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Antifungal and anti-biofilm activity of designed derivatives from kyotorphin

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ABSTRACT

Kyotorphin (KTP, L-tyrosyl-L-arginine) is an endogenous analgesic neuropeptide first isolated from bovine brain in 1979. Previous studies have shown that kyotorphins possess anti-inflammatory and antimicrobial activity. Six kyotorphins—KTP-NH_2, KTP–NH_2–DL, ibuprofen-conjugated KTP (IbKTP), IbKTP-NH_2, N-methyl-D-Tyr-L-Arg, and N-methyl-L-Tyr-D-Arg—were designed and synthesized to improve lipophilicity and resistance to enzymatic degradation. This study assessed the antimicrobial and antibiofilm activity of these peptides. The antifungal activity of kyotorphins was determined in representative strains of Candida species, including *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258, and six clinical isolates—*Candida dubliniensis* 19-S, *Candida glabrata* 217-S, *Candida lusitaniae* 14-S, *Candida novergensis* 51-S, *Candida parapsilosis* 63, and *Candida tropicalis* 140-S—obtained from the oral cavity of HIV-positive patients. The peptides were synthesized by standard solution or solid-phase synthesis, purified by RP-HPLC (purity >95 %), and characterized by nuclear magnetic resonance. The results of the broth microdilution assay and scanning electron microscopy showed that IbKTP-NH_2 presented significant antifungal activity against Candida strains and antibiofilm activity against the clinical isolates. The absence of toxic activity and survival after infection was assessed after injecting the peptide in larvae of *Galleria mellonella* as experimental infection model. Furthermore, IbKTP-NH_2 had strong antimicrobial activity against multidrug-resistant bacteria and fungi and was not toxic to *G. mellonella* larvae up to a concentration of 500 mM. These results suggest that IbKTP-NH_2, in addition to its known effect on cell membranes, can elicit a cellular immune response and, therefore, is promising for biomedical application.

1. Introduction

Infections caused by microorganisms strongly resistant to antimicrobials represent a global public health problem, leading to an increase in mortality rates, hospitalization time, and health care costs (Neidell et al., 2012). Infections caused by yeast species of the genus Candida are associated with high mortality rates (Gudlaugsson et al., 2003), which highlights the need to develop new antifungal agents. Candida is a commensal heterogeneous group of fungal species, and some species are dimorphic (Sanches et al., 2019). Several Candida species can infect vital organs, including the central nervous system (CNS) (Li et al., 2017), given their ability to cross the blood–brain barrier (BBB) (Jong et al., 2001; Wu et al., 2019). Moreover, Candida overgrowth may cause a wide range of infections in immunocompromised patients, from superficial candidiasis to septicemia. *Candida albicans* and, to a lesser extent, *Candida glabrata*, *Candida parapsilosis, Candida krusei*, and *Candida tropicalis*, are the most pathogenic species (Sullivan et al., 2004; Turner and Butler, 2014). Biofilms of Candida spp.

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may confer high levels of resistance and aggravate chronic inflammatory diseases, especially those located in the oral, vaginal, and intestinal mucosa, as well as acute and systemic infections caused by Candida spp (Harriott and Noverr, 2011; Van De Veerdonk et al., 2010; Douglas, 2003). The use of implantable biomaterials has increased human life expectancy by reestablishing vital functions, but at the same time, it exacerbates this problem, and Candida infections associated with biomaterials are a major clinical problem (Busscher et al., 2012; Trentin et al., 2015).

Systemic antifungal therapies are usually active against fungi that are released from the cells dispersed by biofilm (planktonic cells), but are partially effective against biofilms (Sanches et al., 2019). Bioactive peptides have emerged in recent years as an alternative strategy for treating antimicrobial-resistant bacteria and fungi (Dosler et al.,2016; Fjell et al., 2012; Ribeiro et al., 2013; Scarsini et al., 2015). As signaling molecules involved in many physiological functions, peptides present an opportunity for therapeutic development that mimics natural pathways. Moreover, the relationship between peptide structure and function has been studied extensively. Peptides have a high application potential in human health for diagnosis and therapy given their multiple biological functions, reduced size, low immunogenicity, high chemical stability, and fast production due to recent advances in chemical synthesis (Hayashi et al., 2012; Lau and Dunn, 2018).

It has been shown that antimicrobial peptides (AMPs) can prevent and/or eradicate bacterial biofilms independently of the antimicrobial activity against free planktonic microorganisms (De la Fuente et al., 2014; Ribeiro et al., 2015; Pletzer et al., 2016). Kyotorphin (KTP, Arg–Tyr) is an endogenous analgesic neuropeptide (Takagi et al., 1979). KTP has been found in the brain and can act as a biomarker for Alzheimer's disease (Perazzo et al., 2017). However, the inability of KTP to cross the BBB and/or high susceptibility to enzymatic degradation may limit its pharmacological potential (Perazzo et al., 2016). Ribeiro et al. (2011a) designed several kyotorphins by C-terminal amidation (KTP-NH2), N-terminal conjugation to ibuprofen, and C-terminal amidation (IbKTP-NH2). Furthermore, kyotorphins with D amino acids can reach the CNS, have anti-inflammatory activity, and are less susceptible to enzymatic degradation (Ribeiro et al, 2011a, 2011b; Conceição et al., 2015). Ribeiro et al. (2012) showed that KTP-NH_2 presents high antimicrobial activity, which opens perspectives for new studies.

The present study further assessed the antifungal and antibiofilm activity of kyotorphins. The antifungal activity was analyzed in two reference strains, *C. albicans* ATCC 10231 and *C. krusei* ATCC 6258, and six clinical isolates—*Candida dubliniensis C. glabrata, C. parapsilosis, Candida novergensis*, *Candida lusitaniae*, and *C. tropicalis*. IbKTP-NH_2 presented significant broad-spectrum antimicrobial activity against the analyzed strains, and scanning electron microscopy (SEM) showed that this peptide had antibiofilm activity against Candida spp. Toxicity was evaluated by injecting the peptide in larvae of the greater wax moth, *Galleria mellonella* as an *in vivo* model. IbKTP-NH_2 exhibited strong antimicrobial activity against multidrug-resistant fungi and was not toxic to larvae at a concentration of up to 500 μM. These results suggest that IbKTP-NH_2, in addition to its known effect on cell membranes, can elicit a cellular immune response and therefore is promising for biomedical applications.

