based on a release of red fluorescence, which results from the interaction between PI and a cell's DNA. However, PI can only pass through a damaged membrane, and healthy membranes do not allow PI´s movement. So, red fluorescence indicates damage to the membrane. As expected, *C. neoformans* cells treated with DMSO-NaCl control solution presented no red fluorescence indicating their healthy membranes (Fig. 1 A and A1). The same result was found in cells treated with ITR 1x (Fig. 1 B and B1). Even with diluted concentrations and a reduction in activity (Table 1), all synthetic peptides Mo-CBP3-PepII 5xD, Mo-CBP3-PepII 10xD, RcAlb-PepII 1x, RcAlb-PepII 5xD, RcAlb-PepIII 5x, alone, induced pore formation on *C. neoformans* cells (Fig. 1 C to G1).

As expected, all combinations made by peptides and ITR induced pore formation in the membrane of *C. neoformans* cells (Fig. 1 H to L1). However, interestingly, all combinations presented a higher number of cells with red fluorescence, except for Mo-CBP3-PepII 10xD (Fig. 1 D and D1).

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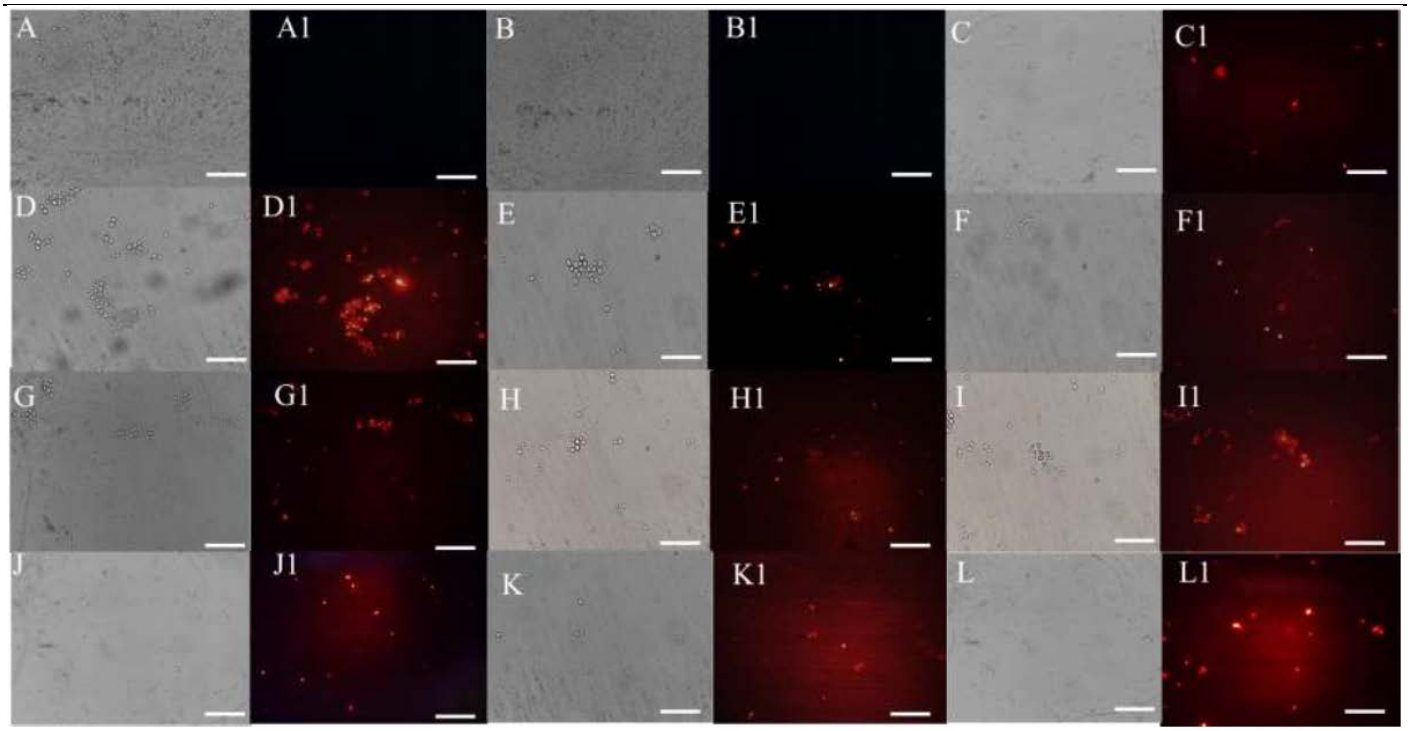

