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Sphingolipid biosynthetic pathway is crucial for growth, biofilm formation and membrane integrity of *Scedosporium boydii*

Rodrigo Rollin-Pinheiro¹, Victor Pereira Rochetti¹, Mariana Ingrid Dutra da Silva Xisto¹, Livia Cristina Liporagi-Lopes², Beatriz Bastos³, Antonella Rella⁴, Ashutosh Singh⁴, Sonia Rozental³, Maurizio Del Poeta^{4,5,6} & Eliana Barreto-Bergter^{*,1}

¹Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-902, Brazil

²Departamento de Análises Clínicas e Toxicológicas, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-902, Brazil

³Departamento de Parasitologia e Biologia Celular, Instituto de Biofísica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-902, Brazil

⁴Molecular Genetics & Microbiology Department, Stony Brook University, NY 11794, USA

⁵Division of Infectious Diseases, Stony Brook University, NY 11794, USA

⁶Veterans Affairs Medical Center, Northport, NY 11768, USA

*Author for correspondence: Tel.: +552139380358, eliana.bergter@micro.ufrj.br

Aim: Glycosphingolipids are conserved lipids displaying a variety of functions in fungal cells, such as determination of cell polarity and virulence. They have been considered as potent targets for new antifungal drugs. The present work aimed to test two inhibitors, myriocin and DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol, in *Scedosporium boydii*, a pathogenic fungus which causes a wide range of disease. **Materials & methods:** Mass spectrometry, microscopy and cell biology approaches showed that treatment with both inhibitors led to defects in fungal growth and membrane integrity, and caused an increased susceptibility to the current antifungal agents. **Conclusion:** These data demonstrate the antifungal potential of drugs inhibiting sphingolipid biosynthesis, as well as the usefulness of sphingolipids as promising targets for the development of new therapeutic options.

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Scedosporium species are filamentous fungi widely found in nature, especially in human-impacted areas [1,2]. *Scedosporium boydii* is one of the species associated with fungal infections in immunocompromised and immunocompetent patients. Scedosporiosis is cosmopolitan and has been reported in North and South America, Europe, Asia and Oceania [3]. It causes wide-spectrum diseases, ranging from cutaneous and subcutaneous tissue infections and traumatic inoculation to invasive and disseminated cases as result of conidia inhalation [4].

Glycosphingolipids, a class of lipid molecules present on cell surfaces and in membranes, play crucial roles in fungal growth and virulence. They are composed of a sphingoid backbone linked to a fatty acid chain through an amide bond and the presence of a sugar unit [5]. Disruption of genes, such as *gcs1* (glucosylceramide synthase), *mts1* (C9-methyltransferase) and *sld* ($\Delta 8$ desaturase), has provided knowledge that highlights the importance of glycosphingolipid synthesis for fungal cells. Mutants of *Cryptococcus neoformans*, *Aspergillus nidulans*, *Fusarium graminearum*, *Penicillium digitatum* and *Candida albicans* displayed decreased virulence in animal and plant infection models, deficient fungal growth and hyphal elongation, defects in alkali tolerance, higher susceptibility to membrane stressors and reduced extravesical secretion [6–10]. Molecules that directly bind to glycosphingolipids on fungal surfaces, such as defensins produced by plants (MsDef1 and RsAFP2) and monoclonal antibodies against glucosylceramides, are known to alter cell shape, conidia germination and yeast-to-hyphae transition, as observed for

C. albicans, *Pichia pastoris*, *Fonsecaea pedrosoi*, *Colletotrichum gloeosporioides*, *Pseudallescheria boydii* and *Scedosporium apiospermum* [11–16].

Inhibitors of sphingolipid biosynthetic pathways have also been already tested in some pathogenic fungi. Myriocin and sphingofungin are inhibitors of serine palmitoyltransferase, important in the first step of sphingolipid biosynthetic pathway, and have been shown to impair *Candida* and *Aspergillus* species biofilm formation [17,18]. Fumonisin B1 and australifungin are ceramide synthase inhibitors displaying antifungal activities in *Cryptococcus*, *Candida* and *Aspergillus* species [19]. Inositolphosphoryl ceramide (IPC) synthases are inhibited by aureobasidin A, khakrefungin and galbonolide, which possess antifungal activity against *C. albicans* and *C. neoformans* by causing ceramide accumulation to toxic levels [20,21]. D-threo-1-phenyl-2-decanoylamino-3-morpholino-1-propanol (D-threo-PDPM) blocks glucosylceramide synthase which is involved in the last step of glucosylceramide synthesis, resulting in reduced hyphal germination and colony growth in *A. nidulans* and *A. fumigatus* [22].

The development of new therapeutic approaches to treat fungal diseases is an urgent need, especially for invasive infections, since the current antifungal options are limited. In this context, fungal sphingolipids have been considered a potential target for new antifungal drugs because their structures and biosynthetic pathway show crucial differences compared with those of mammals [23]. This work aimed to evaluate the effects of sphingolipid inhibition on *S. boydii* growth, morphology, membrane integrity and susceptibility to other antifungal drugs, by treating cells with two sphingolipid inhibitors, myriocin and DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP).

Materials & methods

Microorganism & growth conditions

Scedosporium boydii CBS 120157, a clinical strain isolated from the lung of a patient with leukemia, was kindly provided by Sybren de Hoog from the Westerdijk Fungal Biodiversity Institute, Utrecht, The Netherlands. Cells were maintained in modified Sabouraud media (0.5% yeast extract, 1% peptone and 2% glucose). To produce conidia, a 7-day culture on Sabouraud-agar plates at room temperature was scraped off in phosphate-buffered saline (PBS) and the collected conidia were filtered and washed twice with sterile PBS.

Myriocin (Sigma-Aldrich, M1177) and PPMP (Sigma-Aldrich, P4194) were added to RPMI 1640 media (Sigma-Aldrich, R8758, MO, USA).

