RESEARCH LETTER

Effects of microplusin, a copper-chelating antimicrobial peptide, against Cryptococcus neoformans

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Abstract

Microplusin is an antimicrobial peptide isolated from the cattle tick Rhipicephalus (Boophilus) microplus. Its copper-chelating ability is putatively responsible for its bacteriostatic activity against Micrococcus luteus as microplusin inhibits respiration in this species, which is a copper-dependent process. Microplusin is also active against Cryptococcus neoformans (MIC₅₀ = 0.09 μM), the etiologic agent of cryptococcosis. Here, we show that microplusin is fungistatic to C. neoformans and this inhibitory effect is abrogated by copper supplementation. Notably, microplusin drastically altered the respiratory profile of C. neoformans. In addition, microplusin affects important virulence factors of this fungus. We observed that microplusin completely inhibited fungal melanization, and this effect correlates with the inhibition of the related enzyme laccase. Also, microplusin significantly inhibited the capsule size of C. neoformans. Our studies reveal, for the first time, a copper-chelating antimicrobial peptide that inhibits respiration and growth of C. neoformans and modifies two major virulence factors: melanization and formation of a polysaccharide capsule. These features suggest that microplusin, or other copper-chelation approaches, may be a promising therapeutic for cryptococcosis.

Introduction

Cryptococcus neoformans affects both immunocompetent and immunocompromised individuals, especially patients with advanced HIV infection, with transplanted organs or treated with high doses of corticosteroids (Perfect & Casadevall, 2002). The fungus is responsible for over 600 000 deaths per year worldwide (Park et al., 2009) and is the primary cause of death for systemic mycoses in HIV-infected patients in Brazil (Park et al., 2009; Prado et al., 2009). In general, cryptococcal infections are treated with an initial administration of amphotericin B in combination with fluconazole followed by azole derivatives, such as fluconazole (Perfect et al., 2010). The inconvenience of these therapies lies in their negative side effects for the patient, and to a lesser extent, the development of drug resistance by the fungus (Perfect & Casadevall, 2002; Dan & Levitz, 2006).

The ability of C. neoformans to infect humans is related to several virulence factors and the two most important are the melanin synthesis (Zhu & Williamson, 2004) and the production of a polysaccharide capsule (Zaragoza et al., 2009). Melanin synthesis depends on laccase activity, a copper-containing oxidase that requires exogenous catecholamines as substrate (Williamson et al., 1998; Zhu & Williamson, 2004). Melanization protects the fungus against oxidative stress, extremes of temperature, enzymatic degradation, and antimicrobial compounds (reviewed in Nosanchuk & Casadevall, 2003, 2006). The polysaccharide capsule protects C. neoformans against phagocytosis and induces strong immunomodulatory responses that promote immune evasion and survival...
within the host (reviewed in Zaragoza et al., 2009). Capsule enlargement occurs by self-aggregation of glucuronoxylomannan (GXM) fibers that represent 90–95% of capsular contents. The cross-linking between the anionic polysaccharide chains of GXM depends on the presence of divalent cations, such as calcium II and magnesium II (Nimrichter et al., 2007).

Several studies have shown a relation between copper homeostasis and virulence of C. neoformans. Essential metabolic enzymes, such as Cu/Zn superoxide dismutase and the high-affinity iron transporter Fet3 have been described as copper-dependent proteins in C. neoformans (Davis-Kaplan et al., 1998; Cox et al., 2003). High concentrations of exogenous copper induce laccase expression and production of melanin in C. neoformans, and CnLac1 lacasse gene induction by copper is regulated by the copper-dependent transcription factor 1 (CUF1) (Jiang et al., 2009). Also, the expression of the high-affinity fungal copper transporter CTR4 in C. neoformans is upregulated by CUF1 in conditions of low copper availability, such as the environment of infected macrophages or within brain tissue (Waterman et al., 2007). Mutants for Cuf1 display a severe growth defect and a decrease in laccase activity (Waterman et al., 2007). Moreover, the copper transporter CTR4 is also regulated by the transcription factor Rim101 and rimA C. neoformans mutants are unable to produce large capsules (O’Meara et al., 2010).

