*Article*

# Behind the Curtain: *in silico* and *in vitro* Experiments Brought to Light New Insights into the Anticryptococcal Action of Synthetic Peptides

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**Abstract:** *Cryptococcus neoformans* threaten the health, causing cryptococcal meningitis and pneumonia, especially in immunosuppressed patients, which can be fatal. Recently, our research group evaluated and studied the mechanisms of action of four synthetic peptides (SP) against *C.* neoformans. Here, *in silico* and *in vitro* analyses help deepen understanding of peptides' mechanisms of action. The interaction of the peptides with a membrane receptor was analyzed by docking analysis, in addition to ROS overproduction and the modulation of redox metabolism, inhibition of ergosterol biosynthesis, and release of cytochrome *c*. Out of four, three peptides interacted with membrane receptor PHO36 altering its structure and function and leading to a higher accumulation of $O_2$- and $H_2O_2$. *C. neoformans* cells treated with SP presented a reduction in the activity of antioxidant enzymes, corroborating ROS accumulation. However, in the presence of the antioxidant ascorbic acid, some peptides could not induce this oxidative stress and have the activity against *C. neoformans* affected. Curiously, two of these SPs still maintained the activity against C. neoformans and even induced the membrane pore formation as revealed by propidium iodide uptake assay, revealing their mechanism of action is ROS-independent. Additionally, SPs inhibited the biosynthesis of ergosterol, which corroborates the pore formation on the membrane of *C. neoformans* cells, inhibited the lactate dehydrogenase activity affecting the cell metabolism, and induced the release of Cyt *c* from the mitochondria inducing death by apoptosis in the cryptococcal cells. Our findings strongly suggest that SPs act by multiple mechanisms, making it difficult for *C. neoformans* to acquire resistance highlighting the potential of SPs as alternative molecules in treating infections caused by *C. neoformans*.

**Keywords:** redox system; cryptococcal meningitis; oxidative stress; ergosterol; resistance

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## 1. Introduction

Currently, the treatment against bacterial and fungal infections is limited due to the increased resistance of pathogens to drugs [1]. *C. neoformans* is a good example of a multidrug-resistant pathogen causing dangerous infections worldwide [2]. Cryptococcosis and cryptococcal meningitis caused by *C. neoformans* mainly affect people with compromised immune systems. The number 278,000 infections yearly in HIV-positive patients worldwide is estimated, leading to 181,000 deaths annually [3].

The high level of resistance presented by *C. neoformans* narrows the number of drugs that can be used in treatments. For example, *C. neoformans* presents intrinsic resistance to caspofungin, an inhibitor of the enzyme (1→3)-β-D-glucan synthase and nevertheless perturbs the turnover of the fungal cell wall [1,3,4]. Therefore, combined treatment of Amphotericin B (AmB) and flucytosine (FC) are commonly used to treat cryptococcosis infections [4]. However, prolonged exposure results in the emergence of cryptococcal populations resistant to this treatment, in addition, to the toxicity of those drugs [5].

To cope with this problem imposed by *C. neoformans*, synthetic antimicrobial peptides (SAMPs) have emerged as promising alternative molecules due to their mechanism of action, which is generally associated with membrane pore formation. This mechanism makes it difficult for microorganisms to acquire resistance and low toxicity and allergenicity [6–8]. Recently, our research group reported the anti-cryptococcal potential of SAMPs *Rc*Alb-PepII, *Rc*Alb-PepIII, PepGAT, and PepKAA at low concentrations [7]. Studies of mechanisms of action revealed that SAMPs induced membrane pore formation, DNA degradation, and apoptosis in *C. neoformans* cells [7].

Here, an *in silico* and *in vitro* approach provided new insight into the mechanism of action (*Rc*Alb-PepII, *Rc*Alb-PepIII, PepGAT, and PepKAA) peptides against *C. neoformans*. *In silico* analysis revealed that out of four, three peptides interacted with the PHO36 receptor of *C. neoformans* inducing conformational alteration on it. *In vitro* analysis showed a high accumulation of ROS in *C. neoformans* treated with peptides. In further experiments, it was analyzed that peptides caused a disbalance in redox enzymes and lactate dehydrogenase activity in *C. neoformans* cells. Additionally, peptides induced the decoupling of cytochrome *c* release from mitochondrion and inhibited ergosterol biosynthesis. Together, these results strengthen the potential application of those peptides against *C. neoformans* infections.