Sphingolipid profiling of treated cells

Scedosporium boydii conidia (10^5) were grown in RPMI 1640 medium at 37°C for 12 h in the presence of myriocin (4, 8 and 16 µg/ml) or PPMP (32, 64 and 128 µg/ml). Control cells were grown in RPMI 1640 supplemented with 2% methanol, the solvent used to dissolve both inhibitors. After incubation, the cultures were centrifuged and lipids were extracted from cell pellets by adding ethanol:H₂O:diethylether:pyridine:1 M NH₄OH (15:15:5:1:0.018; v/v) for 1 h at 60°C [19]. Partitioning of neutral lipids was performed using methanol:chloroform (2:1; v/v) for 1 h at 37°C [24] followed by base hydrolysis. Electrospray ionization mass spectrometry of the extracted lipids was used to measure sphingolipid content [25].

Inhibitors effect on fungal growth

The effect of myriocin and PPMP in *S. boydii* growth was evaluated as described previously [26]. Conidia (10^5) were grown in a 10 ml culture of RPMI 1640 medium at 37°C in the presence of myriocin (4, 8 and 16 µg/ml) or PPMP (32, 64 and 128 µg/ml). Control cells were grown in RPMI 1640 supplemented with 2% methanol. Aliquots were collected at different time points, diluted and plated onto Sabouraud's agar and incubated at 30°C for 72 h to count colony-forming units (CFUs).

A Cytation 5 Imaging Reader (BioTek, VT, USA) was used to measure optical density (O.D.) at 660 nm and to photograph germinating conidia. Conidia (10^5) were grown in 24-well plates in the same conditions as described above and O.D. was measured every hour during 48 h. Additionally, each well was photographed every hour to monitor germination.

Biofilm formation & quantification

Scedosporium boydii biofilms were obtained as described previously [27]. Conidia (10^7) were incubated in 96-well plates at 36°C for 1.5 h (adherence phase). Then, RPMI 1640 media supplemented with 2% glucose and 20% fetal bovine serum (Gibco, MD, USA) was added in the presence of myriocin (4, 8 and 16 µg/ml) or PPMP

(32, 64 and 128 µg/ml). Control cells were grown in RPMI 1640 supplemented with 2% methanol. After 24 h of incubation at 37°C, biofilm formation was evaluated using three parameters: violet crystal to measure overall biomass, safranin to analyze extracellular matrix and XTT for metabolic activity [28].

Filipin staining

Scedosporium boydii conidia were stained with filipin [29]. Cells were firstly incubated in RPMI 1640 media for 12 h at 37°C, in the presence of myriocin (16 µg/ml) or PPMP (128 µg/ml). Control cells were grown in RPMI 1640 supplemented with 2% methanol. Germinated conidia were further stained with 50 µg/ml of filipin (Sigma-Aldrich, F9765) for 2 h at room temperature protected from light. After washing twice with PBS, cells were visualized using a fluorescence microscope (Axioplan Imager 2, Carl Zeiss).

Transmission electron microscopy

Conidia of *S. boydii* (10^5) were treated with myriocin (16 µg/ml) or PPMP (128 µg/ml) for 12 h at 37°C. Control cells were grown in RPMI 1640 supplemented with 2% methanol. After the incubation, cells were washed in 0.01 M PBS (pH 7.2) and fixed overnight in 2.5% glutaraldehyde and 4% formaldehyde in 0.1 M cacodylate buffer. The samples were washed in the same buffer, postfixed in 1% osmium tetroxide and 1.25% potassium ferrocyanide for 40 min, and dehydrated in a series of ethanol solutions with increasing concentrations (30, 50, 70, 90, 100% and 'ultra-dry' ethanol) for 30 min at each concentration. Then, the cells were embedded in Spurr's resin. At last, ultramicrotomy (LEICA U6) was used to cut ultra-thin sections of 60 nm that were stained with uranyl acetate and lead citrate for observations under transmission electron microscopy (TEM; ZEISS 900; FEI, Eindhoven, The Netherlands).

Susceptibility to antifungal agents

Scedosporium boydii susceptibility to different antifungal agents was performed in the presence of subinhibitory concentrations of myriocin (4 µg/ml) and PPMP (64 µg/ml). Fluconazole, amphotericin B and caspofungin were used as antifungals at concentrations ranging from 1.57 to 100 µg/ml. The checkerboard method to detect synergistic interactions was used according to EUCAST guidelines [30]. Cells were incubated at 37°C for 72 h and minimal inhibitory concentration (MIC) was evaluated at 600 nm using a spectrophotometer (iMark, Bio-Rad, CA, USA). An XTT reduction assay evaluated cell viability. Fractional inhibitory index (FIC) was calculated according to the following formula: (MIC combined/MIC drug A alone) + (MIC combined/MIC drug B alone). Results were classified as: strongly synergistic effect, FIC of <0.5; synergistic effect, FIC of <1; additive effect, FIC of 1; no effect, FIC between 1 and 2; antagonistic effect, FIC of 2 [31].

Susceptibility to membrane stressors

Scedosporium boydii cells were pretreated with subinhibitory concentrations of myriocin (4 µg/ml) or PPMP (64 µg/ml) for 24 h at 37°C in RPMI 1640 media. SDS (120 µg/ml) and NaCl (5%) were added to the media and, after another 24 h-incubation, cell viability was measured by XTT-reduction assay.

Results & discussion

Myriocin & PPMP alter sphingolipid levels in *S. boydii*

Since myriocin and PPMP are sphingolipid inhibitors, we determined whether the treatment affected lipid profiles in *S. boydii* cells. The sphingolipid biosynthetic pathway, highlighting the steps in which myriocin and PPMP act, is summarized in Supplementary Figure 1. Dihydrosphingosine, one of the first intermediates of the biosynthetic pathway, was reduced by both myriocin and PPMP (Figure 1A).

Regarding the glucosylceramide branch of the pathway, myriocin at 4, 8 and 16 µg/ml strongly decreased dihydroceramide (α OH-Cer) levels, but did not alter 9-methyl-4,8-sphingadienine (α OH- Δ 8-9Me-Cer), (Figure 1B). PPMP treatment strongly decreased α OH-Cer at 32, 64 and 128 µg/ml, but α OH- Δ 8-9Me-Cer only accumulated in cells treated with 128 µg/ml of PPMP (Figure 1D), indicating that the amount of GlcCer was reduced even in the absence of α OH- Δ 8-9Me-Cer accumulation. Interestingly, the final product glucosylceramide is differently affected by both inhibitors. While PPMP reduced GlcCer production at all concentrations, myriocin only led to changes in GlcCer levels at 16 µg/ml, suggesting that higher concentrations are needed for myriocin to decrease GlcCer levels (Figure 1B & D).

