Post-antifungal effects of the antifungal compound 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate on Aspergillus fumigatus

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The post-antifungal effect (PAFE) of the antifungal compound 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate (DHP) upon Aspergillus fumigatus was investigated. The conidia of A. fumigatus were exposed to DHP at concentrations of 1× and 4× MIC90 for variable times at 37 °C. Amphotericin B (AmB)-treated or drug-free controls were included in the study. DHP as well as AmB exposure resulted in prolonged lag phases of the turbidimetric growth curves. Both the treatments gave rise to delayed growth, with lag phases of 11 h upon treatment with a concentration of 4× MIC90 for 4 h. Furthermore, it was observed that DHP inhibited the expression of three A. fumigatus secretory proteins of 18, 42 and 55 kDa. One protein of 42 kDa was found to be a metalloprotease, which is an important virulence factor. Analysis of time-dependent antigenic profiles showed the early expression of high-molecular-mass antigens. Expression of low-molecular-mass antigens started after 24 h culture. The antigens of A. fumigatus that are expressed during the early phase of growth were observed to be adversely affected after treatment with DHP. Although the mechanism of action of DHP to inhibit these proteins/antigens is unknown, the observations may be valuable to understand their role in the virulence of the pathogen, as well as the antigen-mediated responses caused by A. fumigatus.

INTRODUCTION

Aspergillus fumigatus is a ubiquitous pathogen that causes a variety of diseases, especially in immunocompromised patients, such as in cancer patients, bone marrow transplant recipients, solid-organ transplant recipients (Kaiser et al., 1998), and HIV-infected patients (Mylonakis et al., 1998).

A. fumigatus is the Aspergillus species encountered most frequently, although other species have been reported to cause disease. Amphotericin B (AmB) has been in use for a long time for the treatment of invasive aspergillosis, although its clinical response is limited. Alternatively, triazoles, antifungals with good in vitro activity against Aspergillus, have been used successfully as a first-line treatment in patients with invasive disease (Boogaerts et al., 2001; Dannaoui et al., 1999). However, the available antifungal agents are not ideal; indeed, invasive aspergillosis can lead to death in more than half of all patients (Lin et al., 2001). Therefore, there have been continued efforts to develop novel potent antifungal molecules. We have previously described 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate (DHP) as a novel antifungal agent isolated and characterized from Datura metel L. (Dabur et al., 2004). It shows in vitro as well as in vivo antifungal activities (Dabur et al., 2005b), but its mode of action on the pathogen is not known.

A suppression of fungal growth that persists after limited exposure to azoles, AmB and echinocandins has been described (Minguez et al., 1994; Turndidge et al., 1994; Ernst et al., 2000). The post-antifungal effect (PAFE) may have clinical relevance to design the dose for new antifungal agents such as DHP. The antimicrobials having a long PAFE may be administered less frequently in comparison with the drugs having a short PAFE. Therefore, DHP was investigated for its PAFE against A. fumigatus in comparison with AmB.

The genome of A. fumigatus has around 10 000 genes which regulate the expression of a number of antigenic and/or allergenic proteins. The secretory proteins are of prime interest because they have the capability to degrade the host tissue and induce a variety of immunopathologic responses. Therefore, the present study was undertaken to study the PAFE of DHP vis-à-vis the expression of secretory proteins and antigens by A. fumigatus. The proteins inhibited by DHP may represent important targets within the pathogen as well as crucial factors involved in the pathogenesis of A. fumigatus.

Abbreviations: AmB, amphotericin B; DHP, 2-(3,4-dimethyl-2,5-dihydro-1H-pyrrol-2-yl)-1-methylethyl pentanoate; PAFE, post-antifungal effect.
METHODS

Isolates. Seven clinical isolates of A. fumigatus along with a standard strain (ITCC 4517) were used in this study.

Antifungal agents. The compounds DHP and AmB were dissolved in DMSO and aliquots of the stock solution were stored at −70 °C until used. Dilutions of DHP and AmB were made in the appropriate growth medium in order to obtain the required final concentrations.

Antifungal susceptibility assay. The antifungal susceptibility assays were performed according to the standard guidelines described in document M-38P of the National Committee for Clinical Laboratory Standards (1998).