Figure 1. Fluorescence images showing membrane pore formation on *C. neoformans* cells. (A and A1) Control solution of DMSO-NaCl; (B and B1) treated with ITR 1x; (C and C1) treated with *Mo*-CBP3-PepII 5xD; (D and D1) treated with *Mo*-CBP3-PepII 10xD; (E and E1) treated with *Rc*Alb-PepII 1x; (F and F1) treated with *Rc*Alb-PepII 5xD; (G and G1) treated with *RcAlb*-PepIII 5xD; (H and H1) treated with *Mo*-CBP3-PepII 5xD + ITR 5xD; (I and I1) treated with *Mo*-CBP3-PepII 10xD + ITR 5xD; (J and J1) treated with *Rc*Alb-PepII 1x + ITR 1x; (K and K1) treated with *Rc*Alb-PepII 5xD + ITR 5xD; (L and L1) treated with *RcAlb*-PepIII 5xD + ITR 5xD. Membrane pore formation was measured by propidium iodide uptake assay. Bars: 100 μm; ITR: Itraconazole. 1x is the solution without dilution; 5xD is the solution 5-times diluted; 10xD is the solution 10-times diluted.

3.3. ROS overaccumulation and Apoptosis in C. neoformans cells

In addition to pore formation, it was evaluated the ability of induction of ROS over-production and apoptosis (Figs. 2 and 3). The control cells treated with DMSO (Fig. 2 A and A1) and ITR (Fig. 2 B and B1) presented no ROS accumulation. Among peptides alone, only RcAlb-PepII 5xD (Fig. 2 E and E1) did not induce ROS overaccumulation in *C. neoformans* cells. Regarding the combination, only the combination made by Mo-CBP3-PepII 5xD + ITR 5xD (Fig. 2 H and H1) did not induce over-accumulation. Interestingly, the combination made by RcAlb-PepIII 5xD + ITR 5xD (Fig. 2 L and L1) induced ROS overaccumulation.