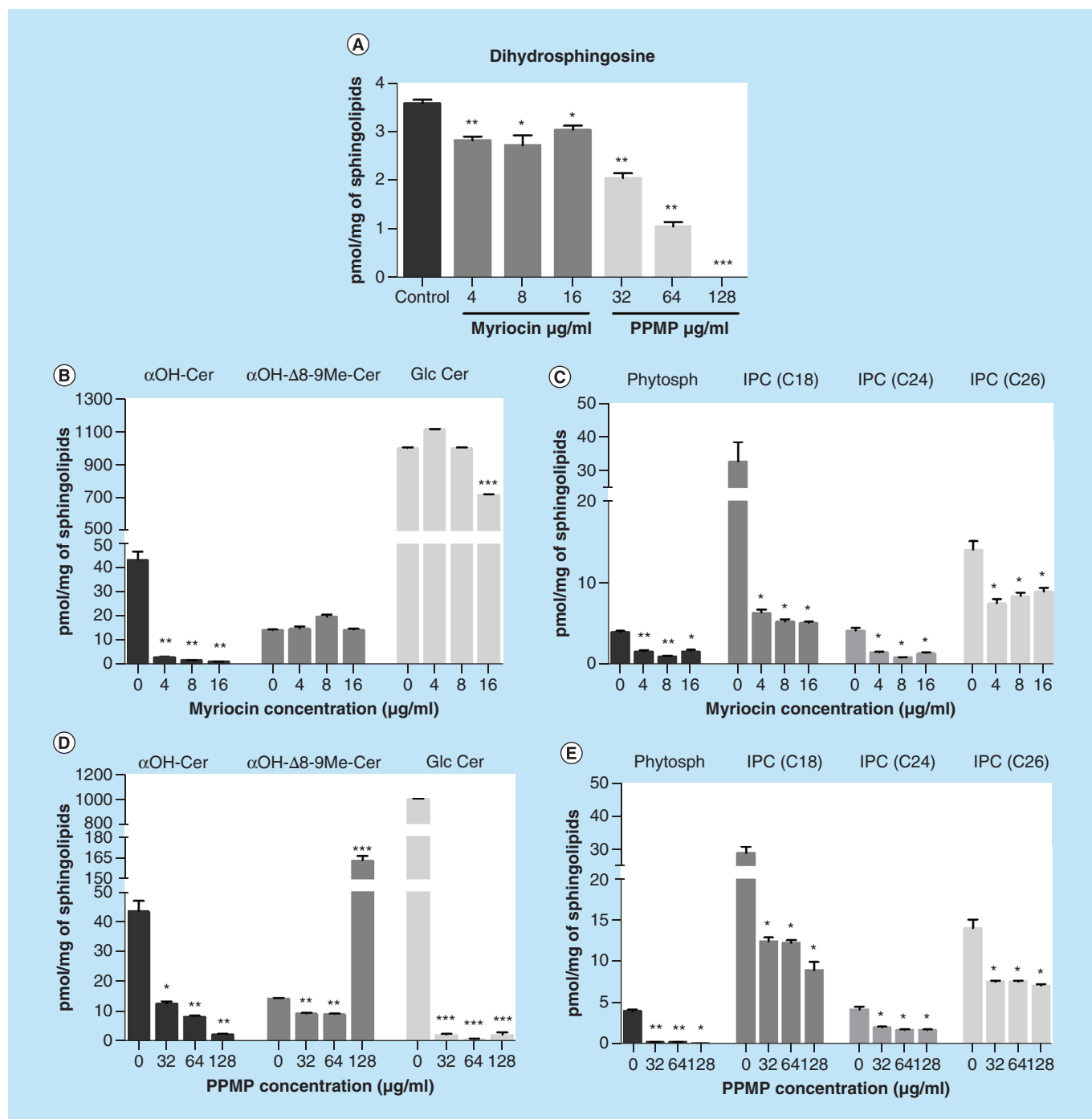


Figure 1. Quantification of sphingolipid intermediates. Cells were incubated for 12 h in the presence of myriocin (4, 8 and 16 µg/ml) or PPMP (32, 64 and 128 µg/ml). Control cells were grown in RPMI 1640 supplemented with 2% methanol, the solvent used to dissolve both inhibitors. After lipid extraction, the samples were analyzed by mass spectrometry and the values were normalized to the total amount of extracted sphingolipids. The following intermediates of sphingolipid biosynthetic pathway were analyzed: Dihydrosphingosine (A), Glucosylceramide branch intermediates (B & D) and IPC branch intermediates (C & E).

*p < 0.05; **p < 0.03; ***p < 0.001.

αOH-Cer: Dihydroceramide; αOH-Δ8-9Me-Cer: 9-methyl-4,8-sphingadienine; GlcCer: Glucosylceramide; IPC: Inositolphosphorylceramide; Phytosph: Phytosphingosine; PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.