Microplusin is a copper II and iron II chelating peptide isolated from the cattle tick Rhipicephalus (Boophilus) microplus (Fogaca et al., 2004; Esteves et al., 2009; Silva et al., 2009). The peptide is formed as a single globular domain with five α-helices, and although we have not yet determined the residues involved in its copper-biding site, our data has suggested that N-terminal residues and His-74 are the main candidates. Moreover, microplusin has a broad antimicrobial spectrum of activity against several Gram-positive bacteria and fungi (Silva et al., 2009). Our data suggest that the antibacterial activity of microplusin against Micrococcus luteus is related to its copper-chelating activity. In fact, we observed that microplusin affects bacterial respiration, a process that involves several heme-copper oxidases (Silva et al., 2009). Among the fungi previously evaluated, microplusin was active against C. neoformans, with an MIC50 (minimal inhibitory concentration that prevented 50% of the growth) of 0.09 μM. In the present work, we demonstrate that microplusin is a fungistatic peptide that negatively affects the respiration of C. neoformans. In addition, microplusin showed inhibitory activity against two important virulence factors, melanization and polysaccharide capsule formation. Our results suggest that the anticyptococcal action of microplusin is strongly related to its copper-chelating ability.

Materials and methods

Microplusin

In all experiments, we used recombinant microplusin obtained as previously described (Esteves et al., 2009). Briefly, a mid-log phase culture of Escherichia coli (strain BL21) containing the microplusin cDNA/pRSET-A plasmid (Invitrogen) was induced with 0.8 mM IPTG (isopropyl β-D-thiogalactoside) during 4 h. Cells were harvested at 10 000 g for 10 min at 4 °C, suspended in phosphate-buffered saline 1 (PBS 1; 500 mM NaCl, 20 mM NaH2PO4; pH 7.5) and lysed by sonication (Branchon Digital Sonifier, Model 450). The bacterial lysate was centrifuged once again and the recombinant fusion protein was purified using a HisTrap™ Quelating HP column (Amersham Biosciences) equilibrated with 100 mM Ni2SO4. Elution was performed with 500 mM imidazole in PBS 1 and the fusion protein was dialyzed against 100 mM Tris-HCl, pH 8.5. To cleave the His6-tag from microplusin, fusion protein was incubated with trypsin type-III from bovine pancreas (Sigma-Aldrich) at an enzyme/protein molar ratio of 1 : 50 for 18 h at 37 °C. Recombinant microplusin was purified by RP-HPLC using a semi-preparative C18 column (Vydac™, 300 Å, 10 mm × 250 mm) with a linear gradient of 2–60% acetonitrile in acidified water over 60 min at a flow rate of 1.3 mL min−1. The molecular mass of recombinant microplusin was analyzed on a LCQ Duo™ mass spectrometer (ThermoFinnigan).

Microorganisms

The following strains of C. neoformans were used in this work: serotype A strains T,444 (Barbosa et al., 2007) and H99 (Frasés et al., 2007), and serotype D strain B3501 (Frasés et al., 2007). The strains were cultivated for 48 h while shaking at 30 °C in Sabouraud dextrose medium (Difco Laboratories). Yeast cells were harvested by centrifugation, washed three times with saline phosphate buffer 2 (PBS 2: 137 mM NaCl, 2.6 mM KCl, 10 mM NaH2PO4, 1.8 mM KH2PO4; pH 7.4) and quantified using a Neubauer chamber. For all experiments, except for oxygen consumption measurements, cell density was set for 1 × 104 yeast cells per mL of medium. All experiments in this work were repeated at least twice to generate triplicate sets.

Antifungal activity assay

Cryptococcus neoformans strain H99 was incubated with or without 10 μM of microplusin (MP-treated and non-MP treated, respectively) in potato dextrose broth (PDB)
formed as values of percentage of growth inhibition.

