Expression Turnover Profiling To Monitor the Antifungal Activities of Amphotericin B, Voriconazole, and Micafungin against Aspergillus fumigatus

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Eight highly expressed candidate genes were selected for mRNA profiling to monitor the transcriptome kinetics of Aspergillus fumigatus strains exposed to antifungal drugs as potential biomarkers of live cells to assess treatment efficacy. Mycelia were treated with fungicidal drugs amphotericin B and voriconazole, as well as the fungistatic drug micafungin. Transcription was monitored at 0, 4, 8, and 24 h posttreatment. The expression turnover profile provides a possible tool to assess antifungal therapy effects.

Invasive aspergillosis (IA) has emerged as the leading cause of mortality (4, 8, 9) in immunocompromised patients (7), and Aspergillus fumigatus accounts for a majority of these infections (7, 8). The difficulty in rapidly diagnosing IA has led to the development of a variety of biomarkers of the disease, including direct detection of fungal-specific nucleic acids. This has led to the standardization of Aspergillus PCR by the European Aspergillus PCR Initiative (EAPCRI) (11). Nucleic acid detection is used for primary identification, but there has been little description of the approach to monitor the efficacy of therapy, largely because it is difficult to distinguish between live and dead cells in most molecular diagnostic assays. The underlying technological problem is a reliance on stable targets, such as DNA or structural RNAs, in the diagnostic nucleic acid assays. This can be problematic, as live organisms may respond to drugs, but the resulting nucleic acid released may be cleared slowly from airways and tissue due to underlying pathophysiology. Thus, there can be the appearance that infecting organisms are either partially viable or refractory to therapy. To address this issue, we have explored using mRNA profiling as a reporter for fungal viability. These transcripts are inherently less stable over time and therefore can be used to assess Aspergillus viability. A large set of microarray data designed to examine the effects of antifungal drugs on A. fumigatus (3) was manually screened to select candidate gene targets for the real-time reverse transcription (RT)-PCR profiling, and highly expressed genes that have rapid turnover following drug exposure were identified.

The clinical A. fumigatus wild-type strain R21 was used in all experiments. Amphotericin B (AMB) (Sigma, St. Louis, MO) and voriconazole (VRC) (Pfizer, New York, NY) were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), and micafungin (MCF) (Astellas Pharma USA, Inc., Rahway, NJ) was dissolved in sterile double-distilled water. The 6.4-mg/ml stocks for all three drugs were aliquoted and stored at −80°C. The frozen stocks were thawed at room temperature prior to the experiments.

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TABLE 1 Gene targets and primers for real-time RT-PCR

<table>
<thead>
<tr>
<th>Locus ID*</th>
<th>Gene target</th>
<th>Protein</th>
<th>Primer direction</th>
<th>Sequence (5′–3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Afu1g17250</td>
<td>RodB</td>
<td>Conidal hydrophobin RodB</td>
<td>Forward</td>
<td>AAGTTCCTGCTGCTTGCTCTCT</td>
</tr>
<tr>
<td>Afu2g01170</td>
<td>Gel1</td>
<td>1,3-Beta-glucanosyltransferase Gel1</td>
<td>Forward</td>
<td>GCACTGCTGTTGAGGAGGT</td>
</tr>
<tr>
<td>Afu6g00690</td>
<td>HYP1</td>
<td>Conserved hypothetical protein</td>
<td>Reverse</td>
<td>CGGAACGAGGTTATCAACGA</td>
</tr>
<tr>
<td>Afu2g02150</td>
<td>S10a</td>
<td>40S ribosomal protein S10a</td>
<td>Forward</td>
<td>CCGCACAGGAGAGAGAAGA</td>
</tr>
<tr>
<td>Afu1g15440</td>
<td>Ags1</td>
<td>α1,3-Glucan synthase, putative</td>
<td>Reverse</td>
<td>ACGGTCGCCACCCACTC</td>
</tr>
<tr>
<td>Afu3g13110</td>
<td>ESTRP</td>
<td>Extracellular serine-threonine rich protein</td>
<td>Forward</td>
<td>ATGGAATCGCCACCACCATAC</td>
</tr>
<tr>
<td>Afu5g02330</td>
<td>F1</td>
<td>Major allergen Asp F1</td>
<td>Reverse</td>
<td>AAGGATGGCGAACGACAGAGA</td>
</tr>
<tr>
<td>Afu6g02280</td>
<td>F3</td>
<td>Allergen Asp F3</td>
<td>Forward</td>
<td>CAGGGAAGTTGCGAGGTTT</td>
</tr>
</tbody>
</table>

*ID, identification.
and used within 24 h. *A. fumigatus* cultures were maintained on potato dextrose agar (PDA) slants. Conidia were collected from *A. fumigatus* cultures growing on PDA in sterile saline with 0.01% Tween 20. After being washed in sterile saline and counted under a microscope using a hemocytometer, 100 μl of a conidium suspension (5 × 10⁷ CFU/ml) was inoculated into 50 ml of liquid yeast extract-peptone-dextrose (YPD) medium, followed by incubation at 37°C and shaking at 200 rpm for 16 h. The mycelium cultures were then treated with the antifungal drugs at different doses, and the same amount of DMSO/H₂O was added to the no-drug control. The final drug concentrations in the mycelium cultures were 1 and 16 μg/ml for AMB, 0.5 and 16 μg/ml for VRC, and 16 μg/ml for MCF. Mycelia were harvested at 0, 4, 8, and 24 h posttreatment by filtration through Miracloth (CalBiochem, La Jolla, CA), washed thoroughly with sterile water, snap-frozen in liquid nitrogen, and disrupted in RNeasy lysis buffer (Qiagen, Valencia, CA) on a FastPrep instrument (MP Biomedicals), followed by total RNA extraction using the RNeasy minikit (Qiagen).