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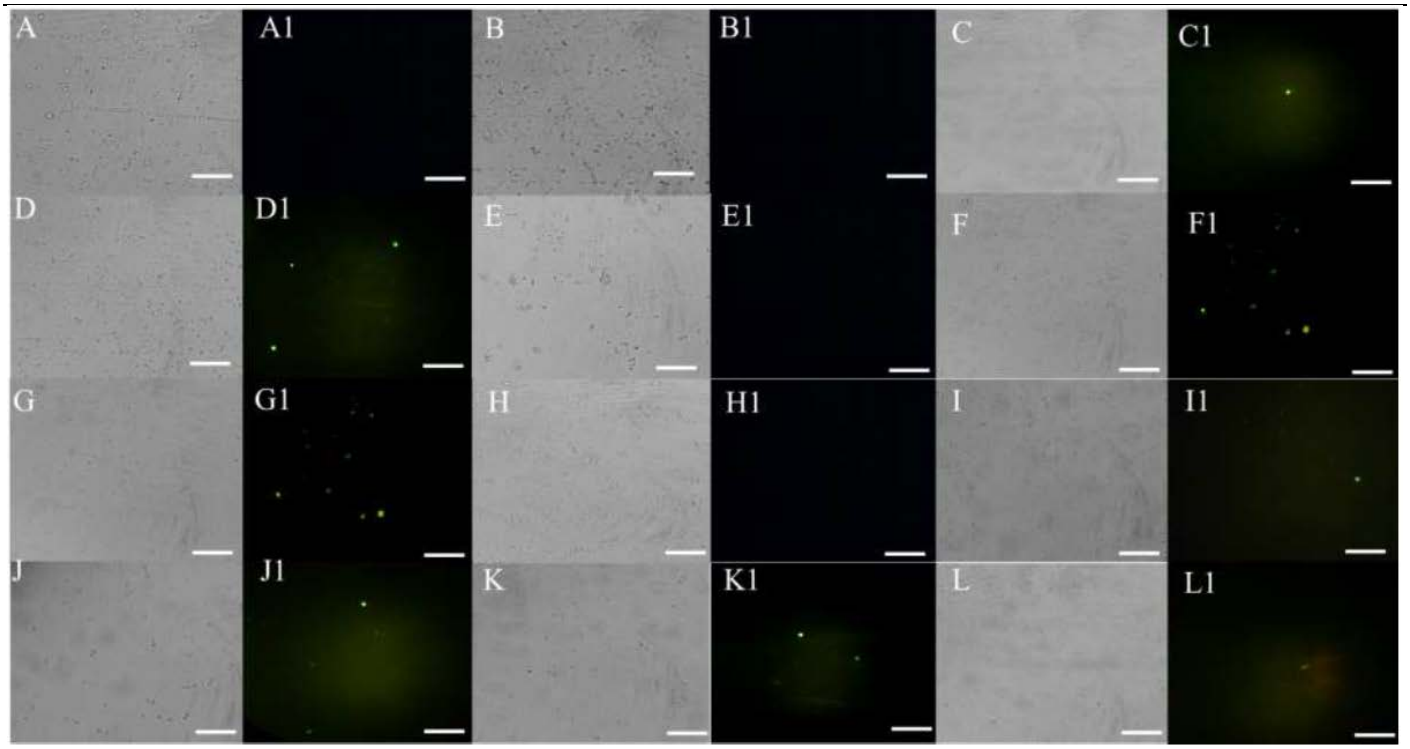

Figure 2. Fluorescence images showing ROS overaccumulation formation on *C. neoformans* cells. (A and A1) Control solution of DMSONaCl; (B and B1) treated with ITR 1x; (C and C1) treated with *Mo*-CBP3-PepII 5xD; (D and D1) treated with *Mo*-CBP3-PepII 10xD; (E and E1) treated with *Rc*Alb-PepII 1x; (F and F1) treated with RcAlb-PepII 5xD; (G and G1) treated with RcAlb-PepIII 5xD; (H and H1) treated. with Mo-CBP3-PepII 5xD + ITR 5xD; (I and I1) treated with Mo-CBP3-PepII 10xD + ITR 5xD; (J and J1) treated with RcAlb-PepII 1x + ITR 1x; (K and K1) treated with RcAlb-PepII 5xD + ITR 5xD; (L and L1) treated with RcAlb-PepIII 5xD + ITR 5xD. ROS overaccumulation was measured by DCFH-DA assay. Bars: 100 μm; ITR: Itraconazole. 1x is the solution without dilution; 5xD is the solution 5-times diluted; 10xD is the solution 10-times diluted.

