Antifungal activity of 6-quinolinyl N-oxide chalcones against Paracoccidioides

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Background: Chalcones are an important class of natural compounds that have been widely applied as synthons in synthetic organic chemistry and possess diverse and interesting biological properties.

Methods: We conducted tests with the synthetic substances 6-quinolinyl N-oxide chalcones 4c and 4e to determine their antifungal activity against several isolates of Paracoccidioides spp. and their activity in a murine model. We also determined whether the chalcones interacted with other drugs or interfered with the morphology of Paracoccidioides brasiliensis (Pb18) yeast cells.

Results: We verified that the substances were active against Paracoccidioides spp., but we did not show an interaction with the drugs tested when only the fractional inhibitory concentration index values were considered individually. We observed that the substances induced in vitro morphological changes. Compounds 4c and 4e showed activity similar to itraconazole in treated mice, as demonstrated by their ability to reduce the number of cfu recovered from the lungs. Histopathological analysis showed that animals treated with 4c presented fewer areas containing inflammatory infiltrate and larger areas of preserved lung tissue, whereas animals treated with itraconazole showed accumulation of inflammatory infiltrate and some granulomas. Mice treated with 4e exhibited inflammation that compromised the tissue.

Conclusions: The results presented in this paper confirm the antifungal potential of the chalcones tested. The chalcone 4c was the more effective at controlling the disease in mice and this compound could be a candidate for future studies of the treatment of paracoccidioidomycosis.

Keywords: paracoccidioidomycosis, murine model, morphological changes

Introduction

Fungi belonging to the genus Paracoccidioides are the agents of paracoccidioidomycosis (PCM). The treatment of PCM is generally protracted (≥1–2 years in many cases) and in the absence of therapy, PCM is fatal. Moreover, according to Travassos and Taborda,1 even after treatment with antifungals there is no assurance of complete clearance of the fungus. This leads to the need for new, safe and effective antifungal compounds for the treatment of PCM. We recently reported that a series of 6-quinolinyl N-oxide chalcones showed interesting antifungal activity against Paracoccidioides brasiliensis (isolate Pb18). The 6-quinolinyl N-oxide chalcones 4c and 4e showed the highest antifungal activity and therefore we selected these compounds for the present study.

Materials and methods

Experimental substances

The N-oxide chalcones 4c and 4e (Table 1) were synthesized in accordance with Tavares et al.2

Fungi and inoculum

In this study, we used 14 isolates of P. brasiliensis and 3 isolates of Paracoccidioides lutzii, all of which were members of the collection of...
Universidade Federal de Minas Gerais. The fungi were maintained in a chemically defined medium (McVeigh & Morton) and subcultured after 7 days of growth at 37°C. The inoculum was prepared as described by Cruz et al.\(^8\)

**Determination of MICs**

A bioassay of all the Paracoccidioides isolates was performed following the CLSI M27-A3 guidelines\(^8\) with modifications suggested by Nakai et al.\(^3\) and Johann et al.\(^8\). The data are representative of three independent experiments.

**Minimum fungicidal concentrations (MFCs)**

The MFC of each compound tested was determined as described by Espinel-Ingroff.\(^7\)

**Time–kill curve procedures**

A time–kill curve was generated for *P. brasiliensis* (Pb18). This test was performed in accordance with Klepser et al.\(^8\). All of the kill curve experiments were performed in triplicate.

**Chequerboard microtitre assay**

Eight serial 2-fold dilutions of compounds 4c and 4e, amphotericin B, trimethoprim/sulfamethoxazole and itraconazole were prepared in the same way as in the MIC test. A chequerboard pattern was prepared in accordance with Cuenca-Estrella.\(^8\)

**Scanning and transmission electron microscopy**

Yeast cells of the isolated Pb18 strain were grown in subinhibitory concentrations of compounds 4c and 4e. The cells were prepared as described previously.\(^10\) Analyses were performed with a DSM 950 microscope (Zeiss, Germany) and an EM 10 microscope (Zeiss, Germany) in the Center for Acquisition and Image Processing, UFMG, Brazil.