PAFE. A. fumigatus was grown at 35 °C for 5 days by subculturing onto Sabouraud dextrose agar (SDA) to obtain sporulation. Conidia were collected in Sabouraud dextrose broth/0.05% Tween 20. The turbidity of suspensions was measured spectrophotometrically at 530 nm, and transmission was adjusted to 80–82%, corresponding to 0.5 × 10⁴–4.5 × 10⁴ c.f.u. ml⁻¹. Conidial viability was checked by plating serial dilutions onto SDA plates. The conidial suspensions were incubated at 37 °C for 4 or 1 h with different concentrations of DHP. After incubation, the conidia were centrifuged at 4000 g for 10 min and washed three times with Sabouraud dextrose broth/0.05% Tween 20. After washing and resuspension, conidia were plated onto SDA plates to determine viable counts. From the resuspended conidia, 200 μl was placed in microplates, which were incubated at 37 °C in a computerized spectrophotometer (SpectraMax 190, Molecular Devices). Growth of fungi was monitored automatically as the change in turbidity at 405 nm at 1 h intervals for 48 h. All assays were performed in triplicate and the data analysed. The PAFE was quantified using the formula T−C, where T is the first significant increase in the initial turbidity of drug-exposed conidia after removal of the drug, and C is the first significant increase in the turbidity of the control.

Morphological studies. Growth of A. fumigatus was examined visually at 96 h on SDA plates, as well as in the microplate wells, using a microscope, in order to observe morphological changes and correlate them with the changes in turbidity.

Preparation of culture filtrate and protein purification. A. fumigatus was cultured in asparagine broth, a synthetic medium (Dabur et al., 2004). The medium was dispensed into 250 ml flasks and sterilized at 115 °C/10 p.s.i. for 15 min. The flasks were divided into two sets, test and control, of two flasks each. The flasks of the test set were inoculated with conidia of A. fumigatus exposed to DHP and incubated at 37 °C in a biological oxygen demand (BOD) incubator for 48 h. Solvent-treated cultures were used as a control. Culture filtrates from both the sets were collected at different time intervals. After centrifugation (5000 g, 10 min), the filtrates were subjected to lyophilization. The concentrated culture filtrates were dialysed against 10 mM sodium acetate buffer (pH 6.2) with three changes of buffer over a period of 24 h.

SDS-PAGE. The bicinchoninic acid assay was performed to estimate the concentrations of proteins in the samples. The protein concentrations of the samples were equalized by diluting the samples with 10 mM sodium acetate buffer (pH 6.2). The proteins were separated using 12.5% polyacrylamide gels and protein bands were visualized by staining the gels with silver stain. After destaining, the gels were washed with distilled water and stored at 4 °C for further analysis.

Western blotting. SDS-PAGE was performed as above, and the proteins were electrotransferred onto PVDF sheets overnight at 30 V in 50 mM Tris/HCl (pH 8.0) buffer containing 200 mM glycine and 20% ethanol. The blots were immunolabelled with immune sera obtained from aspergillosis patients.

Protein sequencing. Western blotting was performed as above and blots were visualized with Ponceau S. The down-regulated protein bands were removed with a surgical blade and subjected to sequencing. The N-terminal amino acid sequence of the protein was determined by using a Procise 490 automated Edman degradation sequencer (Perkin-Elmer). The N-terminal amino acid sequence of the protein obtained was submitted to a BLAST search for sequence homology with the proteins of A. fumigatus and other species.

RESULTS AND DISCUSSION

The MIC₉₀ of DHP ranged between 21.87 and 43.75 μg ml⁻¹ against A. fumigatus isolates, as reported previously (Dabur et al., 2005a). PAFEs were determined from the growth curves of A. fumigatus after 4 h treatment with DHP or AmB, as described by Vitale et al. (2002). The growth curve of drug-exposed conidia was shifted to the right compared with that of the control (Fig. 1). Treatment with DHP for 4 h at 4× MIC₉₀ increased the lag phase from 9 to 11 h (Table 1). PAFEs have been found to be dependent on several factors, such as the concentration of the antifungal agent, the time for which the pathogen was exposed to the drug, and the characteristics of the drug used. In this study, it was found to depend mainly on the concentration of antifungal agent used against A. fumigatus.