2. Material and methods

2.1. Yeast strains and culture conditions

Two reference strains—*C. albicans* ATCC 10231 and *C. krusei* ATCC 6258—and six clinical isolates—*C. dubliniensis* 19-S, *C. glabrata* 217-S, *C. lusitaniae* 14-S, *C. novergensis* 51-S, *C. parapsilosis* 63, and *C. tropicalis* 140-S—were evaluated in this study. The clinical isolates were obtained from lesions of oropharyngeal candidiasis (strains 28 and 63) or saliva of HIV-positive patients of the Institute of Infectology Emílio Ribas (other strains) (Junqueira et al., 2012). All strains were stored in 20 % (v/v) glycerol at −80 °C. Before the assays, cells were subcultured on HiChrome agar (HiMedia Laboratories, Mumbai, India) and grown at 37 °C for 48 h. Observing the respective colors of each colony and thus avoid contamination between different strains (Santos et al., 2016).

2.2. Peptides

The kyotorphins H-D-Tyr-L-Arg-NH_2 (KTP–NH_2–DL), H-L-Tyr-D-Arg-NH_2 (KTP–NH_2–LD), H-D-Tyr-D-Arg-NH_2 (KTP–NH_2–DD), H–N-methyl-L-Tyr-D-Arg (Me-KTP–NH_2–LD), H–N-methyl-L-Tyr-L-Arg-NH_2 (Me-KTP-NH_2), L-Tyr-L-Arg-NH_2 (KTP-NH_2), ibuprofen-Tyr-Arg (IbKTP), and ibuprofen-Tyr-Arg-NH_2 (IbKTP-NH_2) were produced on a small scale through standard solid-phase synthesis using 9-fluorenylmethoxycarbonyl (Fmoc)/tertbutyl (t-Bu), as described by Perazzo et al. (2016) and Ribeiro et al. (2011a and b). All peptides were purified by RP-HPLC, and purity was assessed by mass spectrometry and nuclear magnetic resonance. Peptide stocks were prepared by resuspending the kyotorphin derivatives in 5 % dimethyl sulfoxide (DMSO) in deionized water, and aliquots were collected and analyzed.

2.3. Determination of the minimum inhibitory concentration (MIC)

The antifungal activity of kyotorphins was determined using a standardized broth microdilution method according to the Clinical and Laboratory Standards Institute (Amižić et al., 2017). Briefly, a cell suspension of each Candida species was adjusted to 10^3 CFU/ml using brain heart infusion (BHI) broth (Sigma). Aliquots (100 μl) of the suspension were dispensed in polystyrene, flat-bottomed, 96-well microtiter plates. Peptide stock solutions were diluted in BHI broth, and 100 μL of the dilution was added to the wells at a final concentration ranging from 25 μM to 1000 μM. The plates were incubated at 37 °C for 24 h. Absorbance was measured at an optical density of 590 nm using a microplate reader (Synergy H1 Hybrid Reader, BIOTEK, USA). The assays were repeated independently three times using different cell suspensions.

2.4. Determination of biofilm inhibition and eradication

Biofilm inhibition and eradication assays were performed as previously described (Rosenblatt et al., 2017; Scarsini et al., 2015; Wang et al., 2018). Representative isolates of KTP-susceptible Candida strains were cultured overnight in BHI medium, washed with 0.9 % NaCl, and adjusted to a density of 10^6 cells/ml using BHI broth. To evaluate the behavior/performance of these strains on the first step of cell adhesion, 100 μL of the cell suspension was added to 96-well polystyrene microplates together with 100 μL of the peptide solution at a concentration of 500 μM or 1000 μM to assess the inhibition of biofilm formation. To investigate the impact of KTP on mature biofilms (eradication activity), the cells were allowed to adhere to the plates for 24 h at 37 °C and then incubated with the peptide for 24 h. The growth medium was aspirated, and the biofilms were washed with 100 μL of 0.9 % NaCl to remove non-adherent cells. Fresh BHI medium, followed by 100 μL of KTP (500 μM or 1000 μM) diluted in BHI medium, was added to the biofilms. After an additional treatment for 24 h, adherent cells were detached by scraping, and biofilm formation was measured by counting viable cells (CFU/ml). CFU count was performed in a similar manner, as described previously (Santos et al., 2016). All

experiments were performed in triplicate and repeated independently three times.

2.5. Analysis of biofilms by SEM

Biofilms bound to polystyrene discs were analyzed by SEM. The discs were inoculated with Candida strains and incubated statically in BHI medium at 37 °C for 24 h, as described in section 4. To determine biofilm inhibition and eradication, the samples were washed, fixed in 2.5 % (v/v) glutaraldehyde for 1 h, dehydrated at increasing concentrations of ethanol, and dried overnight (Costa et al., 2011). The samples were sputter coated with gold using an INSPECT S50 SEM (FEI Corporation, Japan) at 15.0 kV in high vacuum mode and observed at different magnifications.

2.6. Effect of peptide toxicity on G. mellonella

This study used the methodologies described by Mylonakis et al. (2005), with modifications. Last-instar larvae of *G. mellonella* were used in the experiments. Sixteen *G. mellonella* larvae with similar weight and size (250–350 mg) were used in each of four treatment groups. A volume of 10 μL of each different peptide concentrations were inoculated in each group. Two control groups (one was inoculated with PBS, and the other was treated with 5 % DMSO in ultrapure water) were included and served as a control for overall viability. The larvae were treated and counted for 7 consecutive days in three independent experiments (Santos et al., 2016).

2.7. Kaplan–Meier survival analysis

We initially determined the lethal concentration of *C. albicans* inoculum by injecting serial 2-fold dilutions of the fungal suspension in larvae. For this purpose, yeast cells were centrifuged and washed with 0.9 % NaCl. Cell density was standardized to 10^9 cells/ml by spectrophotometry (590 nm), and a concentration of 10^5, 10^6, or 10^7 cells/larvae were used in the assays. Ten microliters of the standardized suspension was injected in the last pair of prolegs of each larva to determine the most lethal cell density. The larvae were kept in Petri dishes at 37 °C, and survival was monitored for 7 d.

To evaluate the effects of kyotorphins on Candida infection, 10^6 yeast cells (10 μL) suspended in medium containing different concentrations of IbKTP-NH_2 were injected into the hemocoel of larvae through the last left proleg. In the control groups, 10^6 cells (in 10 μL) were suspended in PBS and injected in the last right proleg of each larva. Larvae were incubated at 37 °C, and survival was monitored for 7 d.