**Sorbitol protection assay**

This assay was performed with compounds 4c and 4e for isolate Pb18 as described previously.\(^11\) MICs were read after 10 days at 37°C.

**Animals**

Four-to-six-week-old male BALB/c mice were obtained from the biotherium of the Centro de Pesquisas René Rachou (Belo Horizonte, Brazil). Mice were used in the animal models conducted in accordance with the Ethics Committee for Animal Experimentation (CETEA/UFMG), protocol no. 100/2010.

**Fungus**

Yeast cells of the virulent Pb18 strain were cultured in YPD medium at 37°C for 7 days. The inoculum was prepared in accordance with Marques et al.\(^12\)

**Animal experiments and treatment**

A total of 25 mice, divided into groups of 5, were used in each experiment: Groups 1, 2 and 3: animals were infected and treated with compounds 4c, 4e or itraconazole, respectively; Group 4: positive control (infected but not treated); and Group 5: negative control (not infected and treated with PBS). The animals were infected via the intratracheal route as previously described by Santos et al.\(^13\) The mice were treated by intraperitoneal injection of 4c, 4e and itraconazole for 2 weeks after infection and in all treatments the doses used were 5 mg/kg of body weight per day. This experiment was repeated three times.

To investigate potential toxicological effects, uninfected mice were treated with 5 and 15 mg/kg/day 4c and 4e for 15 days. Visual appearance, weight of animals and histopathological tissue from the liver were analysed.

**Assay for organ cfu**

The number of viable microorganisms in the lungs, liver and spleen from the experimental and control mice was determined by counting the cfu in accordance with Maluf et al.\(^14\)

**Histopathology**

The lungs, liver and spleen were collected, placed into phosphate-buffered formalin and prepared as described previously.\(^12\) From the histopathological analysis, a score of the infectious process was built to evaluate the histopathological parameters characteristic of these organs.

**Statistical analysis**

The program GraphPad Prism\(^6\) version 5 (GraphPad Software) was used for the statistical analysis. The different groups were analysed using analysis of variance and for multiple comparisons the Newman–Keuls test was used. The level of significance used was 0.05.

**Results**

The MIC\(_{50}\) and MIC\(_{90}\) values of compound 4c were 7.8 and 23.4 mg/L, of 4e were 7.8 and 10.4 mg/L, of itraconazole were 0.007 and 0.062 mg/L and of trimethoprim/sulfamethoxazole were 11.7 and 150 mg/L, respectively. The differences in the MICs of the compounds tested, itraconazole and trimethoprim/sulfamethoxazole were statistically significant (P<0.05) (Table S1, available as Supplementary data at JAC Online). Regarding the MFC, we found that the fungicidal activity coincided with the MIC or 2×MIC. We found that 4c and 4e were able to reduce by 70% the number of viable cells in the first 100 h of incubation (Figure S1). In tests performed with the Pb18 isolate of the interaction between the experimental substances and amphotericin B, itraconazole and trimethoprim/sulfamethoxazole, no interaction was observed.

Scanning and transmission electron microscopy showed that treatment with substance 4c caused flaking of the cell envelope and some cells showed leakage of the cytoplasmic content, retraction of the plasma membrane, several cytoplasmic vacuoles and cytoplasmic clumping. We observed some wilted, burst cells, folds in the plasma membrane, changes in cell morphology and cytoplasmic disorder in cells treated with 4e (Figure S2). No alteration was observed in the value of the MIC in the presence of sorbitol.

