Antifungal activity of altenusin isolated from the endophytic fungus *Alternaria* sp. against the pathogenic fungus *Paracoccidioides brasiliensis*

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**ABSTRACT**

**Background:** Altenusin is a biphenyl derivative isolated from different species of fungi, which presents several biological activities.

**Aims:** We report the antifungal activity of the altenusin isolated from the endophytic fungus *Alternaria* sp., against clinical isolates of *Paracoccidioides brasiliensis*, and its action on cell walls of *P. brasiliensis* and the nonpathogenic yeast *Schizosaccharomyces pombe*.

**Methods:** In vitro antifungal activity of altenusin was evaluated using the broth microdilution method against 11 strains of *P. brasiliensis* and one strain of *S. pombe*. The effects of the altenusin on the cell wall were estimated using the sorbitol protection assay.

**Results:** The altenusin presented strong activity against *P. brasiliensis* with MIC values ranging between 1.9 and 31.2 \(\mu\)g/ml and 62.5 \(\mu\)g/ml for *S. pombe*. Our results demonstrated that the MIC values for altenusin were increased for *P. brasiliensis* Pb18 and for *S. pombe* when the medium was supplemented with sorbitol. Additionally, *S. pombe* cells treated with altenusin were more rounded in shape than untreated cells.

**Conclusions:** Altenusin showed activity against clinical strains of *P. brasiliensis* at the concentration tested, and this compound probably affects fungal cell walls. These findings suggest that altenusin could act through the inhibition of cell wall synthesis or assembly in *P. brasiliensis* and *S. pombe*, and could be considered as a lead compound for the design of new antifungals.

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**Actividad antifúngica de la altenusina aislada del hongo endofítico *Alternaria* sp. frente al hongo patógeno *Paracoccidioides brasiliensis***

**RESUMEN**

**Antecedentes:** La altenusina es un derivado bifenilo aislado de diferentes especies de hongos, que presenta una diversidad de actividades biológicas.

**Objetivos:** Describimos la actividad antifúngica de la altenusina aislada del hongo endofítico *Alternaria* sp. frente a aislamientos clínicos de *Paracoccidioides brasiliensis*, y su acción sobre las paredes celulares de *P. brasiliensis* y la levadura no patogena *Schizosaccharomyces pombe*.

**Métodos:** Se valoró la actividad antifúngica de la altenusina in vitro usando un método de microdilución en caldo frente a 11 cepas de *P. brasiliensis* y una cepa de *S. pombe*. Los efectos de la altenusina sobre la pared celular se estimaron utilizando un análisis de protección con sorbitol.

**Resultados:** La altenusina presentó una potente actividad frente a *P. brasiliensis* con valores de concentración inhibidora mínima (CIM) que variaron entre 1.9 y 31.2 \(\mu\)g/ml y de 62.5 \(\mu\)g/ml para *S. pombe*. Los resultados del presente estudio demostraron que los valores CIM de la altenusina aumentaron para Pb18 de *P. brasiliensis* y para *S. pombe* cuando el medio se suplementó con sorbitol. Además, las células de *S. pombe* tratadas con altenusina adoptaron una forma más redondeada que las no tratadas.

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Altenusin is a biphenyl derivative isolated from different species of fungi, which presents antioxidant properties and the ability to inhibit several enzymes, such as myosin light chain kinase, sphingomyelinase, acetylcholinesterase, and HIV-1 integrase. Altenusin also exhibits broad antimicrobial activity against several drug-resistant pathogens with minimum inhibitory concentration (MIC) values of 3.12 to 125 μg/ml. Cota et al. isolated altenusin from the endophytic fungus Alternaria sp. UFMGCB 55 associated with the plant Trixis vauhieri DC (Asteraceae), and this compound presented the ability to inhibit the enzyme trypanothione reductase (TryR) from Trypanosoma cruzi, an enzyme involved in the protection of Trypanosoma and Leishmania species against oxidative stress.