Yeast growth parameters for MP-treated and MP-treated strains H99 in PDB medium supplemented with serial dilutions of microplusin (25–0.19 μM) with or without 2.5 μM of CuCl₂·H₂O. C. neoformans without any treatment or treated only with 2.5 μM of CuCl₂·H₂O was used as the yeast growth parameters for MP-treated and MP-treated + copper conditions, respectively. After 48 h of incubation at 30 °C, yeast growth was evaluated by absorbance readings at 595 nm and the values obtained were transformed as values of percentage of growth inhibition.

Melanization assay
A previously described protocol was used to evaluate the effect of microplusin on melanization (Martinez et al., 2007). Briefly, in 96-well microplates, C. neoformans strains H99 and B3501 were incubated with or without serial dilutions of microplusin (50–0.09 μM) in chemically defined medium (CD; 15 mM glucose, 10 mM MgSO₄, 29.4 mM KH₂PO₄, 13 mM glycine, and 3 μM thiamine; pH 5.5) containing 1 mM of L-dopa as a substrate for melanization. The cultures were maintained at 30 °C, protected from light, and melanization was monitored visually during the incubation period. In addition, the effect of copper supplementation on melanization of MP-treated yeast cells was evaluated by incubating the C. neoformans strain B3501 in CD with L-dopa and 2.5 μM of CuCl₂·H₂O.

L-dopa autopolymerization assay
The autopolymerization assay was done following a methodology previously described (Nosanchuk et al., 2001). Briefly, a solution of 1 mM of L-dopa in PBS 2 was incubated with different concentrations of microplusin (50–0.38 μM) and kept at room temperature. After 3 and 20 days of incubation, absorbance was measured at 270 nm in an Ultraspec 2000 spectrophotometer (Pharmacia Biotech). A L-dopa 1 mM solution in PBS 2 was used as control for 100% of autopolymerization.

Laccase activity assay
The effect of microplusin on laccase activity was investigated by a quantitative assay using the oxidation of 2,2’-azino-bis(3-ethylbenzothiazoline-sulfonic acid) (ABTS; Sigma) (Martinez et al., 2007). Briefly, C. neoformans strain H99 was grown in asparagine medium [AM; 0.1% asparagine, 10 mM Na₂PO₄ (pH 6.5), 0.01% MgSO₄, 50 mM CaCl₂] with 0.15% glucose for 24 h at 30 °C. Yeast cells were harvested by centrifugation, washed twice with PBS 2, washed once with AM without glucose, and suspended in the AM supplemented with 25 μM of microplusin. A control without microplusin was also prepared. After 48 h of incubation at 30 °C, yeast cells were collected by centrifugation, washed twice in PBS 2 and incubated in an ABTS 1 mM solution in PBS 2 for 24 h. To measure ABTS oxidation, yeast cells were removed by centrifugation and the absorbance of the supernatants was measured at 405 nm.

Capsule enlargement assay
To evaluate the effect of microplusin on capsule enlargement, C. neoformans strains H99, B3501, and T1444 were suspended in capsule inducing medium [10% Sabouraud dextrose media (Sab; Difco Laboratories), 50 mM MOPS (Sigma, St. Louis, MO), pH 7.3; (Zaragoza & Casadevall, 2004)] and incubated in 96-well microplates with serial dilutions of microplusin (25–0.78 μM) for 48 h at 37 °C. Control cultures without microplusin were also performed. Yeast cells were harvested by centrifugation and stained with India ink (Becton Dickinson, NJ). Cells were observed in an Axiovert 200 M inverted microscope and photographed with an Axiocam MR camera controlled by the AXION VISION 4.4 software (Carl Zeiss Micro Imaging, NY). Images were analyzed using the IMAGE J software (W. S. Rasband, National Institutes of Health, Bethesda, MD) (http://rsb.info.nih.gov/ij/) and the capsule size was defined as the distance between the cell wall and the outer border of the capsule (Barbosa et al., 2007).