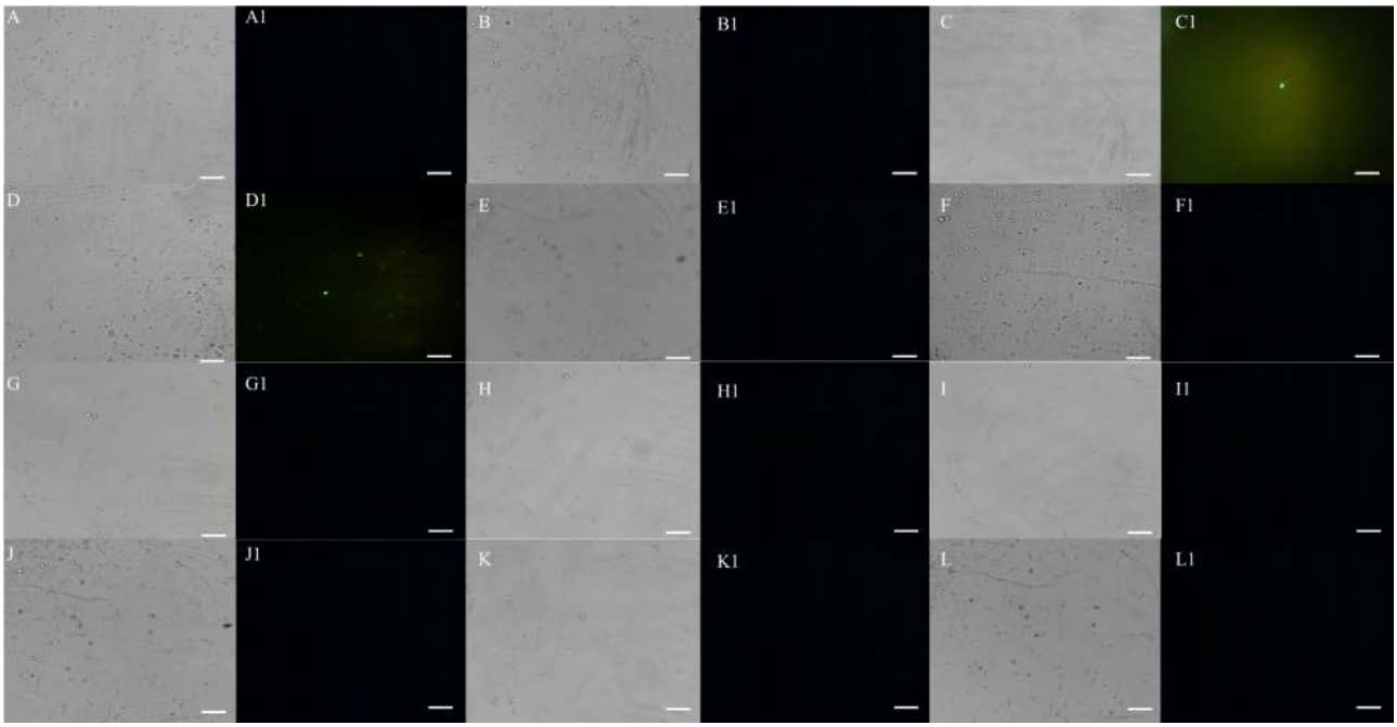

Figure 3. Fluorescence images showing 3/7-mediated apoptosis n on *C. neoformans* cells. (A and A1) Control solution of DMSO-NaCl; (B and B1) treated with ITR 1x; (C and C1) treated with Mo-CBP3-PepII 5xD; (D and D1) treated with Mo-CBP3-PepII 10xD; (E and E1) treated with RcAlb-PepII 1x; (F

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and F1) treated with RcAlb-PepII 5xD; (G and G1) treated with RcAlb-PepIII 5xD; (H and H1) treated with Mo-CBP$_3$-PepII 5xD + ITR 5xD; (I and I1) treated with Mo-CBP$_3$-PepII 10xD + ITR 5xD; (J and J1) treated with RcAlb-PepII 1x + ITR 1x; (K and K1) treated with RcAlb-PepII 5xD + ITR 5xD; (L and L1) treated with RcAlb-PepIII 5xD + ITR 5xD. Apoptosis was measured by CellEvent ki assay. Bars: 100 μm; ITR: Itraconazole. 1x is the solution without dilution; 5xD is the solution 5-times diluted; 10xD is the solution 10-times diluted.

Regarding apoptosis induction, only *C. neoformans* cells treated with Mo-CBP3-PepII 5xD and Mo-CBP3-PepII 10xD (Fig. 3 C-C1 and D-D1) presented green fluorescence as indicative of apoptosis even yet was slight. All the other treatments could not induce apoptosis in *C. neoformans* cells.