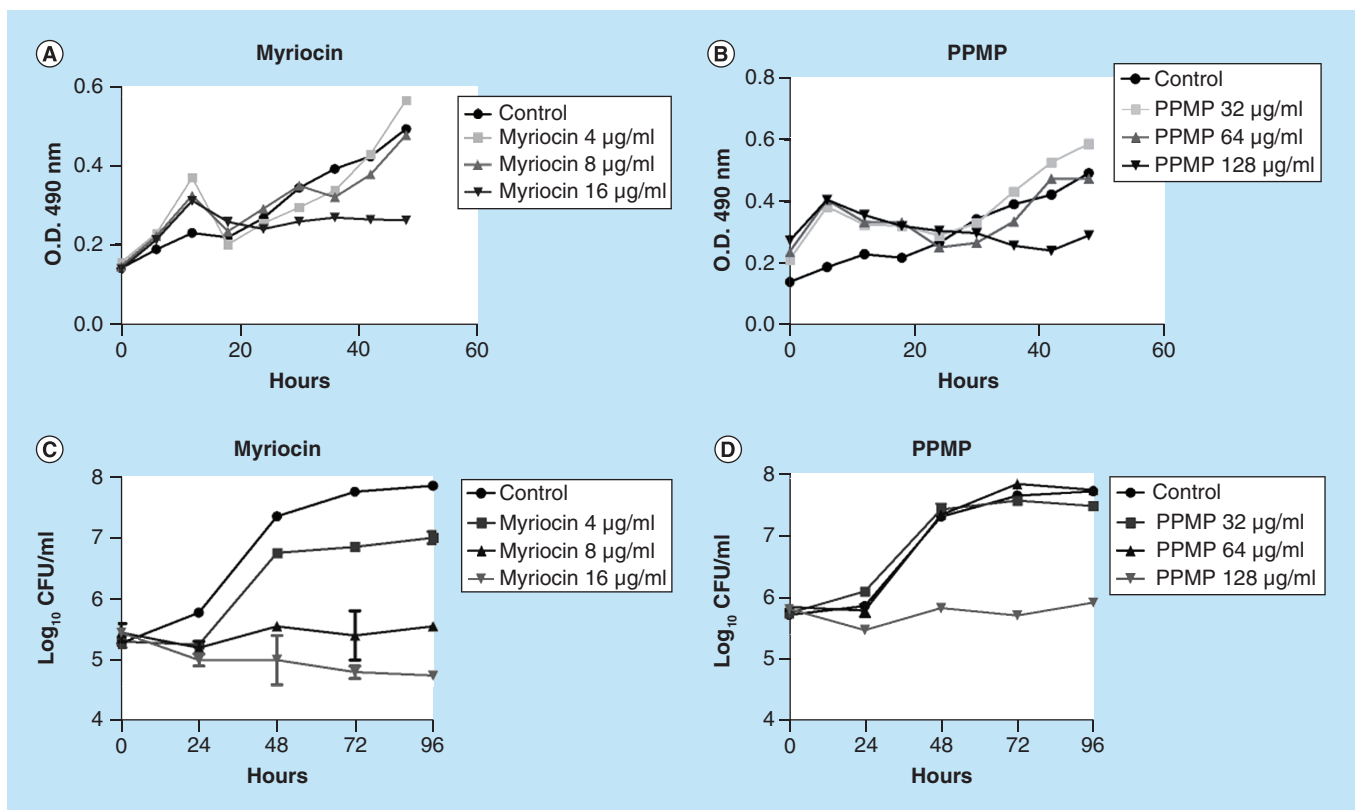


Figure 2. Effect of myriocin and DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol on fungal growth. Conidia were incubated in RPMI in the presence of myriocin (4, 8 and 16 µg/ml) or PPMP (32, 64 and 128 µg/ml). Control cells were grown in RPMI 1640 supplemented with 2% methanol. Growth measurement was performed at different time points by optic density (A & B) and CFU counting (C & D).

CFU: Colony-forming unit; PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.

Intermediates of IPCs were also analyzed. Both myriocin and PPMP at all concentrations used led to a reduction in phytosphingosine and IPCs (C18, C24 and C26) (Figure 1C & E). All these data indicated that sphingolipid inhibitors modify the pattern of the biosynthetic pathway and alter sphingolipid content in *S. boydii*.

Singh and colleagues observed in *C. neoformans* that dhSph, dhCer/phytoCer and GlcCer represent 1.2–2.6, 49–80 and 16–45%, respectively, of the total sphingolipid content [25]. These data corroborate our measurements, in which αOH-Cer, IPCs and GlcCer were the most abundant molecules in *S. boydii*, and also those most reduced by myriocin and PPMP, suggesting that a high concentration of these intermediates is important for sphingolipid biosynthesis.

Scedosporium boydii growth & biofilm formation is reduced by myriocin & PPMP treatment

In order to check the effect of sphingolipid inhibitors on *S. boydii* growth, conidia were grown in RPMI 1640 supplemented with myriocin (4, 8 and 16 µg/ml) and PPMP (32, 64 and 128 µg/ml). O.D. at 600 nm was measured hourly for 48 h. Myriocin at 16 µg/ml and PPMP at 128 µg/ml decreased cell density, whereas lower concentrations of both inhibitors did not affect fungal growth (Figure 2A & B).

CFUs were counted to evaluate fungal viability in the presence of both inhibitors. Myriocin at 8 and 16 µg/ml and PPMP at 128 µg/ml impaired fungal growth compared with the control, since CFU did not increase during the incubation time (up to 96 h) (Figure 2C & D).

To understand whether these inhibitors alter fungal differentiation, conidial germination was assayed by incubating conidia for 12 h at 37°C in the presence or absence of myriocin and PPMP. Compared with the control in which mature hyphae developed after 12 h (Figure 3A & B), myriocin-treated cells showed only short germ tubes (Figure 3C, E & G), indicating an impairment of the germination process. PPMP treatment resulted in longer

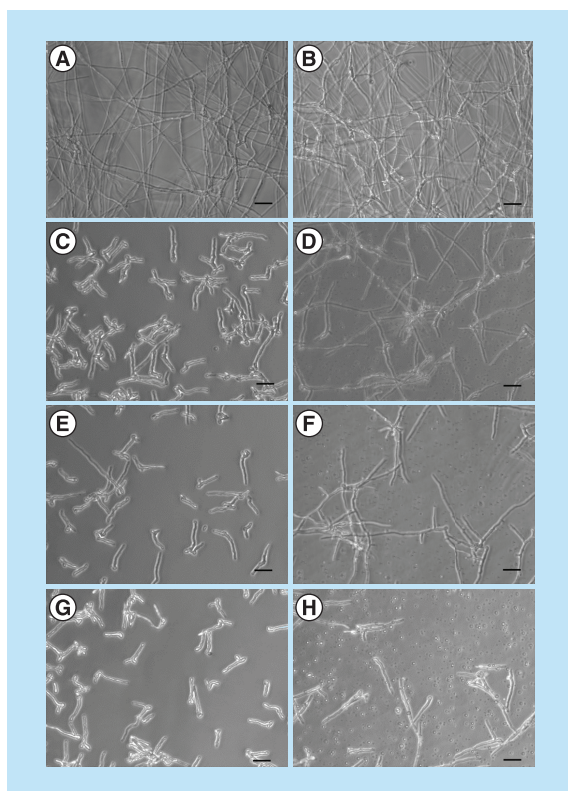


Figure 3. Germination assay of *Scedosporium boydii* conidia. Cells were incubated for 12 h in RPMI in the presence of myriocin or PPMP. Control cells were grown in RPMI 1640 supplemented with 2% methanol (A & B). (C, E & G) *S. boydii* incubation with myriocin at 4, 8 and 16 $\mu\text{g/ml}$, respectively. (D, F & H) *S. boydii* incubation with PPMP at 32, 64 and 128 $\mu\text{g/ml}$, respectively. After the incubation time, the plates were visualized using an optical microscope. PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.

germ tubes than myriocin treatment, but hyphae failed to mature as observed in the control (Figure 3D, F & H). These data suggested that sphingolipids are crucial for hyphae elongation and consequently, for fungal growth.