Oxygen consumption measurements
Oxygen consumption of C. neoformans was measured polarographically at 30 °C using a computer-interfaced Clark-type electrode in PDB media in a final volume of 1 mL and at 6 × 10⁶ yeast cells mL⁻¹ of cell density. C. neoformans was grown at 30 °C in PDB either for 24 h without 10 μM microplusin (non-MP treated) or for 48 h with 10 μM microplusin (MP-treated) to use the fungus at the same concentration in culture, around 3 × 10⁷–5 × 10⁸ yeast cells mL⁻¹. The electrode was first stabilized at zero oxygen consumption in fresh PDB with constant stirring in the thermo-balanced chamber at 30 °C before the fungal suspension was transferred to the chamber. Recordings of respiration rate were initiated after closing the chamber with an air-tight lid. At least 10 min

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(Silva et al., 2009) for 72 h at 30 °C. After this period, cells were washed and quantified as previously described. One hundred cells from each experimental condition in PBS 2 at a final volume of 100 μL were plated on Sabouraud agar medium (Difco Laboratories). After 48 h at 30 °C, the number of colony-forming units (CFU) was determined. To investigate the effect of copper on the growth of MP-treated C. neoformans, a liquid growth inhibition assay was prepared as previously described (Silva et al., 2009) by incubating C. neoformans strain H99 in PDB medium supplemented with serial dilutions of microplusin (25–0.19 μM) with or without 2.5 μM of CuCl₂·H₂O. C. neoformans without any treatment or treated only with 2.5 μM of CuCl₂·H₂O was used as the yeast growth parameters for MP-treated and MP-treated + copper conditions, respectively. After 48 h of incubation at 30 °C, yeast growth was evaluated by absorbance readings at 595 nm and the values obtained were transformed as values of percentage of growth inhibition.

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after initiating the recording of basal respiration, 4 μM of the uncoupler carbonyl cyanide m-chlorophenylhydrazone, 4 μM of the alternative oxidase (AOX) inhibitor salicylhydroxamic acid (SHAM) and/or 4 μM of the complex III respiratory inhibitor antimycin A (AA), or 1 μM of the complex IV respiratory inhibitor potassium cyanide (KCN) were added to the chamber containing *C. neoformans*. Values are represented as the rate of O2 consumption in nanomoles min⁻¹ ± SD.

**Statistical analysis**

Statistical analysis was performed using PRISM version 5 (GraphPad Software). The results were compared by Student’s *t*-test or two-way ANOVA test according to the data.

**Results**

**Microplusin has a fungistatic effect against *C. neoformans***

In a previous study, we showed by absorbance readings (*A*₅₉₅nm) that 0.09 μM of microplusin inhibited 50% of the growth of *C. neoformans* (Silva et al., 2009). However, we did not determine whether microplusin was fungicidal or fungistatic. We addressed this question by incubating *C. neoformans* (strain H99) with 10 μM microplusin. After 72 h incubation, the number of MP-treated yeast cells was 10-fold lower compared with non-MP treated cells (Fig. 1a). A similar result was obtained after 48-h incubation with microplusin (data not shown). To determine the viability of *C. neoformans* after exposure to MP, 100 yeast cells (MP-treated and non-MP treated systems) were plated onto Sabouraud agar medium. Although there was a trend toward a reduction in CFU after 48-h incubation in MP-treated cells, the CFU determinations were not significantly different (*P*-value = 0.1710) between the two culture conditions (Fig. 1b). Hence, microplusin predominantly has a fungistatic effect against *C. neoformans*. In addition, supplementation of PDB medium with 2.5 μM of CuCl₂·6H₂O significantly impaired microplusin’s activity against *C. neoformans* (Fig. 2). The protective effect of copper depended on the concentration of microplusin, as the inhibitory activity of the compound was most pronounced at microplusin concentrations ≥ 1.56 μM.