## 2. Results

### *2.1. ROS accumulation in C. neoformans cells*

Recently, we showed that the synthetic peptides *Rc*Alb-PepII, *Rc*Alb-PepIII, PepGAT, and PepKAA presented an $MIC_{50}$ against *C. neoformans* cells, respectively, of 25, 0.04, 0.04, 0.04, and 0.04 μg $mL^{-1}$ [7]. In the same study, some mechanisms of action were evaluated. Here in this study, new information about the mechanism of action is presented. All the experiments were performed at $MIC_{50}$ for all peptides.

The first step analyzed whether the synthetic peptides can induce the accumulation of different types of ROS. The first analysis was done to evaluate the accumulation of anion superoxide ($O2^{\bullet-}$) (Fig. 1). The experiment was made using nitro blue tetrazolium (NBT), which in the presence of $O2^{\bullet-}$ is converted into formazan with blue color. As expected, the control cells of *C. neoformans* (Fig. 1 – DMSO panel) presented no blue dots indicating no conversion of NBT in formazan and, thus, no accumulation of $O2^{\bullet-}$. In contrast, the *C. neoformans* cells treated with all synthetic peptides at $MIC_{50}$ presented a blue color suggesting the conversion of NBT by high levels of $O2^{\bullet-}$ into formazan (Fig. 1 panel of peptides blue dots – black arrow). Additionally, the quantification of formazan corroborates the data of light microscopy. All treatments presented the statistical significance of control.

In further experiments was evaluated the accumulation of $H_2O_2$ induced by peptides in *C. neoformans* cells (Fig. 2). The control cells treated with DMSO solution presented no

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accumulation of $H_2O_2$ (Fig. 2). In contrast, all peptides induced ROS accumulation in *C. neoformans* cells. Based on the brightness fluorescence, peptides RcAlb-PepIII, PepGAT, and PepKAA presented a higher accumulation of ROS than RcAlb-PepII.

*2.2. Synthetic peptides alter the activity of enzymes of ROS metabolism*

The detection of both $O2^{\bullet-}$ and $H_2O_2$ in *C. neoformans* cells treated with synthetic peptides led us to investigate the activity of enzymes involved in redox metabolism. The first enzyme analyzed was superoxide dismutase (SOD). As expected, control cells of *C. neoformans* presented the highest activity of SOD (4.98 AU $mgP^{-1}$). In contrast, *C. neoformans* cells treated with peptides RcAlb-PepII and RcAlb-PepIII presented no SOD activity. Cells of *C. neoformans* treated with PepGAT and PepKAA still presented SOD activity, but the activity values were 3 and 4 times lower than control cells (Fig. 3A).

Regarding the catalase activity (CAT), the control cells presented the highest activity compared to treated cells (Fig 3B). As happens to SOD, RcAlb-PepIII did not present CAT activity (Fig. 3B). In this case, PepKAA has no CAT activity detected. RcAlb-PepII and PepGAT presented CAT activity 3 and 5 times lower than control cells (Fig 3B).

For ascorbate peroxidase (APX), only cells treated with PepGAT presented no APX activity (Fig. 3C). The cells treated with DMSO (control) presented the highest activity (3.43 AU $mgP^{-1}$). In the case of the other peptides, RcAlb-PepIII, PepKAA, and RcAlb-PepII presented an APX activity, respectively, 7.4, 4, and 3.43 times lower than *C. neoformans* cells treated with DMSO (Fig. 3C).

*2.3. Anticryptococcal activity of peptides is affected by ascorbic acid*

To shed light on the role of ROS in the activity of synthetic peptides against *C. neoformans*, the activity was run out in the presence of an antioxidant agent, ascorbic acid (10 mM) (AsA, Fig 4). As reported above, all the experiments in this study were performed with $MIC_{50}$ concentration. As shown in Fig. 4A, in the absence of AsA, the peptides still present the $MIC_{50}$ activity (Fig. 4A white bars). However, in the presence of AsA, in which all ROS were consumed, all peptides have the activity affected. The most affected was RcAlb-PepII, which completely lost its activity (Fig. 4A dashed bars). The other peptides still presented some activity, but the activity was below 20%. To prove the absence of ROS, a microscopic fluorescence analysis was done in the presence of AsA and revealed that no ROS were produced.

As Aguiar et al. [7] revealed, all peptides can induce pore formation. Here, we aimed to evaluate if this pore formation is ROS-dependent. The fluorescence microscopy in the propidium iodide uptake assay in the presence of AsA revealed that some peptides lost the ability to induce pore formation. RcAlb-PepII, which entirely lost the activity in the presence of AsA (Fig. 4A), also lost the ability to induce pore formation in *C. neoformans cells* (Fig. 5). Likewise, PepGAT did not induce pore formation in *C. neoformans* cells in the presence of AsA (Fig. 5). However, although peptides have their activity affected in the presence of AsA, RcAlb-PepIII and PepKAA still maintain the ability to induce pore formation in *C. neoformans* cells suggesting this mechanism is not dependent of ROS (Fig. 5).