The larvae were considered dead when they did not Respond to touch stimuli, and larval death or the transition to pupa were assessed at the end of the experiment.

2.8. Statistical analysis

Survival curves of *G. mellonella* were plotted, and statistical analysis was performed by analysis of variance. Tukey's test was used to compare the results of hemocyte count. CFU count in larval hemolymph was analyzed using Student's t-test. Data were analyzed using GraphPad Prism (GraphPad Software, Inc., California, CA, USA), and a *p*-value of 0.05 was considered significant.

3. Results

3.1. Determination of MIC

The antifungal activity of kyotorphins against *C. albicans* and *C. krusei* was assessed *in vitro*. Among the tested peptides at a concentration of 50–1000 μM, only IbKTP-NH_2 was active against both strains, whereas KTP-NH2 presented inhibitory activity against *C. albicans* (Table 1). Therefore, IbKTP-NH_2 was used in subsequent experiments.

The antifungal effects of IbKTP-NH_2 on representative clinical isolates of Candida spp were also investigated. The antimicrobial activity of IbKTP-NH_2 was significant for all Candida spp, except for *C. glabrata* and *C. dubliniensis*, for which the peptide was ineffective up to a minimum fungicidal concentration (MFC) of 1000 μM (Table 2).

The MFC of IbKTP-NH_2 against susceptible Candida spp isolates was determined to ascertain whether the results of the antifungal susceptibility test were correlated with killing capacity. For all Candida spp isolates, the MFC values of IbKTP-NH_2 were similar to MIC values, indicating significant candidacidal activity (Fig. 1).

3.2. Analysis of biofilm inhibition and eradication

Candida spp biofilms may be involved in the persistence or worsening of chronic inflammatory diseases, and since biofilms confer increased resistance to antifungal agents (Mathé and Van Dijck, 2013 and Tobudic et al., 2012), we determined the anti-biofilm and antifungal activity of the two highest concentrations of IbKTP-NH_2 in preformed (24 h) biofilms. In inhibition and eradication assays, the activity of IbKTP-NH_2 was highest against *C. krusei* (inhibition of 93 % and eradication of 100 % at 1000 μM), followed by *C. glabrata*, *C. albicans*, *C. tropicalis*, and *C. lusitaniae* (Table 3 and Fig. 2).

The efficacy of IbKTP-NH_2 against preformed (24 h) biofilms of Candida spp was evaluated by CFU count after 24 h of treatment. Biofilms of *C. glabrata and C. krusei* were weakly inhibited by 500 μM IbKTP-NH_2 but strongly inhibited by 1000 μM IbKTP-NH_2 when compared to untreated biofilms (Fig. 2). Moreover, 1000 μM IbKTP-NH_2 significantly reduced the number of CFUs for *C. albicans*, *C. glabrata, C. krusei, C. novergensis*, and *C. tropicalis* in preformed biofilms and completely eradicated the biofilms produced by *C. albicans, C. krusei* and *C. novergensis* (Fig. 3).

3.3. Analysis of biofilm structure

SEM was used to examine the biofilm architecture of different Candida species in the presence and absence of IbKTP-NH_2. Special attention was given to cell morphology and spatial organization on the substrate, and the amount of extracellular polymeric substances (EPS) on and between cells in these biofilms. SEM images of control biofilms (panel A) and biofilms incubated with IbKTP-NH_2 -inhibition assay and eradication assay - are shown in Figs. 4 and 5, respectively. In the control groups, all Candida strains adhered to polystyrene plates and developed mature biofilms.

In inhibition and eradication assays, the treated groups were compared with the control groups to assess structural and morphological changes in cells and biofilms. SEM showed a reduction in the number of cells and, consequently, biofilm density in IbKTP–NH_2–treated cultures. Moreover, the amount of extracellular matrix (ECM) usually decreased in these biofilms. IbKTP-NH_2 caused changes in biofilm structure, including irregularities in the ECM and increased spacing between cells, except in *Candida norvegensis*. The formation of hyphae and pseudohyphae was suppressed in *C. albicans*, *C. lusitaniae*, *C. norvegensis*, *C. parapsilosis*,

Table 1
Minimum inhibitory concentration (MIC) of kyotorphins in two Candida strains.

Microorganism	KTP	KTP-NH_2	KTP-NH_2 DL	Me-KTP-LL	Me-KTP-DL	IbKTP	IbKTP-NH_2
MIC_{50} (μM)							
Candida albicans (ATCC 10231)	–	885	–	–	–	–	430
Candida krusei (ATCC 6258)	–	–	–	–	–	–	707
MFC (μM)							
Candida albicans (ATCC 10231)	–	–	–	–	–	–	1000
Candida krusei (ATCC 6258)	–	–	–	–	–	–	1000

The MIC_{50} values were adjusted by non-linear regression. Error values were not displayed because 100 % survival was not reached, which limited determining the peptide concentration necessary to cause 50 % survival. MFC values were obtained by measuring survival rates. (–) represents MIC values > 1000 μM.

Table 2
Minimum inhibitory concentration (MIC) of kyotorphin IbKTP-NH_2 in Candida strains.

Microorganism	IbKTP-NH_2	
	MIC_{50} (μM)	MFC (μM)
Candida albicans (ATCC 10231)	523	1000
Candida krusei (ATCC 6258)	523	1000
Candida dubliniensis (clinical isolate 19-S)	506	–
Candida glabrata (clinical isolate 217-S)	1000	–
Candida lusitaniae (clinical isolate 14-S)	755	–
Candida norvegensis (clinical isolate 51-S)	525	1000
Candida parapsilosis (clinical isolate 63)	550	–
Candida tropicalis (clinical isolate 140-S)	500	1000

The MIC_{50} values were adjusted by non-linear regression. Error values were not displayed because 100 % survival was not reached, which prevented determining the peptide concentration necessary to achieve 50 % survival. MFC values were obtained by measuring survival rates. (–) represents MIC values > 1000 μM.

and *C. tropicalis*. This change is relevant because the filamentation process has a key role in maintaining the three-dimensional architecture of biofilms and is a hallmark of virulence (Camarillo-Márquez et al., 2018; Madhavan et al., 2018; Monteiro et al., 2013; Panariello et al., 2018; Seneviratne et al., 2009).

The morphology of control cells — the presence of oval spores (blastospores) and elongated pseudohyphae with a smooth surface and intact cell membrane — and IbKTP–NH_2–treated cells was compared. Several alterations were observed in the treated groups, including damage to the cell wall and membrane, pore formation, surface roughness, cell shrinkage, wrinkles and ripples, reduced number of shoots, cell membrane invagination, and decreased number of hyphae and pseudohyphae, which are in line with a previous study (Madhavan et al., 2018).