**Effect of microplusin on oxygen consumption**

To test whether the copper depletion promoted by microplusin affected complex IV functioning, and therefore electron flow through classical respiratory pathway, we measured oxygen consumption of *C. neoformans* in the presence of different inhibitors of the electron transport complexes. In non-treated *C. neoformans*, electrons flow largely via the classical pathway, since inhibition of either complex III/cytochrome *c* reductase with 4 μM AA or complex IV/cytochrome oxidase with 1 μM KCN decreased oxygen consumption by ~70% (Fig. 3). In these non-treated fungi, inhibition of the AOX of the alternative respiratory pathway with 4 μM SHAM did not affect the rate of oxygen consumption, although it fully abrogated respiration in combination with AA. Microplusin completely altered the respiratory profile of *C. neoformans*. The basal oxygen consumption in MP-treated cells was approximately 40% lower than that in non-MP treated cells. In treated fungi, AA or KCN did not further disturb the rate of oxygen consumption, while SHAM fully impaired respiration, which implies that the classical electron transport pathway was either damaged or absent.
and that respiration of *C. neoformans* is entirely driven by the alternative pathway.

**Microplusin inhibits *C. neoformans* melanization**

As laccase is a copper-dependent oxidase responsible for melanization (Zhu & Williamson, 2004) in *C. neoformans*, we investigated whether the copper-chelating properties of microplusin might have a negative effect on this process. Microplusin inhibited melanization of the strains H99 and B3501 at concentrations ≥ 3.12 μM (Fig. 4a). When we supplemented a culture of *C. neoformans* strain B3501 with 2.5 μM of CuCl₂·6H₂O, we observed that the presence of this metal caused a twofold reduction in the antimelanization activity of microplusin (Fig. 4b). Similar results were obtained with *C. neoformans* strain H99 (data not shown). Moreover, we observed that microplusin reduced the laccase activity of *C. neoformans* strain H99 by almost 50% (Fig. 4c).

In parallel, we evaluated whether microplusin could reduce L-dopa autopolymerization in a manner similar to glyphosate, a compound whose antimelanization activity in *C. neoformans* has been described (Nosanchuk et al., 2001). Microplusin did not inhibit the autopolymerization of L-dopa and even increased this process at concentrations ≥ 6.25 μM (Fig. 4d).

**Microplusin inhibits the production of *C. neoformans* polysaccharide capsule**

Several enzymes are involved in the formation of the polysaccharide capsule in *C. neoformans* (reviewed in Zaragoza et al., 2009) and microplusin might affect this process by copper depletion, as copper is a co-factor for some of these enzymes. Our results revealed that microplusin impeded capsule enlargement of *C. neoformans* (strain T1444) in a dose-dependent manner (Fig. 5a). We also observed that 25 μM of microplusin significantly inhibited the capsular enlargement of H99 and B3501 (Fig. 5b and c).

**Discussion**

Our main hypothesis was that microplusin could negatively affect *C. neoformans* by copper depletion, which would be consistent with the importance of copper homeostasis for this fungus (Davis-Kaplan et al., 1998; Cox et al., 2003; Zhu et al., 2003; Waterman et al., 2007;
Jiang et al., 2009) and the copper-chelating property of microplusin at a MP : copper II molar ratio of 1 : 1 (Silva et al., 2009). We have shown that microplusin at concentrations ≥ 1.56 μM significantly affected the growth of C. neoformans, similar to the activity of the peptide against M. luteus (Silva et al., 2009). Moreover, the anticryptococcal effect was considerably reversed when 2.5 μM copper was added.

Notably, our results indicate the presence of both classical and alternative respiratory pathways in the C. neoformans electron transport chain and suggest that the effect of microplusin on the growth of the fungi may be related to the damage of the classical respiratory chain, probably at the copper-containing complex IV. Although we cannot entirely discard the effects of Fe²⁺ on microplusin, our assumption that microplusin is preferentially a copper chelator is based on the fact that the four respiratory complexes that have iron as prosthetic groups or bound to the heme group remained functional, whereas complex IV, the only complex that has copper as a prosthetic group, was affected by microplusin. We also show that microplusin stimulated the alternative respiratory pathway in C. neoformans, likely to compensate for the damaged classical electron transport chain. The alternative pathway is not coupled to oxidative phosphorylation and ATP synthesis, and hence, energy production in microplusin-treated yeasts is likely to be deficient. However, uncoupled respiration helps the cells to manage reactive oxygen species production under stress conditions. Similar to complex IV, the assembly and functioning of other copper proteins, such as the antioxidant enzyme Cu-Zn superoxide dismutase (SOD1), might also be compromised in microplusin-treated C. neoformans.