*2.4. Synthetic peptides interfere in other metabolic processes on C. neoformans cells*

Here, it was the ability of peptides to inhibit the biosynthesis of ergosterol on C. neoformans cells (Fig. 6A). As expected, the control cells treated with DMSO did not present any inhibition in ergosterol biosynthesis. In this assay, a control for inhibition was Itraconazole (ITR), inhibiting the biosynthesis of ergosterol at 47%. All synthetic peptides tested presented values of inhibition higher than ITR did. RcAlb-PepII, RcAlb-PepIII, PepGAT, and PepKAA inhibited, respectively, 80%, 85%, 75%, and 89% of the biosynthesis of ergosterol in *C. neoformans* cells (Fig. 6A).

The energetic metabolism of *C. neoformans* cells was also evaluated after treatment with peptides (Fig. 6B and C). First, it was analyzed the ability of peptides to interfere

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with the activity of Lactate dehydrogenase (LHD) from *C. neoformans* cells (Fig. B). Controls cells presented the highest activity of LDH (227.25 UA $mgP^{-1}$) (Fig. 6B). Except for RcAlb-PepII (24.21 UA $mgP^{-1}$) that presented LDH activity 10 times lower than controls cells, in the cells treated with RcAlb-PepIII, PepGAT, and PepKAA no activity of LDH was detected (Fig. 6B).

It was also analyzed if peptides could induce the release of Cytochrome c (Cyt c) from mitochondrial membranes of *C. neoformans* cells (Fig. 6C). As expected, DSMO cannot release Cyt c from mitochondrial membranes of *C. neoformans*. In this experiment, the positive control that induced Cyt c from *C. neoformans* was $H_2O_2$, which presented the highest level of Cyt c decoupling of *C. neoformans* cells (Fig. 6C). All synthetic peptides induced the decoupling of Cyt c from the mitochondrial membrane of *C. neoformans*. However, all these values were below $H_2O_2$ (Fig. 6C). Among peptides, the highest value for Cyt decoupling was presented by PepKAA.

### *2.4. Computational simulations*

Aiming to produce more information about the mechanisms of action of synthetic peptides, we performed a docking analysis to try to explain more about the action of peptides. The protein chosen was the membrane receptor PHO36 from *C. neoformans*. First, the sequence of PHO36 from Saccharomyces cerevisiae was used to find the sequence of PHO36 from *C. neoformans*. After finding the protein sequence, the three-dimensional (3D) model was built using the Swiss model server. Then, ClusPro 2.0 Web Server was used to perform the docking analysis. PHO36 is a transmembrane protein. Based on that, it was only considered for docking analysis the peptides that did not interact in the transmembrane domain, as shown in figure 7 (red dashed lines). Among all peptides tested, RcAlb-PepIII was the only peptide that interacted in the transmembrane domain, so the result was not considered (data not shown).

Contrary to RcAlb-PepIII, all the other peptides interacted with PHO36 in the extracellular domain (Fig. 7). The binding energy of peptides with PHO36 was -632.98 kCal $mol^{-1}$, -678.98 kCal $mol^{-1}$, and 578.12 kCal $mol^{-1}$, respectively, for RcAlb-PepIII, PepGAT, and PepKAA. An analysis of root-mean-square deviation (RMSD) indicates changing in atomic position and then in the 3D structure of PHO36. The values of RMSD were 1.542, 0.876, and 1.247, respectively, for RcAlb-PepIII, PepGAT, and PepKAA. These values indicate that the interaction of peptides with PHO36 changes its structure and thus functions for cells (Fig. 7). The peptides interact with the PHO36 receptor from *C. neoformans* supported by hydrogen bonds and salt bridge type interactions.

## 3. Discussion

*C. neoformans* is a human pathogenic yeast responsible for severe infections, mainly in patients with a compromised immune system, such as those with $HIV^+$, patients with transplanted organs, and in intensive care units [1,2,4]. As *C. neoformans* is resistant to several drugs used in the treatment, it becomes essential to search for bioactive molecules as an alternative to conventional treatment [9,10]. This study was developed based on this emergence to find new molecules to overcome the C. neoformans resistance to drugs. Here, we provide new mechanisms behind the activity of four synthetic peptides against *C. neoformans*.