Microscopic examination of the A. fumigatus conidia showed that germination started after 6 h incubation. Drug treatment delayed the germination of conidia due to a PAFE. The mycelia that developed in control and DHP-treated microwells were examined after 14 h using an inverted microscope (Nikon). The smoothness of the hyphal wall appeared to be affected in DHP-treated cultures. The visual examination of treated cultures showed delayed greyish-green pigmentation in comparison with
the control (Fig. 2). The delayed formation of pigment has been reported to affect the virulence of the pathogen (Langfelder et al., 1998, 2003).

The SDS-PAGE profiles of secretory proteins of *A. fumigatus* grown in the absence or presence of DHP were analysed. DHP treatment was found to down-regulate or inhibit the expression of three proteins of 18, 42 and 58 kDa (Fig. 3). N-terminal amino acid sequencing of the 42 kDa protein gave the sequence ALVPCNPNTV. The sequence of the protein showed 80% homology with a metalloprotease (accession no. 3776613) of *A. fumigatus*. Metalloproteases degrade collagens in human lung tissue (Markaryan et al., 1994; Sirakova et al., 1994). Two other proteins could not be sequenced due to interference from their high level of glycosylation.

The time-dependent antigenic profile of *A. fumigatus* showed that high-molecular-mass antigens were expressed first within a time frame of 10 h in the cultures. However, the lower-molecular-mass antigens were expressed subsequently. The treatment of *A. fumigatus* with DHP inhibited the expression of low-molecular-mass antigens. The expression of the 18 kDa antigen which was expressed along with the high-molecular-mass antigens within 10 h of incubation was found to be inhibited by DHP (Fig. 4).

### Table 1. PAFE of *A. fumigatus* strains after 1 and 4 h exposure to 1× and 4× MIC90 DHP

<table>
<thead>
<tr>
<th>Fungal strain</th>
<th>Drug concn (× MIC90)</th>
<th>PAFE (h)</th>
<th>1.0 h exposure</th>
<th>4.0 h exposure</th>
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<tr>
<td></td>
<td></td>
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<td>AmB DHP</td>
<td>AmB DHP</td>
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<tr>
<td><em>A. fumigatus</em> (187/96)</td>
<td>1×</td>
<td>0</td>
<td>0</td>
<td>&lt;3</td>
</tr>
<tr>
<td></td>
<td>4×</td>
<td>&lt;2</td>
<td>1</td>
<td>&gt;12</td>
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<td>0</td>
<td>0</td>
<td>&lt;2</td>
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<td></td>
<td>4×</td>
<td>&lt;2</td>
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<td>&gt;11</td>
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<tr>
<td><em>A. fumigatus</em> (124/98)</td>
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<td>0</td>
<td>0</td>
<td>&lt;3</td>
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<tr>
<td></td>
<td>4×</td>
<td>&lt;2</td>
<td>2</td>
<td>&gt;12</td>
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<tr>
<td><em>A. fumigatus</em> (12/01)</td>
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<td>0</td>
<td>0</td>
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<tr>
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<td>&lt;2</td>
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<td>0</td>
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<td>0</td>
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Fig. 2. Effect of DHP on the visual morphology of *A. fumigatus* after 96 h. Lane 1, normal growth; lane 2, conidia treated with 1× MIC90 for 1 h; lane 3, conidia treated with 1× MIC90 for 4 h; lane 4, conidia treated with 4× MIC90 for 1 h; lane 5, conidia treated with 4× MIC90 for 4 h. DHP delayed both the germination of conidia and pigment formation in the fungus.

Fig. 3. SDS-PAGE profile of secretory proteins of *A. fumigatus* after 10 h incubation. Lane M, molecular mass marker; lane 1, control; lane 2, *A. fumigatus* treated with 4× MIC90 DHP for 4 h.
Conclusion
The analysis of PAFEs in this study showed that DHP treatment increases the lag phase of fungi. Simultaneously it inhibits the expression of a metalloprotease and of important antigens, i.e. the 18 kDa antigen which is cytotoxic. The in vivo efficacy of the compound in a murine model of aspergillosis has already been established (Dabur et al., 2005a). Therefore, the compound may be employed as a model to develop new antifungal drugs, or can be used directly after further studies to reduce the severity of fungal infections.

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REFERENCES