Microplusin at concentrations ≥ 3.12 μM clearly inhibited C. neoformans melanization as well as reduced laccase activity. This further suggests that the copper-chelating ability of microplusin may affect the loading of copper ions to laccase apoenzyme. In addition, we observed that copper supplementation of the medium prevented the inhibition of melanization by microplusin, according to
1:1 binding ratio (Silva et al., 2009). A correct laccase metallation is reportedly crucial for its biological activity, as shown for the laccase produced by the avirulent Δvph1 mutant of C. neoformans. Defective vesicular acidification disrupts the insertion of copper cofactors into proteins, resulting in the inability of Δvph1 laccase to catalyze phenolic compounds to melanin (Erickson et al., 2001). As expected, addition of 1 mM of the copper chelator BCS to the medium abolished laccase activity not only in the Δvph1 mutant but also in the wild-type strain and copper supplementation, restored laccase activity as well as induced its transcription (Zhu et al., 2003). Therefore, these data support the hypothesis that microplusin sequesters copper and may affect the availability of this metal to copper-dependent enzymes, such as laccase.

The microplusin concentrations that inhibited melanization (≥3.12 μM) also increased the autopolymerization of l-dopa. L-dopa autopolymerization is a process that occurs spontaneously by exposure to light (Mason, 1955). Microplusin probably stimulates the spontaneous autopolymerization of the products derived from l-dopa oxidation; however, this possible action did not interfere with its inhibitory effect on melanization of C. neoformans in the absence of light.

Capsule enlargement in C. neoformans requires extra-cellular deliverance of GXM, which is further incorporated into the fungal cell surface to promote distal capsular growth (reviewed in Zaragoza et al., 2009). The subsequent self-aggregation of polysaccharide molecules occurs by mechanisms that putatively require divalent cations, such as calcium II (Nimrichter et al., 2007). The inhibitory activity of microplusin on capsular enlargement could be due to the interference with aggregation of the building blocks through metal chelation, thereby affecting the correct polysaccharide capsule assembly. However, based on our mass spectrometry analysis, microplusin does not bind calcium II (Silva et al., 2009). Thus, its effect on capsule enlargement most likely results from inhibition of one or more metabolic processes dependent on enzymes that requires copper as a cofactor. Notably, the Δvph1 mutant also had aberrant capsular production (Li & Kaplan, 1998; Erickson et al., 2001).

Fig. 5. Effect of microplusin on the formation of the polysaccharide capsule of Cryptococcus neoformans. (a) Measurement of capsule size in C. neoformans (strain T 1444) incubated with serial dilutions of microplusin (25.0–0.78 μM) for 48 h at 37 °C. (b) Capsular dimensions of C. neoformans (strains H99 and B3501) after incubation with 25 μM microplusin for 48 h at 37 °C. (c) Capsule morphology of C. neoformans (strain H99) after exposure to microplusin. Scale bar: 10 μm. MP-treated: yeast cells treated with microplusin; non-MP-treated: control without microplusin. The experiments were repeated twice with similar results. ***P < 0.05 comparing MP-treated to non-MP-treated yeast cells (control).
In conclusion, microplusin showed a noteworthy fungistatic activity in vitro against *C. neoformans*. We demonstrate that this effect may be related to its inhibitory effect on the classical respiratory pathway of *C. neoformans*. Microplusin also affected the two most important virulence factors of this mycopathogen: the melanization process and the formation of a polysaccharide capsule. These findings are particularly relevant for determining the utility of copper-chelator compounds, like microplusin as a therapeutic for cryptococcosis. However, studies in vivo are crucial to corroborate the efficiency of this peptide or other metal chelators for combating *C. neoformans*.

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