Our SAMPs demonstrated inhibitory activity (MIC50) in a previous study at low concentrations [7]. The mechanisms evaluated at that time were pore formation, DNA damage, apoptosis induction, and damage to the cell wall and cell membrane caused by peptides [7]. Based on previously published results regarding DNA damage and apoptosis induction, we started the analysis by evaluating the redox metabolism in *C. neoformans* cells after contact with peptides (Figs. 1-3). The induction of ROS overaccumulation in microorganisms by peptides is not a surprise, but it could explain a lot about the mechanism of action of peptides [11–14].

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ROS is generic to include many molecules such as singlet oxygen ($^1O_2^-$) superoxide anion ($\bullet O_2^-$), hydroxyl radical ($\bullet OH$), and hydrogen peroxide ($H_2O_2$) [15]. In pathogenic fungi with a controlled production, ROS displays many different beneficial roles to pathogens, such as developmental process, increased virulence, biofilm formation, and infection [15]. On the other hand, ROS is a byproduct of the natural metabolic process in cells. Without a proper scavenger system to balance their levels, ROS could bring damage to cells by interaction with vital molecules such as DNA, lipids, and protein, leading to death [15].

Usually, $H_2O_2$ is the main molecule analyzed in experiments of ROS accumulation induced by peptides in cells because it is more stable and easy to evaluate [11–13]. Here, to better picture the redox state in *C. neoformans* cells, we analyzed the accumulation of $\bullet O_2^-$ (Fig. 1), which is one of the most unstable ROS and is rapidly converted in $H_2O_2$ [15]. Our results revealed a higher accumulation of $\bullet O_2^-$ in *C. neoformans* cells after treatment with peptides (Fig. 1). Uncontrolled accumulation of $\bullet O_2^-$ accelerates the oxidative damage to DNA molecules caused by iron. The $\bullet O_2^-$ induces the increase in iron levels by releasing it from proteins and enzyme clusters. The free iron interacts with DNA molecules oxidizing it, leading to fragmentation [16]. This result is in accordance with our previously published result that *C. neoformans* cells presented fragmented DNA after treatment with the same synthetic peptides [7]. To prevent the damage caused by $\bullet O_2^-$, cells use the SOD enzyme to produce $H_2O_2$, which is more stable than $\bullet O_2^-$ but still lethal [15]. Our results revealed a high accumulation of $H_2O_2$ in *C. neoformans* cells after incubation with peptides (Fig. 2).

Although $H_2O_2$ induces damage to DNA molecules as $\bullet O_2^-$, it usually has other targets such as proteins and lipids. In the case of lipids, $H_2O_2$ causes the oxidation of lipids in the membrane in a process known as lipid peroxidation. This process could lead to membrane destabilization and, consequently, pore formation, increasing membrane permeability [17,18]. In addition, H2O2 also interact with proteins, damaging them and inhibiting their activity [19]. Recently, Branco et al. [19], using a proteomic approach, revealed that *Klebsiella pneumoniae* cells treated with a synthetic peptide presented a high accumulation of $H_2O_2$ followed by an increase in the accumulation of proteins involved in the recovery of proteins damaged by ROS. This result suggests that the higher levels of $H_2O_2$ are involved with protein damage and agrees with our hypothesis.

It is clear that synthetic peptides cause a perturbation in redox homeostasis of $\bullet O_2^-$ and $H_2O_2$ (Figs. 1 and 2). However, more information about somehow peptides do is necessary. Based on that, the activity of scavenger enzymes was evaluated in *C. neoformans* cells. The enzymes evaluated were SOD, CAT, and APX (Fig. 3). First, it is necessary to understand the role of these enzymes in ROS metabolism. SOD enzymes are involved in the conversion of $\bullet O_2^-$ into $H_2O_2$; CAT and APX are responsible for converting $H_2O_2$ into $H_2O$ and $O_2$ [15]. These enzymes are responsible for the tiny balance in ROS levels that tell apart the beneficial and harmful effects of ROS.

As revealed in Fig. 3A, *C. neoformans* cells treated with all peptides presented a reduced activity of SOD. This reduced activity of SOD activity is responsible for two problems: (1) the reduced activity of the SOD enzyme is responsible for low levels of conversion of $\bullet O_2^-$ into $H_2O_2$, leading to accumulation of $\bullet O_2^-$ (Fig. 1 blue dots). (2) Low activity of SOD in *C. neoformans* cells treated with peptides is still associated with the $H_2O_2$ even if it is at low concentration. However, the activity of CAT and APX (Fig. 3B and C), which are involved in scavenging of $H_2O_2$, is also reduced in cells treated with peptides leading to the accumulation of $H_2O_2$ in *C. neoformans* cells (Fig. 2 green fluorescence). Therefore, synthetic peptides by an unknown mechanism insult the balance between SOD (converts $\bullet O_2^-$ into $H_2O_2$) and CAT and APX ($H_2O_2$ in $H_2O$ and $O_2$), producing a scenario that $\bullet O_2^-$ and $H_2O_2$ (Figs. 1 and 2) accumulate at the same in the *C. neoformans* cells potentializing the damage caused by ROS. As far as we know, our study is the first to show ROS accumulation and propose how peptides induce that by negatively modulating the activity of redox enzymes involved in ROS metabolism.