4. Discussion

Cryptococcal disease caused by *C.* neoformans has a high impact on public health worldwide. For example, cryptococcal meningitis causes 181,000 yearly, with a dramatic mortality rate of 100% without the correct treatment [14]. In addition to this alarming problem, the arsenal of drugs to treat infection caused by *C. neoformans* is quite limited. Cryptococcosis infections are treated based on antifungal drugs such as polyenes, pyrimidine analogs, and azoles. To date, one new class of antifungal drugs, echinocandins, was developed, but its effects against *C. neoformans* are disappointing [14].

Azoles such as ITR are gain attention again in the treatment of cryptococcal infection. Azole, such as ITR, has a mechanism of action based on the inhibition of ergosterol biosynthesis. The inhibition of the ergosterol synthesis seems to be caused by the formation of a complex between ITR and the group heme iron of the fungal cytochrome P450 that leads to the inhibition of lanosterol 14α-demethylase [14,15]. Several studies have shown the potential of azoles, such as ITR and fluconazole (FLU), to treat infection caused by *C. neoformans* [16–21]. Nowadays, cryptococcal meningitis therapy uses azole drugs such as FLU. However, in cases in which FLU cannot be given because the intolerance or toxicity, ITR is an acceptable alternative [21,22]. Another advantage of ITR is that it is promising for treating cryptococcosis in patients with and without acquired immunodeficiency syndrome [19].

The development of resistance to ITR by *C. neoformans* brought the need to seek alternatives to continue employing ITR in treatment. One of these alternatives is the combination of ITR with other drugs to improve its activity. The combination of drugs can prevent the emergence of pathogen-resistant [8,9,11,16,17,23]. Some recent studies tried to combine other classes of drugs, such as anti-inflammatory [17], aminoglycosides [16], and even synthetic peptides [8,9,11], with antifungal drugs to enhance their action against *C. neoformans*.

For example, Shrestha et al. [16] reported the combined activity against *C. neoformans* of the aminoglycoside K20 with ITR. Alone, K20 and ITR presented activity against *C. neoformans* at 4 and 780 μg mL^{-1}. In contrast, when combined, the concentration to reach the same activity decreases to 187 and 1 for K20 and ITR [16]. In another study, Rocha et al. [17] evaluated the synergistic effect between ibuprofen and ITR against *C. neoformans*. The authors discussed that alone the MIC50 of ibuprofen against C. neoformans was 512 μg mL^{-1} and ITR was 500 μg mL^{-1} [17]. However, the combination between them reduced the concentrations of ibuprofen and ITR, respectively, to 16 and 125 μg mL^{-1} [17]. The use of synthetic peptides in synergism with an azole is still poorly studied and yet even against *C. neoformans*. As far as we know, the most recent studies with synthetic peptides improving the activity of azoles and polyenes came from our research group [8,9,11]

Recently, two synthetic peptides bioinspired in the *Mo*-CBP$_3$ protein of *Moringa oleifera* have a synergistic antifungal effect with Itraconazole. The peptides *Mo*-CBP$_3$-PepI and *Mo*-CBP$_3$-PepIII were able to act in synergism with ITR on *Candida ssp.* biofilms, with a mechanism of action involving pore formation and overproduction of reactive oxygen

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species [8]. Another study revealed that synthetic peptides PepGAT and PepKAA designed from a chitinase from *Arabidopsis thaliana* enhance 10 times the activity of ITR against the biofilm of *C. albicans* [9].

Here, we tested different combinations of synthetic peptides and ITR against *C. neoformans*. Our results show that synthetic peptides enhanced the antifungal activity of ITR against *C. neoformans* at low concentrations (Table 1). Synthetic peptides Mo-CBP$_3$-PepII, RcAlb-PepII, RcAlb-PepIII, PepGAT, and PepKAA used in this study already presented high activity and have the mechanism of action studied against *C. neoformans* [7]. Our results revealed synthetic peptides induced membrane pore formation, DNA degradation, and apoptosis in *C. neoformans* cells. Among all combinations and peptides, the peptides Mo-CBP$_3$-PepII, RcAlb-PepII, and RcAlb-PepIII were able to improve the activity of ITR even at a concentration 5-times lower than MIC50 (Table 1).