The biofilm structure of *C. glabrata* treated with IbKTP-NH_2—a well-defined, thin, and irregular biofilm embedded in an extracellular polymeric matrix—was different from that of control cells (Fig. 4). *C. glabrata* must also be subject of an adaptive process of apoptosis as demonstrated previously (Camarillo-Márquez et al., 2018), herewith the occurrence of membrane changes induced by the peptide in the first 24 h corroborates CFU data. The amount of EPS was also comparatively smaller at 48 h after treatment, as shown previously (Seneviratne et al., 2009). Cellular changes were also evident.

3.4. *Toxicity of IbKTP-NH_2 to* G. mellonella

Peptide toxicity was assessed in *G. mellonella* larvae (Fig. 6). No significant toxic effect was observed ($p < 0.0001$), except at the concentration of 1000 μM. Therefore, the concentration used in *in vivo* tests was 500 μM.

3.5. *Survival curve analysis*

The susceptibility of *G. mellonella* larvae to *C. albicans* infection at cell densities of 10^5 to 10^7 cells/larva was evaluated. The concentration of 10^6 cells/larvae caused 100 % larval mortality within 24 h after infection and was used in subsequent assays (Fig. 7A).

Survival was 100 % in the control groups, demonstrating that larval viability was not affected by transportation and experimental procedures.

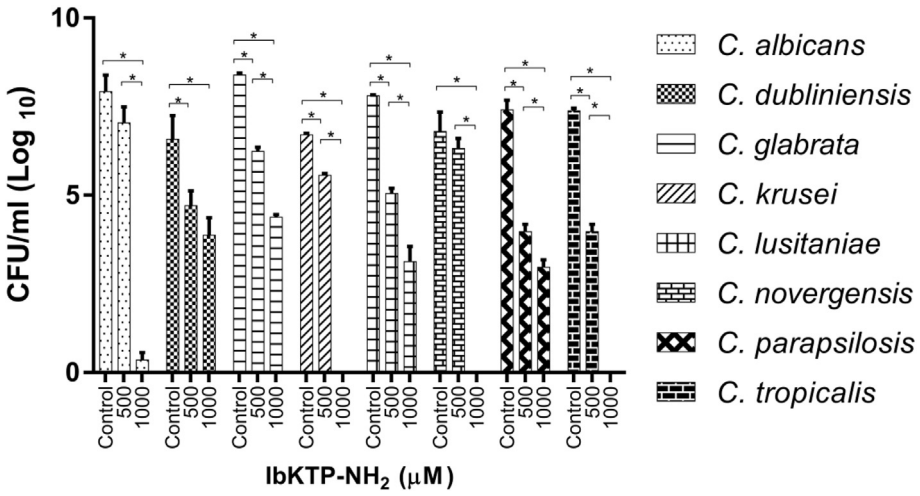

Fig. 1. Antifungal profile of peptide IbKTP-NH_2 in planktonic cells. Mean and standard deviation of cell count (CFU/ml, log_{10}) of Candida spp treated with two peptide concentrations. The control group represents untreated fungi. Data are reported as means ± standard errors of at least three independent experiments. *Significant difference between the groups ($p = 0.05$).

Table 3
Antibiofilm activity of peptide IbKTP-NH_2 in Candida species.

		Inhibition	Eradication
C. albicans	500 μM	19 % ± 0,006*	18 % ± 0,006
	1000 μM	23 % ± 0,011*	100 % ± 0,000*
C. dubliniensis	500 μM	26 % ± 0,017*	24 % ± 0,032
	1000 μM	34 % ± 0,023*	45 % ± 0,016*
C. glabrata	500 μM	12 % ± 0,029	27 % ± 0,008*
	1000 μM	100 % ± 0,000*	74 % ± 0,036*
C. krusei	500 μM	20 % ± 0,053*	19 % ± 0,004*
	1000 μM	93 % ± 0,104*	100 % ± 0,000*
C. lusitaniae	500 μM	30 % ± 0,021*	35 % ± 0,019*
	1000 μM	50 % ± 0,026*	60 % ± 0,055*
C. novergensis	500 μM	20 % ± 0,023*	3 % ± 0,039
	1000 μM	20 % ± 0,023*	100 % ± 0,000*
C. parapsilosis	500 μM	14 % ± 0,038	46 % ± 0,029*
	1000 μM	25 % ± 0,022*	60 % ± 0,029*
C. tropicalis	500 μM	28 % ± 0,011*	46 % ± 0,029*
	1000 μM	51 % ± 0,030*	87 % ± 0,014*

*Significantly different at $p \leq 0.05$.

The effect of 500 μM IbKTP-NK_2 on larval viability was evaluated. The rate of survival of infected larvae was 80 % and 30 % at 24 h and 48 h after treatment, respectively (Fig. 7B), and remained at 20 % throughout the experimental period ($p = 0.0031$).

The number of yeast cells in the hemolymph of *G. mellonella* larvae infected with *C. albicans* was quantified at 0, 6, and 12 h after infection. The results indicated that the number of yeast cells decreased significantly starting 6 h after treatment of infected larvae with 500 μM IbKTP-NH_2 (Fig. 8).

4. Discussion

Infections caused by bacteria, viruses, and parasites are more common than infections caused by pathogenic fungi. However, fungal infections in the CNS are a global health problem (Rizvi et al., 2018), and the most common pathogens affecting the CNS are Candida spp. (Wu et al., 2014). The small size of yeast cells allows access to the microcirculation, potentially causing