Because biofilm is an important structure for fungal virulence, the influence of sphingolipid inhibitors on *S. boydii* biofilm formation was assessed. Myriocin at 8 and 16 $\mu\text{g/ml}$ reduced fungal biomass, metabolic activity and matrix production, whereas PPMP (32, 64 and 128 $\mu\text{g/ml}$) only decreased matrix production, suggesting that sphingolipids are important molecules for biofilm development, especially for the synthesis of a robust extracellular matrix (Figure 4).

Similar effects were observed in *A. fumigatus* and *C. albicans*, with reduced CFU, growth and biofilm formation after 24 h of myriocin treatment [18], as well as disruption of lipid raft organization [17], suggesting that this point of inhibition in sphingolipid biosynthesis is conserved in pathogenic fungi. Several PPMP analogs, such as PDMP ((\pm) - threo - 1 - Phenyl - 2 - decanoylamino - 3 - morpholino - 1 - propanol hydrochloride) and EDO-P4, have been tested in *A. fumigatus* and *A. nidulans* [22]. Each analog displayed a differentiated influence in *Aspergillus* species, indicating that chemical modifications in the structure are a valid approach to increase the effects of these inhibitors.

Similar observations were also reported for *A. fumigatus* mutants lacking glucosylceramide synthase and $\Delta 8$ -desaturase genes, in which the absence of glucosylceramide resulted in deficient conidiation and radial growth [32], showing that the inability to synthesize glucosylceramide lead to similar patterns found when inhibitors are used.

We observed that myriocin and PPMP alter cell membrane differently, especially regarding GlcCer production, suggesting that fungal growth can be affected by a variety of changes in membrane lipids. In fact, studies of *Aspergillus*, *Fusarium* and *Candida* mutant strains presenting gene disruptions in other genes than that coding for GlcCer synthase, such as the genes for delta-8 desaturase and C9-methyltransferase, also display membrane alterations and decreased fungal virulence [7,9,10,32]. These findings indicate that an imbalance of sphingolipid biosynthesis could lead to phenotypic alterations in fungal cells. This could be a reason why myriocin reduced *S. boydii* growth at 16 $\mu\text{g/ml}$ even causing a lesser decrease of GlcCer compared with PPMP.

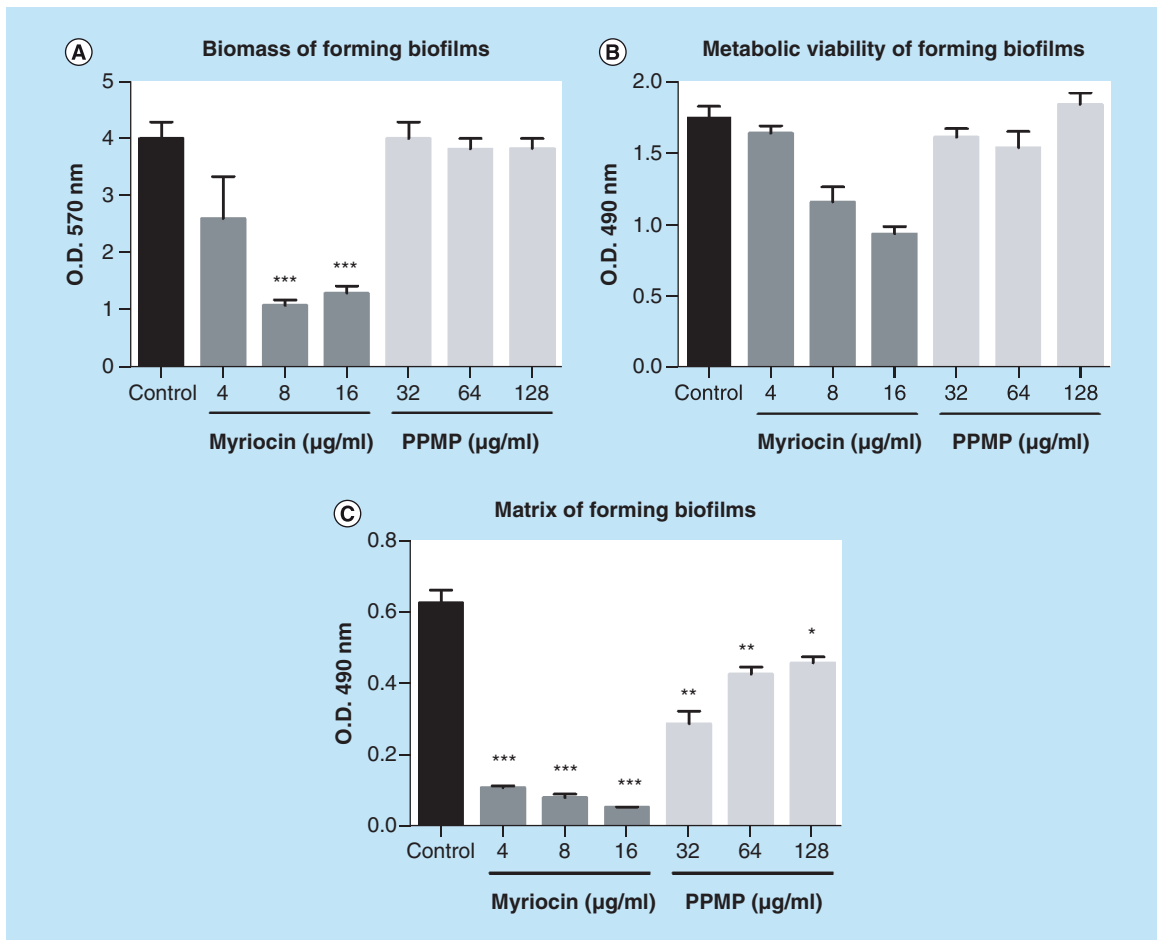


Figure 4. Biofilm formation by *Scedosporium boydii* in the presence of myriocin (4, 8 and 16 $\mu\text{g/ml}$) or DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (32, 64 and 128 $\mu\text{g/ml}$). Control cells were grown in RPMI 1640 supplemented with 2% methanol. Cells were incubated for 24 h and then the following parameters were measured: biomass by crystal violet staining (A); cell viability by XTT-reduction assay (B); and matrix quantification by safranin staining (C).