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There are not many studies with similar results to ours to redox enzymes with peptides on yeasts, even to *C. neoformans*. However, Neto et al. [20] reported that a chitin-binding protein purified from *Moringa oleifera* seeds also caused perturbation in the redox enzymes leading to the accumulation of ROS. In that case, the authors only measured the accumulation of $H_2O_2$.

Our data revealed that ROS plays an important role in the antimicrobial activity of synthetic peptides. However, one question arises: Is the antimicrobial action of peptides fully or partially dependent on ROS? An experiment with the antioxidant AsA provided new clues to this question. In the presence of 10 mM of AsA, all peptides have the activity affected (Fig. 4A). The most affected peptide, RcAlb-PepII, completely lost its activity. Similar results were posted by Neto et al. [20] for an anticandidal protein that had its activity reduced by 60% in the presence of AsA. Fluorescence microscopy (Fig. 4B-F) proved no ROS accumulation in *C. neoformans* treated with peptides in the presence of AsA.

A common mechanism of action of peptides against pathogens is the induction of pore formation on the membrane, increasing membrane permeabilization, loss of internal content, and death [21,22]. The pore formation process depends on many aspects. It could be driven directly by the interaction of peptides with lipids in the membrane or an indirect process driven by ROS species [13,15,18]. In previous work, Aguiar et al. [7] showed that all synthetic peptides could induce pore formation in C. neoformans membrane. Here, as shown, the same peptides have activity in the absence of ROS, which was consumed by AsA. Here, we try to understand if the ability of peptides to form pores is dependent or nor ROS accumulation. To do so, peptides were incubated with *C. neoformans* cells and AsA. After incubation, an iodide propidium uptake assay was done. The result was quite surprising and exciting (Fig. 5). The peptides RcAlb-PepII and PepGAT lost the ability to induce pore formation in *C. neoformans* membranes (Fig. 5). For RcAlb-PepII, the result corroborates the lost of activity in the AsA (Fig. 4A).

The exciting results came to RcAlb-PepIII and PepKAA, which, even in AsA preventing ROS accumulation (Fig. 4), were able to induce pore formation in *C. neoformans* cells. These results suggest that the ability to induce pore formation of these peptides is not ROS-dependent and might be driven by the direct interaction of peptides with the membrane. RcAlb-PepIII and PepKAA are cationic peptides with a net charge, respectively, of +1 and +3 and hydrophobic potential [8,23]. These features are important to pore formation in two steps: 1) positive charge is important to ionic interaction with the negative charge of lipid heads in the membrane, and 2) hydrophobic potential is critical for inserting peptides in the membrane hydrophobic core [8].

In our previous study [7], we observed that the presence of exogenous ergosterol affected the activity of peptides against *C. neoformans*, suggesting that peptides can bind to sterol in fungal membranes [7]. Therefore, we experimented with verifying whether peptides also inhibit ergosterol biosynthesis. In this experiment, the control was the antifungal drug ITR (Fig. 6A). All peptides presented inhibition higher than ITR. ITR is an antifungal drug belonging to the azole class, whose main mechanism is to inhibit the ergosterol synthesis pathway. Our results demonstrate that peptides are more effective in inhibiting biosynthesis than ITR. Recently, the antifungal $MoCBP_2$ protein purified from *M. oleifera* seeds could not inhibit the biosynthesis of ergosterol. New targets and different mechanisms in potential new drugs are important due the resistance fungal to the current antifungal [24].

All our data suggests that synthetic peptides dysregulate the redox metabolism of *C. neoformans* cells. As we know, ROS are natural byproducts of cell metabolism [15]. The energetic metabolism is essential to cell response to environmental insults because it provides energy as NADPH and ATP are used to produce response proteins [25]. Even the importance of energetic metabolism to cells, studies reporting alterations caused by peptides in energetic metabolism are scarce. Here, we try to understand if peptides could cause perturbation in *C. neoformans* production of energy. First, the activity of the LDH

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enzyme in C. neoformans cells was analyzed after the treatment with peptides. All peptides dramatically reduced the activity of LDH (Fig. 6B).