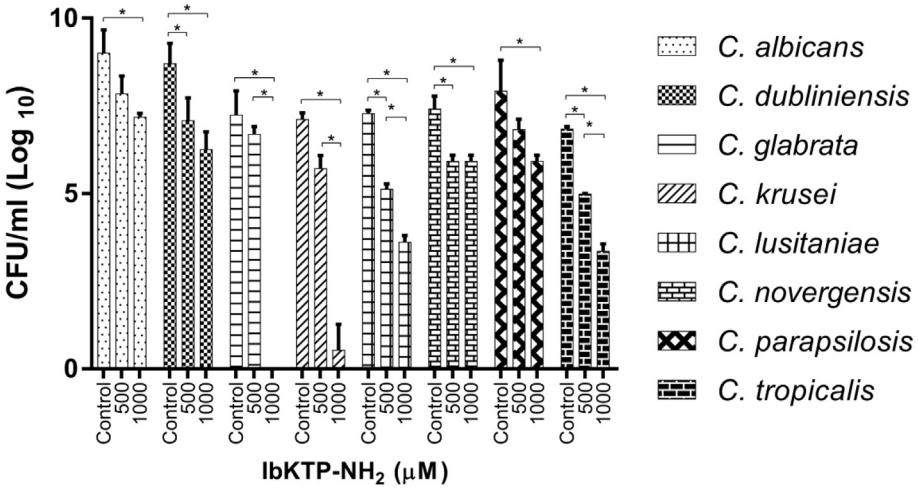

Fig. 2. Inhibition of biofilms of Candida spp. Mean and standard deviation of cell count (CFU/ml, $\log_{10}$) of Candida spp treated with two peptide concentrations. The control group represents untreated fungi. Data are shown as means ± standard errors of at least three independent experiments. *Significantly different at $p = 0.05$.

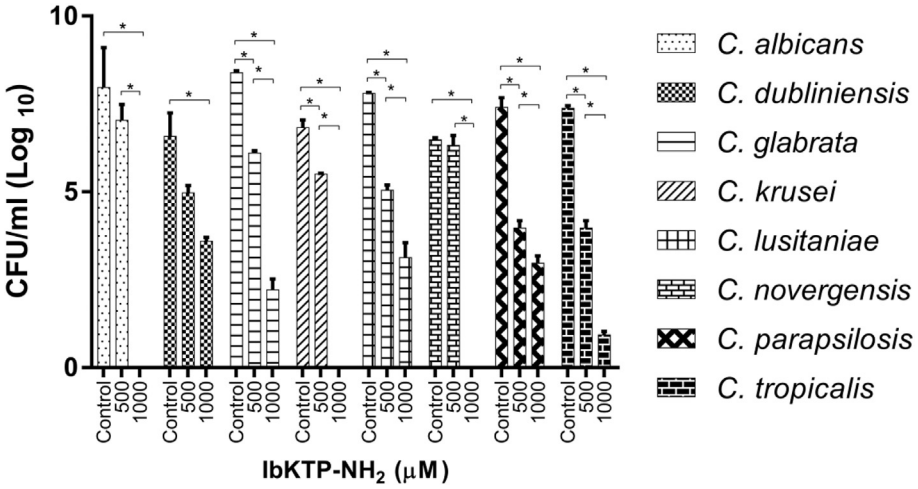

Fig. 3. Eradication of biofilms of Candida spp. Mean and standard deviation of cell count (CFU/ml, $\log_{10}$) of Candida spp treated with two peptide concentrations. The control group represents untreated fungi. *Significantly different at $p = 0.05$.

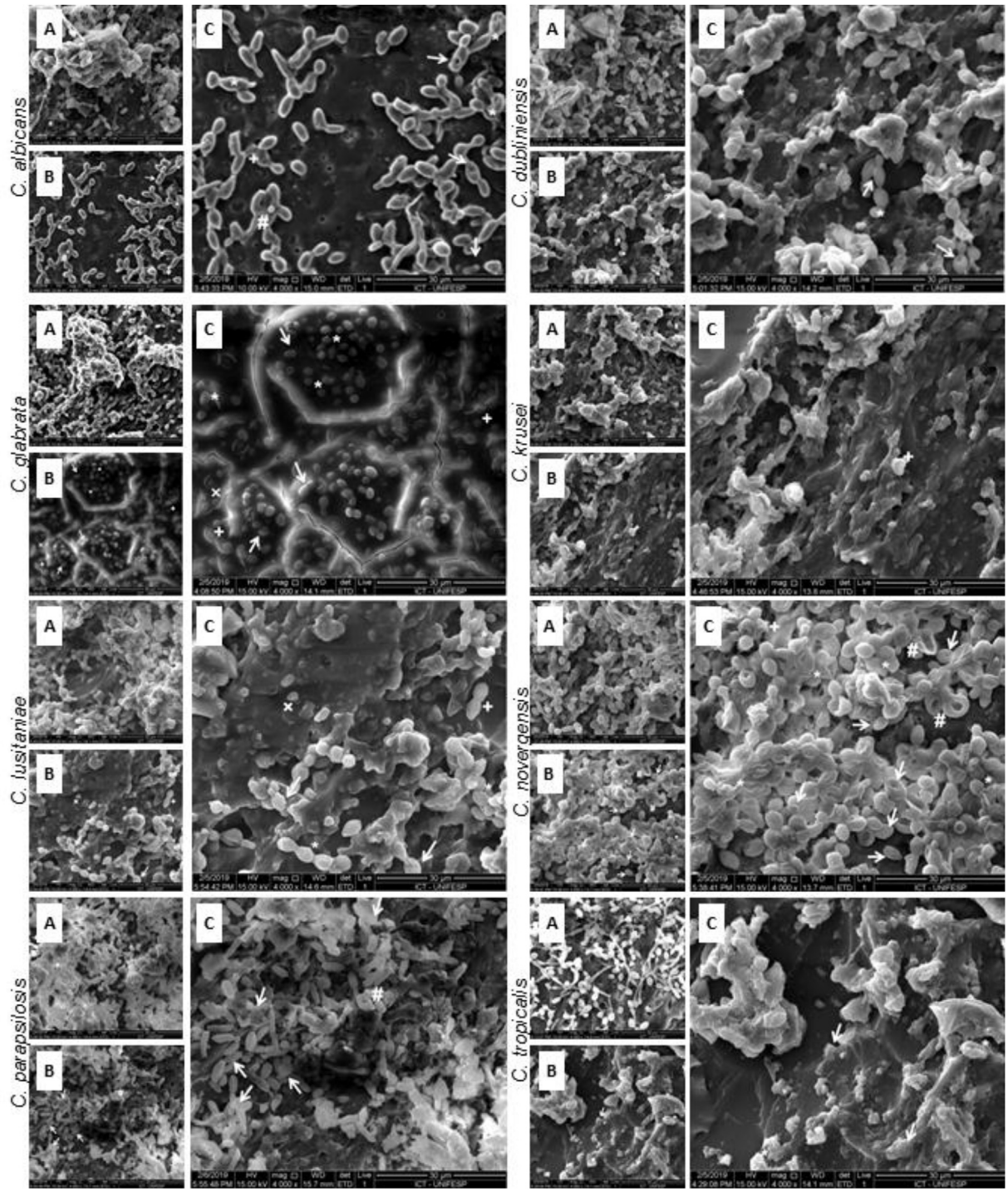

Fig. 4. Scanning electron microscopy showing the inhibition of biofilm formation in Candida spp by peptide IbKTP-NH_2. (A) Biofilm control; (B–C) Biofilm treated with IbKTP-NH_2 (1000 μM); Magnification at 4000x. Morphological changes are indicated by: (→) membrane pore formation, (+) surface roughness, (×) cell size reduction, (*) membrane wrinkles and undulations, (#) membrane invagination.

diffuse meningitis or manifesting as granulomas or abscesses (Gavito-Higuera et al., 2016). Non-albicans Candida species are also found in the CNS, and the most common pathogenic species are *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. krusei* (Storti et al., 2012; Pfaller et al., 2014; Turner and Butler, 2014). These data highlight the importance of designing and developing new antifungal agents to address this significant global health challenge.