*p < 0.05; **p < 0.03; ***p < 0.001.

PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.

Myriocin & PPMP affect lipid raft accumulation in hyphal tips

Lipid raft accumulation is crucial for polarized cell growth because it modulates vesicle recruitment and cell wall synthesis [33,34]. For this reason, *S. boydii* cells were stained with filipin to visualize sterol-enriched microdomains in plasma membrane (Figure 5). Filipin is a polyene macrolide that binds to ergosterol present in lipid rafts and is commonly used in sphingolipid studies [32,35]. Since a defect in *S. boydii* germination was observed in the presence of sphingolipid inhibitors, cells were treated with filipin to check lipid raft organization in fungal membrane after exposure to myriocin and PPMP. Compared with the control, which displayed the expected lipid raft accumulation in the apical region, cells treated with myriocin were homogeneously stained by filipin, showing a lack of accumulation along the plasma membrane (Figure 5). In PPMP treated cells, lipid rafts randomly accumulated along the membrane, suggesting a disorganization in lipid microdomains (Figure 5).

Similar patterns were observed in *A. fumigatus* mutants lacking *gcs1* and *sld* genes, in which filipin staining revealed a cell polarity defect [32]. These data suggest that disrupting glucosylceramide synthesis, either by disrupting genes or by using chemical inhibitors, results in deficient lipid raft accumulation in tip zones which could explain the impairment of fungal germination when filamentous fungi are treated with these inhibitors.

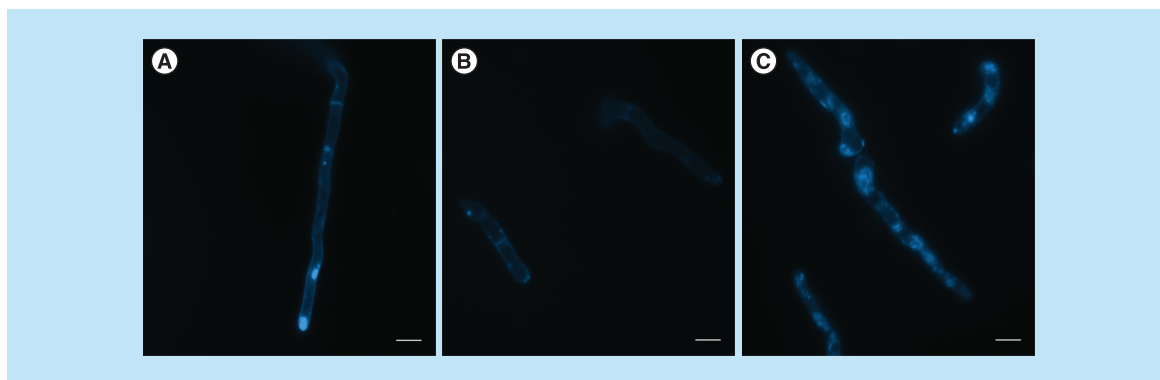


Figure 5. Sterol staining using filipin. *Scedosporium boydii* conidia were incubated for 12 h in the presence of myriocin (16 $\mu\text{g/ml}$) or PPMP (128 $\mu\text{g/ml}$). Control cells were grown in RPMI 1640 supplemented with 2% methanol. After incubation time, cells were fixed and stained with filipin (50 $\mu\text{g/ml}$) for 2 h. Samples were analyzed using a fluorescent microscope. (A) Control. (B) Myriocin. (C) PPMP. PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.

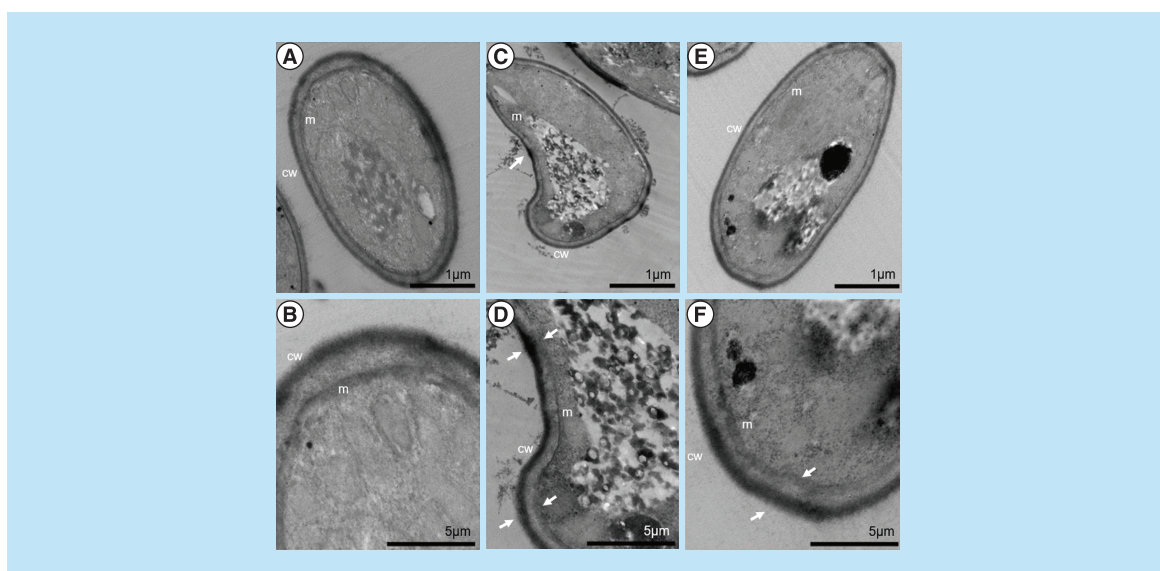


Figure 6. Transmission electron microscopy of *Scedosporium boydii* cells incubated for 12 h in the presence of myriocin (16 $\mu\text{g/ml}$) or PPMP (128 $\mu\text{g/ml}$). Control cells were grown in RPMI 1640 supplemented with 2% methanol. After the incubation time, the samples were processed as described in the methodology section and visualized using a ZEISS 900 microscope. White arrows indicate altered membrane (m) and cell wall (cw). (A & B) Control. (C & D) Myriocin. (E & F) PPMP. PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.