The increase in frequency of immunocompromised individuals among the world’s human population has resulted in an ever-growing number of serious fungal infections, which will require new antimicrobial therapy. The pathogenic fungus Paracoccidioides brasiliensis is the etiologic agent of paracoccidioidomycosis (PCM), a human systemic mycosis for which the portal of entry of the fungus is the respiratory tract via the inhalation of airborne propagules. Although geographically confined, paracoccidioidomycosis constitutes one of the most prevalent deep mycoses in Central and Southern America. Antifungals used in cases of PCM are sulfonamides, amphotericin B, or azoles, mainly itraconazole. Extended periods of treatment are necessary and relapses of the disease commonly occur. In the present work, we report the antifungal activity of altenusin isolated from the endophytic fungus Alternaria sp. UFMGCB 55, against clinical strains of P. brasiliensis, and we also show the activity of altenusin on cell walls of P. brasiliensis and the nonpathogenic yeast Schizosaccharomyces pombe.

Materials and methods

Isolation of altenusin

The biphenyl derivative altenusin (Fig. 1) was isolated from ethyl acetate extract of the endophytic fungus Alternaria sp. UFMGCB 55, which was recovered from leaves of the bioactive plant Trixis vauhieri DC (Asteraceae) as described by Cota et al. This compound was stored at –20 °C at 20 mg/ml.

Table 1

<table>
<thead>
<tr>
<th>Fungal strains</th>
<th>Altenusin</th>
<th>Amphotericin B</th>
<th>Trimethoprim–sulfamethoxazole</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED01*</td>
<td>1.9</td>
<td>0.031</td>
<td>75</td>
</tr>
<tr>
<td>Pb 01*</td>
<td>3.9</td>
<td>0.12</td>
<td>300</td>
</tr>
<tr>
<td>Pb 2b</td>
<td>1.9</td>
<td>0.031</td>
<td>75</td>
</tr>
<tr>
<td>Pb B339*</td>
<td>1.9</td>
<td>0.015</td>
<td>75</td>
</tr>
<tr>
<td>Pb 11*</td>
<td>3.9</td>
<td>0.12</td>
<td>150</td>
</tr>
<tr>
<td>Pb 14*</td>
<td>1.9</td>
<td>0.125</td>
<td>75</td>
</tr>
<tr>
<td>Pb 8b</td>
<td>1.9</td>
<td>0.062</td>
<td>300</td>
</tr>
<tr>
<td>Pb B18*</td>
<td>15.6</td>
<td>0.062</td>
<td>300</td>
</tr>
<tr>
<td>Pb 3c</td>
<td>31.2</td>
<td>0.015</td>
<td>300</td>
</tr>
<tr>
<td>Pb 1578*</td>
<td>1.9</td>
<td>0.062</td>
<td>75</td>
</tr>
<tr>
<td>Pb 4c</td>
<td>31.2</td>
<td>0.062</td>
<td>150</td>
</tr>
<tr>
<td>S. pombe</td>
<td>62.5</td>
<td>0.025</td>
<td>–</td>
</tr>
</tbody>
</table>

* Paracoccidioides brasiliensis isolate representative of the new phylogenetic species Pb-01-like.
* Paracoccidioides brasiliensis isolates representative of the phylogenetic species S1 (Pb-18, Pb-B339, Pb-14, Pb-8 and Pb-11) (reported by Matute et al. as B17, B18, B22, B25, B21, Table S1). Paracoccidioides brasiliensis isolates representative of the phylogenetic species PS2 (Pb-2, Pb-3 and Pb-4) (reported by Matute et al. as V2, B26, B23 Table S1).
* Values were expressed in μg/ml.