Membrane-active peptides are promising broad-spectrum antimicrobial agents (Lee et al., 2019; Torres et al., 2019; Wang et al., 2019). However, few synthetic membrane-active peptides targeting pathogenic fungi have been investigated. Analgesic kyotorphins have been shown to be safe and effective AMPs (Ribeiro et al., 2012). We hypothesized that kyotorphins presented antifungal activity given their anionic nature. In this study, six kyotorphins were tested against two fungal species commonly found in

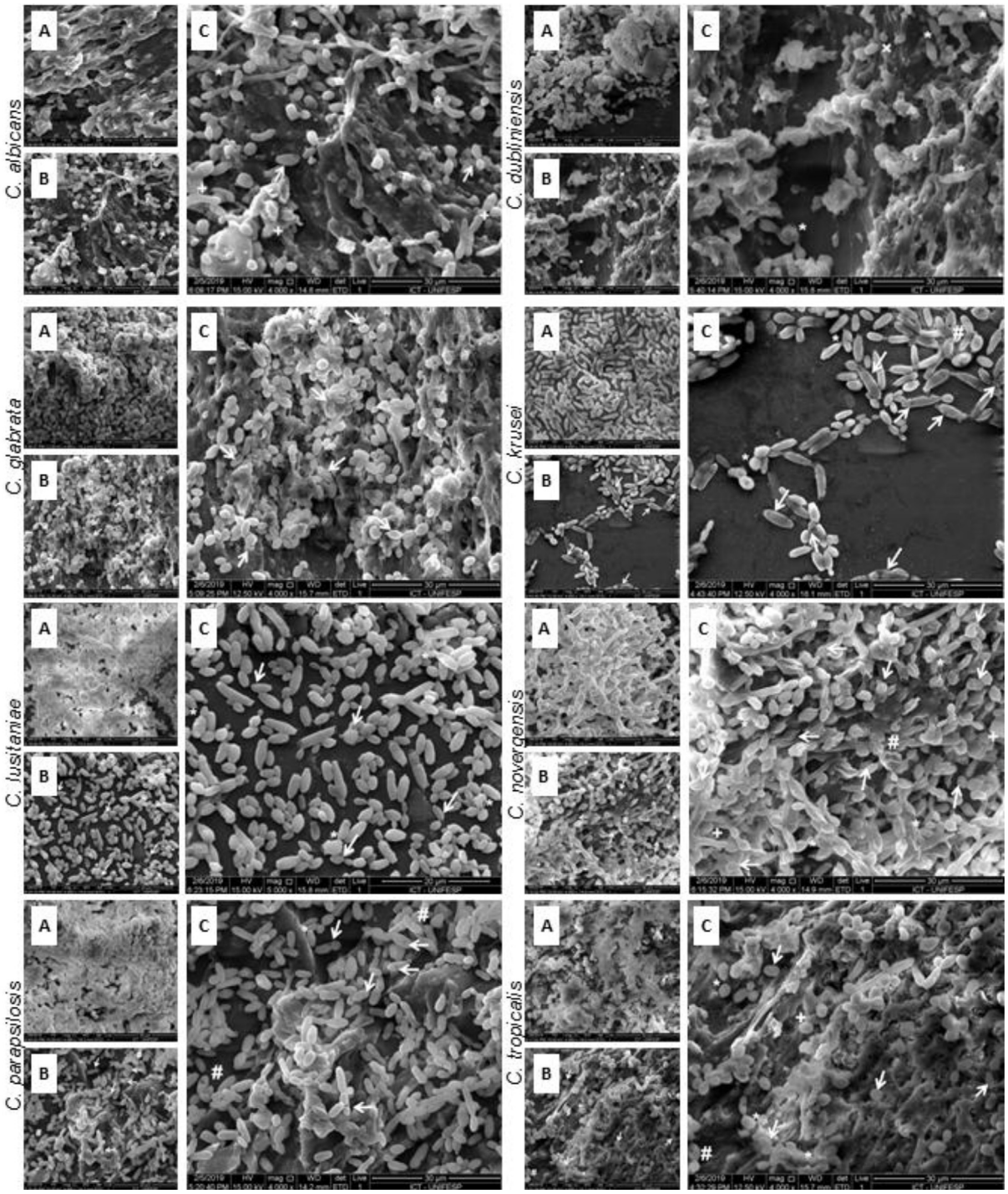

Fig. 5. Scanning electron microscopy showing the eradication of biofilm formation in Candida spp by peptide IbKTP-NH_2. (A) Biofilm control; (B–C) Biofilm treated with IbKTP-NH_2 (1000 μM); Magnification at 4000x. Morphological changes are indicated by: (→) pore formation, (+) surface roughness, (×) cell size reduction, (*) membrane wrinkles and undulations, (#) membrane invagination.

clinical infections, *C. albicans* and *C. krusei* (Deorukhkar and Roushani, 2018). IbKTP-NH_2 was the most active against these pathogens and presented antifungal and antibiofilm activity against a panel of highly pathogenic clinical Candida spp. isolates.

Our results showed that IbKTP-NH_2 had antifungal and antibiofilm activity against *C. albicans* and non-albicans species. Although the MIC of IbKTP-NH_2 was usually higher than that of other AMPs, MIC_{50}, MFC, and minimum biofilm eradication concentration (MBEC) values were species/strain-dependent. The MIC_{50}, MFC, and MBEC values of *C. albicans* strains were similar to those of *C. krusei*, *C. lusitaniae*, *C. novergensis*, and *C. parapsilosis*. Surprisingly, the MBEC results revealed that adherent cells were less resistant to antifungal agents than planktonic cells.