In the animal model assay, cfu were recovered only from the lungs. Analysis of the lung cfu/g of tissue showed a significant reduction in the number of fungal cells recovered from the animal groups treated with itraconazole or compounds 4c or 4e compared with the positive control (untreated infected group) (P<0.05) (Figure 1a). Although differences were observed between the three treatments (itraconazole, 4c and 4e), they were not statistically significant. This result points to similarities in the efficiency of
Table 1. Experimental compounds

<table>
<thead>
<tr>
<th>Substance</th>
<th>Structural formula</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
<th>Log P value</th>
</tr>
</thead>
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<td>C₁₉H₁₅NO₃</td>
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<td>4e</td>
<td><img src="4e.png" alt="Structure" /></td>
<td>C₁₈H₁₂FNO₂</td>
<td>293.29</td>
<td>3.59</td>
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</table>

Figure 1. (a) Lung cfu from BALB/c mice infected intratracheally with 1.0 × 10⁶ yeast cells of the Pb18 isolate of *P. brasiliensis* and treated with 4c, 4e or itraconazole. The control mice were infected with the same number of yeast cells. Each bar represents the average count of fungi in the lungs and the errors bars indicate SDs. Asterisks denote significant differences (P < 0.05) using the multiple-comparison Newman–Keuls test (n = 15). (b) The histopathological score of infection with *P. brasiliensis* (Pb18) in the liver and lungs of BALB/c mice treated with 4c, 4e or itraconazole. Asterisks denote significant differences (P < 0.05) using the multiple-comparison Newman–Keuls test.
the three treatments under the conditions evaluated. The lungs from infected animals that received no treatment showed multiple pulmonary foci of epithelioid granulomatous inflammation and early pulmonary fibrosis by haematoxylin and eosin staining. The lungs of animals treated with 4c showed a few areas containing inflammatory infiltrate and large areas of preserved lung tissue. The lungs from the group of animals treated with 4e showed excessive infiltration and granulomas. Animals treated with itraconazole exhibited an accumulation of inflammatory infiltrate and some granulomas. In the liver, animals treated with itraconazole and 4c showed no visible morphological changes, but the group treated with N-oxide chalcone 4e showed extensive areas of microatresia and hydropic degeneration, similar to untreated animals. The spleen did not show pathological changes (Figure S3). The histopathological score revealed that the inflammatory process of the liver from animals infected but not treated (positive control) was greater than in the groups treated with itraconazole and substance 4c (Figure 1b). The score for compound 4c showed a significant difference compared with untreated animals. Only the lungs of animals that received treatment with 4c showed a pathological score with significant differences in inflammation compared with the control animals. The visual appearance, weight and histopathological tissue from liver of animals treated with both doses of compounds tested were very similar to control group, suggesting a lack of overt toxic effects.

Discussion

This study showed that 4c and 4e are capable of killing fungi in vitro and this activity extends within the genus Paracoccidioides. In yeast treated with compounds 4c and 4e, we found disorder in the plasma membrane and cytoplasm as well as abnormal morphology. Animals treated with 4c and 4e showed a significant reduction in the number of cfu recovered from the lungs when compared with the positive control group that received no treatment. In the animal models, this activity was not significantly different in animals treated with itraconazole, a drug indicated for the treatment of human PCM.15 Marques et al.16 evaluated the potential of the immunizing peptide P10 in BALB/c mice infected with Pb18 and compared this result with treatment with itraconazole (10 mg/kg) and other antifungal drugs. In all tests, immunization with P10 in combination with the other treatments had the best results for controlling infection, with a 60%–80% reduction in lung cfu compared with the control (untreated infected animals). However, P10 immunization or chemotherapy independently achieved a 40%–60% reduction of the cfu. In the present study, treatment with 4e and 4c (5 mg/kg/day) reduced the cfu by ~65% and ~75%, respectively, indicating their ability to control the infection. The histopathological analysis and progression score of the disease in mice showed that compound 4c was better able to control inflammation and resolved the infection with better results than treatment with itraconazole and 4e. In summary, our results show great therapeutic potential for compound 4c, owing to its important antifungal activity in a murine model, without granuloma formation and with preservation of lung tissue.

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figures S1 to S3 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

References