Antifungal activity assays

Maintenance of P. brasiliensis and S. pombe strains

Eleven clinical P. brasiliensis strains, of two established cryptic phylogenetic species (S1 and S2) and one possibly new cryptic species, were used in the biological assays (Table 1). Isolates Pb8, PbB339, Pb14, Pb8 and Pb11 belong to the cryptic species S1, and isolates Pb03, Pb2 and Pb4 belong to the PS2 cryptic phylogenetic species. Isolates Pb01, ED01 and Pb1578 are considered representative of a new phylogenetic species “Pb-01-like”. The strains of P. brasiliensis were maintained at Departamento de Microbiologia de Universidade Federal de Minas Gerais, Brazil, by weekly transfer in solid YPD medium (yeast extract, peptone and dextrose) at 37 °C. The wild type of S. pombe (PN556) was maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK).

Minimal inhibitory concentrations determination

The bioassays with all clinical strains of P. brasiliensis and S. pombe (PN556) were performed following the CLSI M27-A2 guidelines with the modifications suggested by Johann et al. Amphotericin B (AMB) (Sigma, St Louis, USA) and trimethoprim/sulfamethoxazole (SMT/TMP) were included as positive antifungal controls, being the stock solutions prepared in DMSO and water, respectively. Twofold serial dilutions were prepared exactly as outlined in CLSI document M 27–A2.

Minimal fungicidal concentrations determination

The microtitrator plate used to determine MIC values was also used to determine MFC values. The in vitro minimal fungicidal concentrations (MFC) of each compound tested was determined by streaking 10 μl from each well that showed complete inhibition (100% inhibition or a clear well), from the last positive well (growth similar to that of the growth control well), and from the growth...
control well onto YPD plates. The MFC was determined as the lowest drug concentration at which counts lower than three colonies were recovered after cultivation on YPD agar for 10 days at 37°C.\textsuperscript{4,18}

**Sorbitol protection assays**

MIC values were determined using *P. brasiliensis* strain Pb18 and *S. pombe* by the standard broth microdilution procedure as described above. Duplicate plates were prepared: one of them contained altenuisin plus 0.8 M sorbitol as an osmotic support and the other one contained only altenuisin. MICs were determined after 14 days for *P. brasiliensis* and 48 h for *S. pombe*.\textsuperscript{3}

**Cell morphology analysis**

The model organism for cell morphology analysis was the non-pathogenic yeast *S. pombe*. *S. pombe* cell morphology was analyzed by fluorescence microscopy after cell staining with Calcofluor white. The *S. pombe* cells were grown in YES-yeast extract plus supplements: adenine, leucine, histidine, or uracil (100 mg/ml; Sigma) liquid medium to mid log-phase to an \(A_{600}\) of 0.6.\textsuperscript{21} Images were captured with a Leica DM 4000B fluorescence microscope coupled to a cooled Leica DC 300F camera and IM50 software. For analyses of vacuoles, the cells of *S. pombe* were stained with CDCFDA (carboxydichlorofluorescein diacetate; Molecular Probes) and observed under fluorescence microscopy.

**Results**

Preliminary results demonstrated that altenuisin was not active against *Candida albicans* (>250.0 \(\mu\)g/ml) (data not showed), but *P. brasiliensis* strains were susceptible to altenuisin, with MIC values between 1.9 and 31.2 \(\mu\)g/ml. *P. brasiliensis* strains Pb ED01, 2, B339, 11, 14, 8, and 1578 were the most susceptible with MIC values of 1.9 \(\mu\)g/ml whereas the strains Pb03 and Pb04 were less susceptible to altenuisin with an MIC value of 31.2 \(\mu\)g/ml. MIC values found for amphotericin B were between 0.031 and 0.12 \(\mu\)g/ml for the *P. brasiliensis* strains tested, with better activity against the isolates Pb ED01 and Pb2. The drug trimethoprim–sulfamethoxazole presented high MIC values, ranging between 75.0 and 300.0 \(\mu\)g/ml. For all strains of *P. brasiliensis* the MIC values obtained for altenuisin were equal to the MIC values. In addition, altenuisin also presented activity against *S. pombe* with a MIC value of 62.5 \(\mu\)g/ml.