Among kyotorphin derivatives, IbKTP-NH_2 possessed the highest fungicidal activity against attached cells growing to mature structures that constitute early biofilm elements in Candida

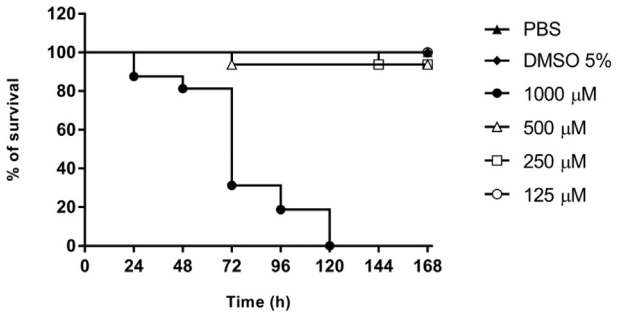

Fig. 6. Toxicity of peptide IbKTP-NH_2 to *G. mellonella* larvae.

species. It has been shown that amphotericin B does not kill all Candida cells in biofilms, and the metabolic activity of biofilms was detected even at high antifungal concentrations (Ramage and Wickes, 2001). IbKTP-NH_2 was the most potent kyotorphin derivative with antifungal activity against biofilm formation and eradication, achieving 100 % inhibition for *C. glabrata*, 97 % inhibition for *C krusei*, and 100 % eradication for *C. albicans*, *C. krusei*, and *C. novergensis*. Recent studies reported that the susceptibility of *C. glabrata* biofilms to two lipopeptides (echinocandins) was lower than that of *C. albicans, C. tropicalis*, and *C. parapsilosis* biofilms. In this respect, naturally-occurring gene polymorphisms may have reduced the susceptibility of Candida species to lipopeptides (Rodrigues et al., 2018).

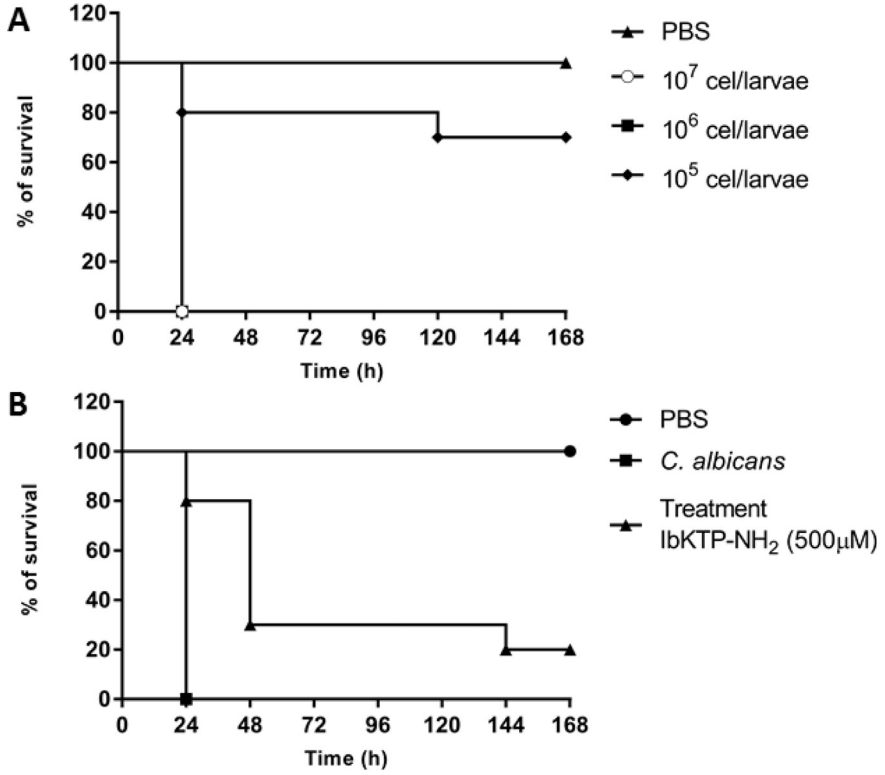

Fig. 7. (A) Survival curve of *Galleria mellonella* larvae infected with *Candida albicans* at densities of 10^5, 10^6, or 10^7 cells per larva. (B) Survival curve of *G. mellonella* larvae infected with *C. albicans* (10^7 cells per larva) and treated with IbKTP-NH_2.

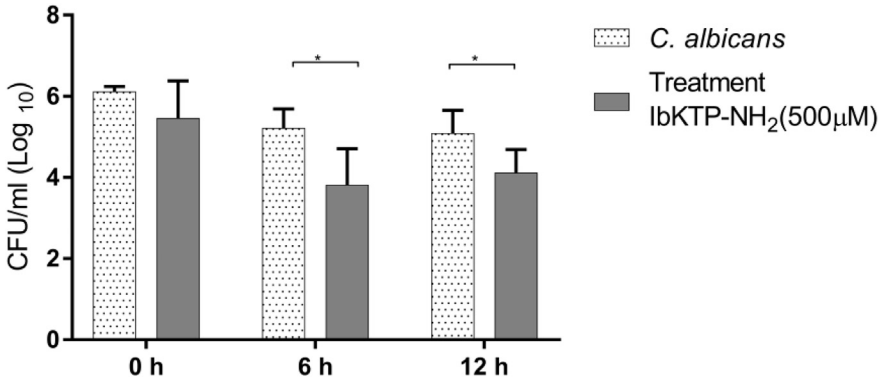

Fig. 8. Number of *C. albicans* cells recovered from the hemolymph of *G. mellonella* larvae treated with 500 μM IbKTP-NH_2 for different times. *Significantly different at $p \leq 0.05$.