From prior microarray data obtained following exposure of *Aspergillus* strains to various antifungal drugs (3), eight highly expressed and abundant candidate gene targets with the potential for clinical diagnostic application (Table 1) were selected for mRNA profiling to monitor the transcriptome kinetics of *A. fumigatus* during exposure to antifungal drugs. Notably, as the specific targets for different antifungal drugs were not found to be significantly downregulated with the addition of the drug in our microarray data, they were not suitable candidates and were not included in this study. Primers (Table 1) were designed using the online software Primer3 (http://frodo.wi.mit.edu/primer3/) to generate amplicons of approximately 250 to 350 bp, and the specificity of the primers was analyzed by the dissociation curve in the real-time RT-PCR. The amplification efficiency of each primer set was evaluated by the standard curve method using 10-fold serially diluted *A. fumigatus* total RNA ranging from 10 ng to 1 pg per assay. Each target was tested in duplicate with two biological replicates, and an 18S rRNA gene was used as the normalizer in the calibration due to the observed stable detection throughout our study. SYBR green one-step RT-PCR assays were performed on an

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**FIG 1** Time-expression profiles of *A. fumigatus* strains exposed to 16 μg/ml of amphotericin B (A), 1 μg/ml of amphotericin B (B), 16 μg/ml of voriconazole (C), 0.5 μg/ml of voriconazole (D), or 16 μg/ml of micafungin (E).
Mx3005P real-time instrument (Stratagene, La Jolla, CA) using the 1-step Brilliant II SYBR green quantitative RT-PCR (qRT-PCR) master mix kit (Stratagene). The thermal cycling conditions consisted of 30 min at 48°C, 95°C for 10 min and 45 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, followed by a dissociation step at 95°C for 1 min, ramping temperature from 55°C to 95°C at the rate of 0.2°C/S. Nuclease-free water was used as the no-template control in all real-time experiments. The $2^{-\Delta\Delta CT}$ (threshold cycle) method was used to analyze the relative changes in gene expression (5), and data were analyzed for significant differences by analysis of variance (ANOVA) using the statistical software package SPSS 11.0 (SPSS Inc., Chicago, IL).

Most of the gene targets showed dramatically decreased expression, as much as 100-fold ($P < 0.001$) over 24 h, following exposure of *A. fumigatus* to 16 μg/ml of amphotericin B (Fig. 1A). The transcript turnover started as early as 8 h posttreatment. Much smaller decreases of the expression of the same genes were observed from the group with the lower dose (1 μg/ml) of AMB treatment (Fig. 1B). Similarly, another fungicidal drug, voriconazole, also demonstrated the turnover profile for most of the genes tested at the dose of 16 μg/ml (Fig. 1C). Compared to AMB, voriconazole exerted an effect on the transcripts that resulted in a delayed turnover pattern, as the most-significant reductions were observed at 24 h posttreatment. Whereas the sharp dropping of the mRNA expression of *A. fumigatus* clearly showed the fungicidal activities of voriconazole at the higher dose, the sublethal level (0.5 μg/ml) or around-the-MIC level of voriconazole exhibited the opposite effect on the transcripts. Differential induction to 0.5 μg/ml of VRC was seen at 4 h of exposure for the tested targets, except HYP1, S10a, and AGS1, and this increase diminished with time during the drug treatment (Fig. 1D). Meanwhile, a slight induction followed by a small decrease in the same transcripts was observed for mycelia treated with 16 μg/ml of the fungistatic drug micafungin (Fig. 1E).

The dose-dependent mRNA expression profiles for both AMB and VRC reflected the killing effect of the drugs. In fact, a similar turning profile for most of the genes was previously reported (1). The different time-killing profiles for AMB and VRC are possibly due to the different mechanisms of the antifungal activities (6). The physical injury in the cytoplasmic membrane created by AMB is direct, and the killing effect is rapid when the drug dose is sufficient. However, the depletion of ergosterol due to the inhibited 14α-demethylase takes time and prolongs the initiation of the lethal effect of voriconazole at a lethal dose. When the lower concentration of VRC was used for the treatment, the time required for the lanosterol depletion was out-competed by the time of fungal cell growth and it induced the stress response of the fungal cells. This was truly reflected by the induced expression of the genes in our study and consistent with the genome-wide transcriptome analyses (2). In contrast, even at a high concentration, the largely fungicidal drug micafungin did not have any profound effect on the transcripts, and only a very small fluctuation was observed for all the targets tested, confirming that turnover reflects viability.

These promising data raise the possibility that down the road, a molecular assay which assesses the viability of infecting organisms following therapy can be developed. This approach could effectively provide data on the susceptibility or resistance of an infecting organism to a drug within the host, eliminating the need for *in vitro* testing.

**ACKNOWLEDGMENT**

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**REFERENCES**