LDH is a crucial enzyme in the carbohydrate metabolic pathway, and it catalyzes the conversion of pyruvate into lactate, regenerating the $NAD^+$ from NADH [26]. This reaction is important to regenerate the $NAD^+$ to maintain the glycolytic pathway and produce ATP and pyruvate to run the Krebs cycle [26]. Another experiment suggests that peptides interfere in the energetic metabolism of *C. neoformans* cells. The analysis of Cyt c decoupling from the mitochondrial membrane induced by peptides indicates that peptides interfere with mitochondria's energy production.

By inducing the decoupling of Cyt c from mitochondrial membrane peptides cause two problems for C. neoformans cells. First, Cyt c is a key molecule in the electron transport chain (ETC) to support ATP synthesis [27]. Inducing the decoupling of Cyt c peptides to destabilize the ETC leads to a depletion in ATP levels of the cell. Second, Cyt c release of mitochondrial membrane acts as a stimulus for cells to start apoptosis. So, peptides may be inducing this event. It is essential to notice that all peptides induced apoptosis in *C. neoformans* cells, as revealed by our previous published study [7].

In an attempt to find possible protein targets for peptides to induce all these damages in *C. neoformans* cells, computational simulations were employed. The target chosen is a transmembrane protein known as PHO36. The PHO36 is a receptor adiponectin-like involved in lipid and phosphate metabolism in yeasts [28]. PHO36 works with RAS proteins in the same pathway involved in several cellular events essential for yeasts' life, such as division, apoptosis, longevity, differentiation, nitrogen, and carbon nutrition [28].

Here, molecular modeling analysis revealed that RcAlb-PepII, PepGAT, and PepKAA interact with PHO36 in the extracellular domain resulting in conformational alteration in its structure. By interacting with PHO36 and changing its structures, peptides inhibit PHO36 function in cells, negatively affecting several cellular processes in yeasts. Additionally, misfunction is related to a stimulus for apoptosis in yeast cells. Lopes et al. [29] recently reported a synthetic peptide interacting with PHO36 from *C. albicans* induced ROS accumulation, DNA fragmentation, and Apoptosis. Our results revealed that RcAlb-PepII, PepGAT, and PepKAA interact with PHO36 and cause the same damage. These results suggest PHO36 as a new target for antimicrobial activity mediated by synthetic peptides.

## 4. Conclusion

The synthetic peptides evaluated in this study display anticryptococcal activity by multiple mechanisms of action. Synthetic peptides interfere with the redox enzymes leading to the accumulation of ROS, which are involved in cell death. It was also shown that some peptides induced pore formation in a ROS-dependent manner, while others did the same in a ROS-independent manner. All peptides caused perturbation in energetic metabolism by inhibiting the activity of LDH and decoupling Cyt c from the mitochondrial membrane. Altogether these results reinforce the potential of these synthetic peptides against *C. neoformans* and bring them to promise to develop new forms of treatment against *C. neoformans* infections.

## 5. Materials and methods

### *5.1. Fungal strains, chemicals, and synthetic peptides*

*C. neoformans* (ATCC 32045) was obtained from the Department of Biochemistry and Molecular Biology at the Federal University of Ceará (UFC), Fortaleza, Brazil. All the chemicals used in the experiments were obtained from Sigma Aldrich (São Paulo, SP, Brazil). The synthetic peptides *Rc*Alb-PepII, *Rc*Alb-PepIII, PepGAT, and PepKAA, were chemically synthesized by the company Chempeptide (Shanghai, China). The quality and purity (≥ 95%) were analyzed by reverse-phase high-performance liquid chromatography (RP-HPLC, Jasco, Easton, MD, USA) and mass spectrometry (Waltham, MA, USA).

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*5.2. Antifungal assay*

The antifungal assay was performed following the methodology [7,30]. *C. neoformans* was cultivated in YPD agar for approximately 15 days, then cells were resuspended in a YPD medium. Because the MIC found previously was 0.04 μg $mL^{-1}$ [7] for all synthetics peptides, that was the concentration chosen to perform all the study of mechanisms. So, 25 μL of YPD with cryptococcal cells ($10^6$ cells $mL^{-1}$) and 25 μL of each synthetic peptide at the final concentration (0.04 μg $mL^{-1}$) were added. Then, the samples were incubated for 24 h at 30 °C before each assay.

*5.3. Detection of ROS overproduction*

To evaluate the peptide-induced ROS generation ($H_2O_2$), it was performed the fluorometric assay with 2′,7′ dichlorofluorescein diacetate (DCFH-DA, Sigma, St. Louis, MI, USA). Briefly, after the antifungal assay as described above, the samples were washed three times with NaCl 0.15 M and centrifuged at 5000 x g for 10 min at 4° C. Next, 9 μL of DCFH-DA was added and incubated in the dark for 20 min at 25 ° C. Then, the samples were washed two times with NaCl 0.15 mM and centrifuged as described. Finally, cryptococcal cells were transferred to slides and observed with a fluorescence microscope (Olympus System BX 41, Tokyo, Japan) with an excitation wavelength of 535 nm and emission wavelength of 617 nm [31].