The mode of action of known AMPs in the analyzed Candida species may include binding to the cell wall, induction of signaling cascades, and interaction with intracellular targets, leading to apoptosis, membrane permeabilization, and receptor-mediated internalization (Thevissen et al., 2004; de Medeiros et al., 2010; Van Der Weerden et al., 2013). A previous study reported that the mode of action of IbKTP-NH_2 against bacteria involved disruption of the integrity of the plasma membrane. In the present study, the effect of IbKTP-NH_2 on the integrity of fungal cell membranes was determined by SEM. The results revealed that all Candida species from the control group had a high capacity to produce biofilms. Furthermore, IbKTP-NH_2 strongly inhibited biofilm formation, evidenced by the inhibition of *C. albicans* and *C. tropicalis* and eradication of *C. krusei*. Pore structures were observed in the cell membranes of all tested fungi. Most Candida species can undergo complex morphological changes, and this feature is frequently linked to the virulence of opportunistic pathogens (Kukamoto et al., 2005; Shapiro et al., 2011). A few Candida species can transition between yeast and filamentous growth states. *C. albicans* can transition between yeast, pseudohyphal, and hyphal growth states under diverse environmental conditions (Shapiro et al. 2011). Other Candida species, such as *C. tropicalis* (Porman et al., 2013) and *C. dubliniensis* (Martins et al., 2007), can assume yeast, hyphal, and pseudohyphal forms, whereas *C. parapsilosis, C. lusitaniae*, and *C. krusei* cannot form true hyphae but can transition between yeast and pseudohyphal forms (Samaranayake and Samaranayake, 1994; Laffey and Butler, 2005; Miller et al., 2006). Our results showed that IbKTP-NH_2 induced a decrease in the number of hyphae and pseudohyphae, especially in *C. albicans*, *C. novergensis*, and *C. krusei*, and a reduction in the size of *C. tropicalis* cells. This result indicates a possible decrease in virulence because the formation of hyphae is related to tissue invasion.

SEM showed morphological changes in cell membranes, including pore formation, invaginations, and surface roughness, which reduced cell viability, and these alterations were probably caused by the peptide. The high number of changes and low cell viability suggest that IbKTP-NH2 acts in the fungal wall, disaggregating the polysaccharide matrix and disturbing the wall structure composed of mannoproteins, β-glucans, and chitin. This process can affect cell wall integrity, increasing surface roughness and decreasing stiffness. Furthermore, there was an overall decrease in the amount of EPS formed by cells. Considering that interactions of EPS are required for biofilm antifungal resistance (Mitchell et al., 2015), a lower amount of EPS in the presence of IbKTP-NH_2 may alter the constitution of polysaccharides, increasing biofilm susceptibility to antifungal therapy. Another notable finding was that IbKTP-NH_2 exhibited a strong antibiofilm activity by significantly reducing the number of cells of all evaluated fungal species.

Candida CNS infections are usually due to *C. albicans* (Sánchez-Portocarrero et al., 2000; Fernandez et al., 2000) but may be caused by *C. parapsilosis* and *C. tropicalis*, which have been gaining notoriety as pathogenic species (Chiou et al., 1994; McCullers et al., 2000; Trofa et al., 2008). *C. glabrata* rarely causes CNS infections. However, the excessive use of immunosuppressive agents and broad-spectrum antifungal drugs has led to the emergence of pathogenic *C. glabrata* strains, especially in HIV and diabetic patients (Fidel et al., 1999). Moreover, some strains are inherently resistant to therapy with azoles, such as fluconazole.

The MIC values indicated that the efficacy of IbKTP-NH_2 was lower than that of marketed drugs and other AMPs. It is worth highlighting that this peptide can cross the BBB, which is a desirable feature of antifungal drugs. Several cell types are present in the BBB, including microvascular endothelial cells, astrocytes, and pericytes. The BBB separates the brain extracellular fluid from blood components, controls and regulates the transport of molecules, maintains brain microenvironment, and protects the brain from toxins and microorganisms (Abbott et al., 2010; Kniesel and Wolburg, 2000). However, fungal species such as Candida spp can cross the BBB through transcellular, paracellular, and Trojan-horse mechanisms and cause serious infections in the CNS (Jong et al., 2001; Pisa et al., 2015; Wu et al., 2019). *C. albicans* can invade human brain microvascular endothelial cells via transcytosis and develop pseudohyphae inside these cells (Jong et al., 2001).

An *in vivo* assay involving the infection of *G. mellonella* larvae with *C. albicans* and treatment of candidiasis was conducted to evaluate the therapeutic potential of IbKTP-NH_2. IbKTP-NH_2 decreased the number of yeast cells in the larval hemolymph. The survival curves showed that IbKTP-NH_2 not only prevented the death of 80 % of the sample but it prolonged the survival of infected larvae that died relative to the PBS-treated control, and 20 % of the treated larvae were alive at the end of the experimental period. This result demonstrates that IbKTP-NH_2 has a high therapeutic potential for treating *C. albicans* infections. Nonetheless, other *in vivo* studies using different models are necessary to confirm these results. Notwithstanding, the present data serve as the basis for reducing the number of animals in future studies because *G. mellonella* is an effective *in vivo* model for this purpose.

IbKTP-NH_2 is less potent than small drug molecules, with MFC values in the range of 500–1000 mg/L for most strains, but can provide a basis for developing promising antimicrobial agents. Most AMPs are cationic and highly cytotoxic, and their toxicity is nonspecific. The toxicity of IbKTP-NH_2 was assessed using *G. mellonella* larvae as an *in vivo* model. Drug toxicity was low up to 2 d after treatment and agreed with the results of treatment of *C. albicans*-infected larvae.

In view of the potential therapeutic application of these peptides, small peptides are chemically simple and their production is economically feasible. Furthermore, these peptides can be suitable for chemical grafting in medical biomaterials. In this respect, ongoing studies aim to identify kyotorphin derivatives with higher antimicrobial activity and high applicability in biomedical devices.

In conclusion, the present study confirmed the antimicrobial properties of kyotorphin derivatives and expanded the spectrum of activity to include the antifungal activity of IbKTP-NH_2, attaching an antifungal propriety for IbKTP-NH_2. The results showed for the first time that this peptide strongly inhibited biofilms produced by *C. albicans* and non-albicans clinical isolates. Compared with KTP-NH_2 (Ribeiro et al., 2012), IbKTP-NH_2 was active against a broader spectrum of microorganisms, was more effective in inhibiting and eradicating fungal growth on the surface of polystyrene plates, and killed Candida cells in 48-h biofilms by membrane permeabilization. These results encourage further investigations on IbKTP-NH_2 in view of its potential antifungal application, especially in CNS infections.

Author contributions

V.M.A. participated in the execution of antimicrobial and SEM experiments, data analysis, and manuscript preparation; E.B., M.H., and V.G.R. synthesized and purified peptides and determined their masses; J.C.J. was involved in data analysis and manuscript preparation; J.D.S. performed antifungal assays; M.A.R.B.C. was involved in the study design; K.C. participated in the study design, data analysis, and manuscript preparation.

Declaration of Competing Interest

The authors declare no conflict of interest.

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