Sphingolipid inhibitors affect *S. boydii* morphology & membrane integrity

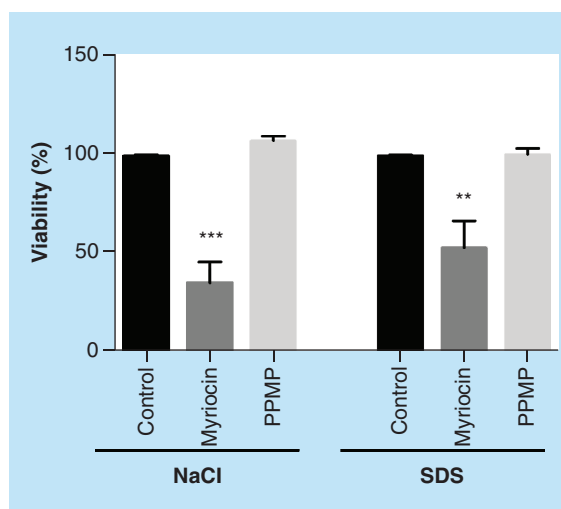
In order to visualize the effect of sphingolipid inhibitors in *S. boydii*, cells were treated with myriocin (16 $\mu\text{g/ml}$) and PPMP (128 $\mu\text{g/ml}$) for 12 h and the samples were processed for TEM. Compared with the control, both treatments resulted in punctual membrane alterations that seemed to be accompanied by a thicker cell wall region, suggesting that the loss of membrane integrity could be followed by a compensation mechanism in the cell wall (Figure 6). Additionally, an intracellular accumulation of electron dense material was observed in both myriocin and PPMP-treated cells.

Myriocin-treated *A. fumigatus* cells displayed similar patterns, as demonstrated by TEM analysis [18]. After 4 h of incubation, plasma membrane became nonhomogeneous, suggesting the loss of bilayer integrity when cells were in contact with myriocin.

Figure 7. *Scedosporium boydii* susceptibility to membrane stressors after incubation with myriocin (16 $\mu\text{g/ml}$) or DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol (128 $\mu\text{g/ml}$). Cells were pre-incubated with the inhibitors for 12 h and then the media were changed for those supplemented with NaCl (5%) or SDS (120 $\mu\text{g/ml}$). After 24 h in the presence of stressors, cell viability was analyzed by XTT-reduction assay.

** $p < 0.03$; *** $p < 0.001$.

PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.



To confirm the hypothesis that plasma membrane is punctually affected by sphingolipid inhibitors, as observed in TEM, *S. boydii* cells were grown in the presence of membrane stressors, such as SDS and NaCl, and cell viability was measured. When previously treated with myriocin (subinhibitory concentration), cells were more susceptible to SDS and NaCl compared with the control, indicating that the reduction of sphingolipid content leads to a more fragile plasma membrane (Figure 7). PPMP treatment did not alter the susceptibility to membrane stressors comparing to the control, suggesting that each inhibitor acts differently in plasma membrane susceptibility (Figure 7). Interestingly, *A. fumigatus* mutants lacking *gcs1* and *sld* genes are more resistant to cell wall stressors, such as calcofluor and Congo red [32], which corroborates with the results found in SEM images, in which a thicker *S. boydii* cell wall was observed in the regions where plasma membrane presented less integrity.

Sphingolipid inhibition influences *S. boydii* susceptibility to antifungal agents

Since sphingolipids are considered a potent target for new antifungal drugs, myriocin and PPMP were used in combination with three different antifungal agents with distinct modes of action: fluconazole, amphotericin B and caspofungin. Myriocin increased *S. boydii* susceptibility to all antifungals tested (Figure 8). The MIC values of fluconazole, amphotericin B and caspofungin decreased from 25, 100 and 25 $\mu\text{g/ml}$, respectively, from when tested alone, to 3.125, 12.5 and 6.25 $\mu\text{g/ml}$, respectively, when combined with myriocin, resulting in a synergistic effect (Supplementary Table 1). In contrast, PPMP did not alter susceptibility of *S. boydii* to the antifungals (Figure 8). Although we are currently not suggesting myriocin and PPMP as therapeutic options, these data add additional support for exploring sphingolipids as targets for future drug development.

Little is known about synergism between sphingolipid inhibitors and antifungal drugs. Studies in *S. apiospermum* using monoclonal antibodies against glucosylceramide revealed that these molecules increase the efficacy of itraconazole, but not of amphotericin B [15], suggesting that targeting sphingolipids and sterol simultaneously could enhance the therapeutic approach. Lazzarini and colleagues tested some synthetic acylhydrazone compounds, which target fungal sphingolipid synthesis, and also displayed synergistic effect with azoles [31]. On the other hand, RsAFP2, a plant defensin that directly targets fungal glucosylceramide, presented an antagonistic effect to caspofungin in *C. albicans* [12]. These data clearly highlight the need for further studies regarding the interaction between sphingolipid inhibitors and antifungal agents.

Myriocin has already been described to be toxic for mammalian cells and to increase host mortality in a *C. albicans* infection model using *Galleria mellonella* [36]. However, a myriocin–nanocarrier solution has been shown to be nontoxic to mice and also to be efficient in reducing pathological inflammation and *A. fumigatus* infection [37], demonstrating that the use of molecules capable to inhibit sphingolipids is a promising field. In this context, our study highlights the effect of two sphingolipid inhibitors, myriocin and PPMP, showing their effect on *S. boydii* growth, lipid biosynthesis and cell surface integrity. Since the resistance of *Scedosporium* species to the current antifungal agents continues to emerge, the recognition of new possible drug targets in these fungi is highly relevant to the medical mycology community.