Altenusin modified MIC values to *P. brasiliensis* and *S. pombe* after addition of sorbitol to the culture medium. The MIC value against *P. brasiliensis* (strain Pb18) in culture medium treated with altenuisin and supplemented with sorbitol was 62.5 \(\mu\)g/ml whereas the MIC was 15.6 \(\mu\)g/ml in the same medium without the addition of sorbitol. MIC values obtained against *S. pombe* were 62.5 \(\mu\)g/ml and 125.0 \(\mu\)g/ml in absence and presence of sorbitol in the medium, respectively. These results suggested that the antifungal activity of altenuisin could affect fungal cell walls. When *S. pombe* cells stained with calcofluor white were observed in fluorescence microscopy, cells treated with altenuisin were more rounded than untreated cells (Fig. 2). Tests to observe whether vacuoles of the cells treated with altenuisin were affected by the compound were negative. Differences between control *S. pombe* cells and cells treated with altenuisin were not observed (Fig. 3).

**Discussion**

We assayed the altenuisin against fungal isolates of *P. brasiliensis* of two distinct cryptic phylogenetic species: S1 (Pb18, PbB339, Pb14, PbB8 and Pb11) and PS2 (Pb03, Pb2 and Pb4).\textsuperscript{11} The cryptic species S1 was more susceptible than the cryptic species S2, with MIC values of 1.9–15.5 \(\mu\)g/ml for the first and MIC values of 1.9–31.2 \(\mu\)g/ml for the second. Altenuisin was also tested against *P. brasiliensis* isolated Pb01, ED01 and Pb1578, which are considered representative of a new phylogenetic species “Pb-01-like”.\textsuperscript{3} Teixeira et al.\textsuperscript{22} recommended the formal description of the “Pb-01-like” (Pb 01, 1578 and ED01) cluster as a new species, *Paracoccidioides lutzii*.\textsuperscript{22} The isolates of the *P. lutzii* group treated with altenuisin presented the highest susceptibility, with MIC values ranging between 1.9 and 3.9 \(\mu\)g/ml. On the basis of the MIC values for altenuisin,
isolates of *P. brasilienis* were around 50 times more susceptible to this compound than to trimethoprim–sulfamethoxazole in vitro assays. Sulfonamides are the first class of drugs available for treating patients with PCM, but long periods of treatment may be required (more than 2 years). In addition, the identical MFC and MIC values presented by altenusin may be important as the altenusin could kill the pathogen in situations when infection occurs in sites not easily accessed by host defenses.

According to Frost et al., a distinctive feature of the specific inhibitors of the fungal cell wall is that the antifungal effect is reversed in media containing an osmotic stabilizer such as sorbitol. Cell wall disruptive and osmotically destabilizing agents lead to cell wall rearrangements that enable fungal cells to survive. Our results suggest that altenusin could act through the inhibition of cell wall synthesis or assembly in *P. brasilienis* and *S. pombe* cells. Cell morphology analysis was performed with *S. pombe*, a yeast that is an excellent model organism for the study of cell walls. The major *S. pombe* glucose polymers of the cell wall are similar to those of *P. brasilienis*, with presence of β-α-(1,3) glucan and α-α-(1,3) glucan. In the present work, the *S. pombe* cells treated with altenusin in sub-inhibitory concentration were smaller in size and more rounded than control (altenusin absence) cells. It is known that β-α-(1,3) glucan synthase inhibitors produce hallmark changes in the morphologies of yeasts and filamentous fungi. According to Frost et al., the target in cell walls is unknown when *C. albicans* presents smaller rounded cells. Altenusin showed inhibition in vitro activity against clinical strains of both phylogenetically cryptic species of *P. brasilienis*, with low MIC values when compared to trimethoprim–sulfamethoxazole. This compound could be considered as a lead compound for the design of new antifungals. Our results suggested that the antifungal activity of altenusin affects fungal cell walls, but new assays will be performed to establish its specific action mechanism.

**Conflict of interest statement**

The authors declare that they have no conflict of interest.

**Acknowledgments**

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