Qualitative and quantitative assays for anion superoxide were performed following the methodology described by Choi et al. [32]. For the qualitative assay, *C. neoformans* cells were treated with the peptides described above. Then, cells were washed with 0.15 M NaCl to remove the excess media. Afterward, cells were incubated with 0.1 mM of nitroblue tetrazolium (NBT) for 3 h at room temperature (22 ± 2 °C) in the dark. Then cells were visualized in a light microscope (Olympus System BX 41, Tokyo, Japan). The quantitative assay was placed in the same way as the qualitative. The difference was that the quantitative assay was performed in 96-well plates and the conversion of NBT to formazan was quantified at 630 nm in an automated microplate reader (Epoke, Biotek).

In addition, the same assay to detect $H_2O_2$ was performed in the presence of 10 mM ascorbic acid (AsA) [20]. Besides, the pore formation in the presence and absence of 10 mM AsA using the Propidium Iodide (PI) influx assay, following the methodology described in [7].

*5.4. Redox system enzyme activity*

5.4.1. Catalase (CAT)

The assay was performed according to [33] to evaluate the catalase activity. After the antifungal assay at the same conditions described previously, cells were washed three times with 0.15 M NaCl, resuspended in 0.05 M sodium acetate buffer pH 5.2, frozen for 24 h, sonicated for 30 min, centrifuged for 10 min, 10000 g at 4 °C and the supernatant collected as described by [20]. 200 μL of samples were incubated with 700 μL phosphate buffer of 50 mM potassium, pH 7.0 at 30 °C for 10 min. Subsequently, 100 μL of 112 mM H2O2 was added, starting the reaction. The reaction medium was transferred to a quartz cuvette (1 cm) and the absorbance was measured in a spectrophotometer (Biochron, Libra S12). The decrease in absorbance at 240 nm was observed at intervals of 10 seconds to 1 minute. A decrease of 1.0 absorbance unit per minute was assumed to be 1 unit of catalase activity (AU).

5.4.2. Ascorbate Peroxidase (APX)

Ascorbate peroxidase activity was evaluated following the methodology previously described by Souza et al. [33]. After the antifungal assay, in tubes of 800 μL of 50 mM potassium phosphate buffer, pH 6.0, containing 0.5 mM of L-ascorbic acid, 100 μL of 2 mM hydrogen peroxide in 100 μL of treated sample or control. Then, they were incubated at 30 °C for 10 minutes. The enzymatic activity was measured through ascorbate

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oxidation, indicating the action of the enzyme, for 1 minute at 10s intervals in the spectrophotometer at the length of wave 290 nm. Ascorbate peroxidase activity was expressed (UA) by reducing 0.01 in absorbance at 290 nm, indicating the use of ascorbate to remove $H_2O_2$ by milligram of protein (UA/mg).

4.4.3. Superoxide dismutase (SOD)

Superoxide dismutase activity was measured according to Souza et al. [33] in 96-well microplates. In triplicate, 1 M potassium phosphate buffer, pH 7.8 (10 µL), 1 mM 2,2',2'',2'''- ethylenediaminetetraacetic acid (EDTA) (20 µL), 10 µL of Triton x 0.25%, 20 µL of 130 mM L-Methionine, 100 µL of samples in deionized water in the presence and absence of peptides (MIC50) and 100 mM of riboflavin (20 µL) were homogenized and kept in the dark for 5 min. After that, aliquots of the reaction medium were placed in a 96-well microplate and read at 630 nm. The microplate was exposed to fluorescent light of 32 W, and the absorbances will be measured at 630 nm in an automated microplate reader (Epoke, Biotek) at intervals of 1 min to 5 min when the reaction was stopped by turning off the light. Blanks consisted of all reagents without yeast extract (replaced by ultrapure water). The enzyme activity was measured as the difference between the absorbance recorded for the light reaction and the corresponding dark reaction (estimated per min). It was expressed in activity units (AU). One unit of SOD activity (1 AU) will correspond to the amount of sample needed to inhibit the photoreduction of NBT by 50%.