Aguiar et al. [7] reported that the MIC50 of Mo-CBP$_3$-PepII against *C. neoformans* was 25 μg mL^{-1}. The result presented in this study revealed that even at a concentration of 2.5 μg mL^{-1} [10xD], Mo-CBP$_3$-PepII was able to improve the activity of ITR at a concentration of 100 μg mL^{-1} [5xD] (Table 1). It is noteworthy to mention that, alone at this concentration, both have no activity (Table 1). Indeed, that was the best combination found in this study. Another good result was found for RcAlb-PepII and RcAlb-PepIII. Both have an MIC50 against *C. neoformans* at 0.04 μg mL^{-1} [7]. Here, both at a concentration of 0.008 μg mL^{-1} [5xD] were able to enhance the activity of ITR at 5xD of its MIC50 concentration (Table 1).

As discussed above, the mechanism of action of ITR is based on the inhibition of ergosterol biosynthesis by inhibiting the activity of lanosterol 14α-demethylase [14]. One of the mechanisms of resistance to ITR in *Cryptococcus spp* is associated with mutations in ERG11, the responsible gene for encoding the lanosterol 14α-demethylase. However, other resistance mechanisms include efflux pumps that decrease the intracellular level of drugs and reduce membrane permeability to ITR [14].

Aguiar et al. [7] revealed that at MIC50 concentration, all peptides could induce pore formation in the *C. neoformans* membrane, as revealed by the PI uptake assay. Surprisingly, at diluted concentration and alone, all peptides induced membrane pore formation (Fig. 2) as revealed by PI uptake assay. The pore formed by peptides in the membrane of *C. neoformans* might be led to a higher concentration of ITR inside the cells, improving its activity. That may explain how peptides enhanced the activity of ITR even at a low concentration, such as 5xD concentration (Table 1). Indeed, cells of *C. neoformans* treated with a combined solution made of peptides and ITR present a higher number of cells with red fluorescence (Fig. 2).

Another important point is that the combinations made by peptides and ITR can also maintain high levels of ROS (Fig. 2) and the treatment with peptides alone. High levels of ROS are lethal to cells because ROS interact with protein, DNA, and lipids, causing loss of function [24]. Additionally, it was investigated the induction of apoptosis mediated by 3/7 caspase (Fig. 3). However, our result revealed that it is not a mechanism employed by the peptides and ITR combined.

The reduction of the concentration of ITR with higher activity is a great result of this study. However, another important point needs to be discussed. It has already been posted that the same synthetic peptides presented here can reduce the toxicity of ITR to human red blood cells (HRBC) [8,9]. As it is known, all drugs present collateral effects on the patient, and ITR is not an exception. Some side effects such as headache, dizziness, vomiting, diarrhea, cardiotoxicity, and hypertension effects [25]. In our previous study, ITR at a concentration of 1000 μg mL^{-1} alone caused 100% hemolysis of A-, B-, and O-type of HRBC [8,9]. However, when combined with the same synthetic peptides tested here, the hemolysis rate decreased to levels below 10% [8,9]. Here, the peptides raised the activity of ITR with a reduced concentration of 100 μg mL^{-1}.

5. Conclusion

Here, synthetic peptides were able to increase the activity of ITR against *C. neoformans* with a concentration 5 times lower than the MIC50. This combined effect occurs because peptides increase the cytoplasmic concentration of ITR by improving its movement through *the C. neoformans* membrane. Therefore, synthetic peptides are potential molecules for clinical application as adjuvants to commercial drugs that are becoming useless.

Author Contributions: All authors made substantial contributions. The conception and design of the study and acquisition of data, analysis, and interpretation were performed by T.K.B.A., R.M.F., N.A.S.N., E.A.M., F.I.R.G., A.C.M.C., C.D.T.F., F.P.M., P.F.N.S. Microscopic analyses were carried out by T.K.B.A., N.A.S.N., and P.F.N.S. Writing or revising the article was done by T.K.B.A., and P.F.N.S. P.F.N.S did final approval and submission. All authors have read and agreed to the published version of the manuscript.

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