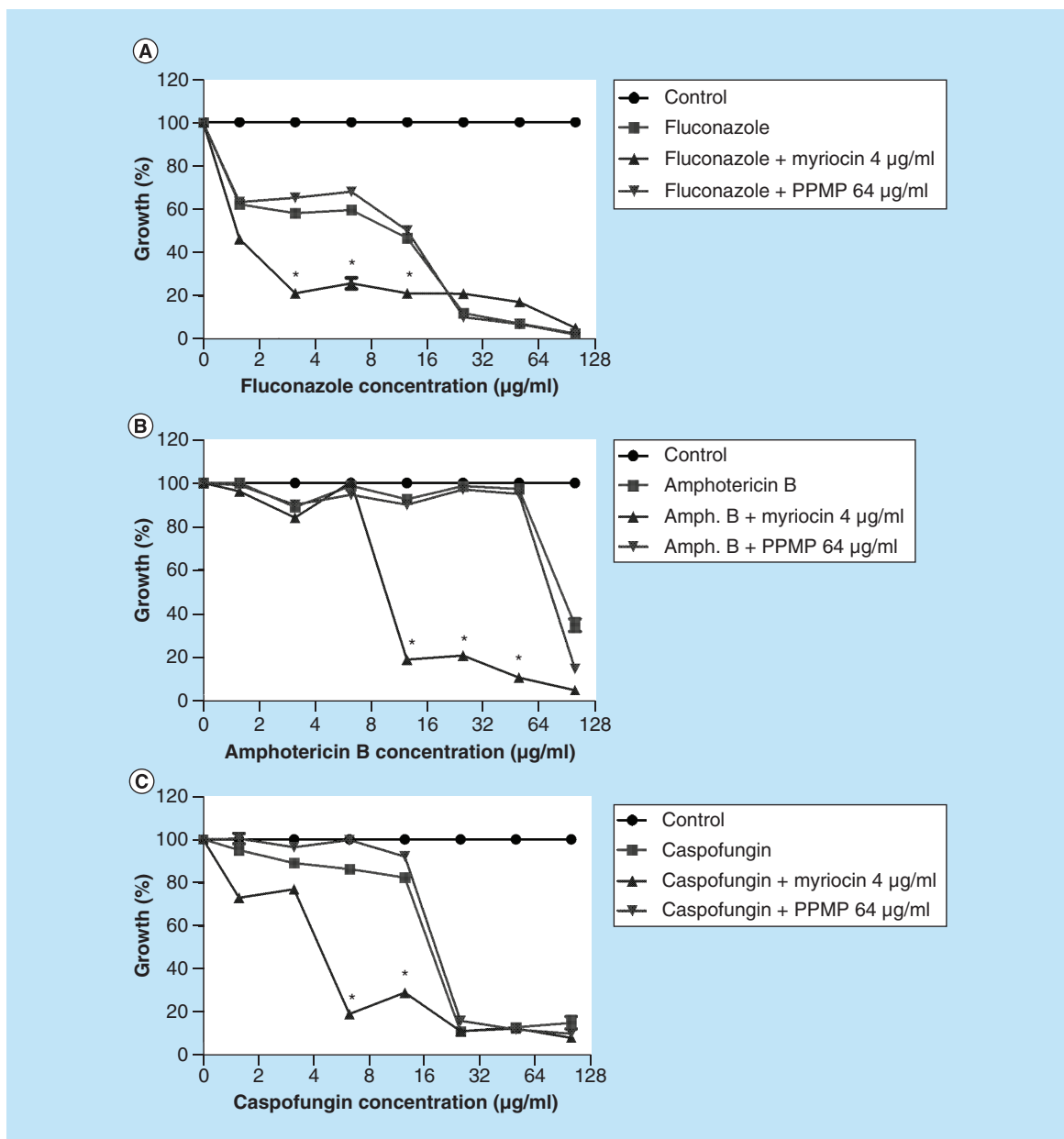


Figure 8. *Scedosporium boydii* susceptibility to antifungal agents in the presence of subinhibitory concentrations of myriocin and DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol. Antifungal drugs (fluconazole, amphotericin B and caspofungin) were tested in serial dilution (100–1.57 µg/ml) in the presence or absence of myriocin (4 µg/ml) and PPMP (64 µg/ml).

*p < 0.05.

PPMP: DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol.

Conclusion

The effect of different inhibitors of sphingolipid biosynthetic pathway was demonstrated in *S. boydii* cells. When treated with myriocin and PPMP, sphingolipids content was reduced, fungal growth was impaired and membrane integrity was affected. Moreover, susceptibility to current antifungal drugs was potentiated when cells were simultaneously exposed to myriocin.

These data highlight the usefulness of sphingolipids as new targets for fungal treatments, as well as the potential of inhibitor molecules for the development of new drugs in therapeutic approaches.

Future perspective

Treatment of fungal infections has become a challenge because the current antifungal drugs used in the clinics present a variety of limitations, such as high toxicity to patients and low efficacy due to fungal resistance. During the last decades, sphingolipids have been considered attractive targets for the development of new antifungal drugs, opening a promising field for development.

The use of plant defensins, monoclonal antibodies, biosynthetic inhibitors and synthetic chemical compounds has demonstrated that targeting sphingolipids from pathogenic yeast and filamentous fungi lead to growth defect and reduced virulence. In this context, a promising route to development of new antifungal drugs could be modifying inhibitor molecules to broaden pathogen spectrum and reduce toxicity for testing in infection models for different pathogenic fungi.

Summary points

- Sphingolipid inhibitors led to a reduction of lipid content in treated *Scedosporium boydii* cells.
- Myriocin and DL-threo-1-Phenyl-2-palmitoylamino-3-morpholino-1-propanol were shown to be efficient in decreasing fungal growth and hyphal elongation.
- Inhibitor mechanism of action was suggested, since lipid raft accumulation and membrane integrity were affected in treated cells.
- Myriocin seems to present a synergistic effect with different antifungal drugs, suggesting a promising use for treatment of resistant strains.
- All data support the potential use of sphingolipid inhibition molecules to treat fungal infections, highlighting the need of more studies which describe new compounds targeting sphingolipids and test fungal virulence.

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