*5.5. Ergosterol biosynthesis inhibition*

The ergosterol biosynthesis inhibition was evaluated following the method described previously by Neto et al. [20]. The cryptococcal cells were cultured in YPD medium in the presence of synthetic peptides ($MIC_{50}$), 5% DMSO, and Itraconazole (1000 µg $mL^{-1}$) for 24 h, 30 °C. Next, cells were centrifuged at 3000g for 5 min, and the pellet was dried and weighed. Then, 2 mL of 25% alcoholic sodium hydroxide solution (m/v) was added to each pellet and vortexed for 1 min. To sterol extraction, it was added of 4 mL of sterile 75% *n*-hexane and vortexed to homogenize. After 400 µL of 100% ethanol was added to 200 µL of sterol extract, mixed, and the absorbance was measured at 230 and 282 nm in quartz cuvettes. Ergosterol content was calculated based on the equations:

Eq. 1: % ergosterol + 24(28) [DHE=(Abs282/290) x F] / pellet weight

Eq. 2: % 23(28) DHE =[(Abs230/518) x F] / pellet weight

Eq. 3: % ergosterol = % ergosterol + 24(28) DHE - % 24(28) DHE

24(28) DHE refers to 24(28) dehydroergosterol, a class of sterol that presents an absorbance reading similar to ergosterol at 282 nm. F, in both equations, represents the factor for dilution in ethanol.

*4.6. Lactate dehydrogenase activity*

The LDH Liquiform™ kit (Labtest Diagnóstica, BR) was used to evaluate lactate dehydrogenase activity, following the manufacturer's instructions.

*4.7. Cytochrome c release*

Because cytochrome *c* release is related to apoptotic events in cells, we evaluated the induction of cytochrome *c* release by peptides following the methodology described in Neto et al. [20]. The cryptococcal cells were incubated with 50 µL of synthetic peptides or H2O2 (10 mM) at 30 °C for 24 h. After this, 100 µL of buffer 50 mM Tris-HCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 6% glucose, pH 7.5 was added to the cell suspension and homogenized. Then, the cells were centrifuged at 2000g at 4 °C for 10 min, and the supernatant was gently collected and packed in microtubes. The pellet was washed in buffer 50 mM Tris, 2 mM EDTA, pH 7.5, and centrifuged at 6000g for 30 sec. The supernatant was discarded, and the mitochondria were suspended in 100 µL of buffer 50 mM Tris, 2 mM EDTA, pH 7.5. Then, 100 µL of cytosolic and mitochondrial cytochrome

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c suspension was treated with 30 mM of ascorbic acid (100 µL) for 5 min at 30 °C. After this, the optical density of the obtained solution was measured using a microtiter plate reader at 550 nm.

*5.8. Bioinformatics assays*

5.8.1. Identification of PHO36 homolog receptor in C. neoformans genome

The *C. neoformans* amino acid sequence for PHO36 was taken homolog genes from the NCBI database (National Center for Biotechnology Information) (http://www.ncbi.nlm.nih.gov) through a basic local alignment search tool (BLAST) using the sequence of Saccharomyces cerevisiae.

5.8.2. Molecular modeling

The three-dimensional (3D) models of the PHO36 from *C. neoformans* were by comparative modeling using the A chain of the revised crystals of the adiponectin receptors (PDB code: 5LXG and 5LWY) was done by Lopes et al. [29], using the SWISS-MODEL (https://swissmodel.expasy.org/interactive) to generate the models for PHO36 from C. neoformans. The Macromolecular X-Ray Crystallography Software was used to fix the Phi and Psi angles of the 3D models. Then, the 3D model was refined using the ModRefiner atomic-level protein structure refinement algorithm was used to minimize the energy of the models (https://zhaglab.ccmb.med.umich.edu/ModRefiner/). The stereochemical quality of the predicted models was evaluated with Molprobity (http://molprobity.biochem.duke.edu/) by Ramachandran plot analysis. Also, the global quality factor and the reliability of the models' folding were evaluated by ERRAT2 (http://servicesn.mbi.ucla.edu/ERRAT/) and Verify3D (http://servicesn.mbi.ucla.edu/Verify3D/), respectively. The overall model quality for the final structures was determined using the Protein Structure Analysis server (https://prosa.services.came.sbg.ac.at/prosa.php). The best 3D model was submitted to the simulation of interaction (receptor and each peptide).

5.8.3. Molecular docking

Molecular docking studies between the synthetic peptides (ligands) and the plasma membrane receptor of *C. neoformans* were performed using the protein-protein ClusPro 2.0 docking server (https://cluspro.bu.edu/login.php), and the output files were analyzed using the PyMol program.

*5.9. Statistical Analysis*

All experiments were performed three times and the values are expressed as the mean ± standard error. GraphPad Prism 5.01 (GraphPad Software company, Santa Clara, CA, USA) for Microsoft Windows was used to run the statistical analyses. All data obtained in the assays were compared using ANOVA, followed by the Tukey test ($p < 0.05$).

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