Intracellular Activity of Voriconazole, Fluconazole, and Itraconazole Against *Candida albicans* in Human Monocytes With and Without Activation by GM-CSF and TNF-

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**ABSTRACT**

**Background:** Disseminated infections caused by *Candida albicans* continue to be associated with high mortality. Monocytes are important host phagocytic cells which, when activated by cytokines, can have increased killing activity. This study compares the intracellular activity of three azoles (fluconazole, voriconazole, and itraconazole) in human monocyte-derived macrophages (MDM) activated or not activated by cytokines.

**Design and Methods:** Human MDM monolayers prepared from heparinized blood of healthy volunteers were infected with *C albicans* (fluconazole-resistant [flu]$^8$] strains 8336-2 and ATCC 64550; fluconazole-susceptible [flu]$^8$] strains T=6, ATCC 56882, and ATCC 90028). Antifungal agents (1 x minimal inhibitory concentration) were added following a 1-h phagocytosis time. Numbers of viable *C albicans* from MDM lysates were determined at 0, 24, and 48 h.

**Results:** For two of the five *C albicans* strains (ATCC 90028 and T=6), all azoles had similar activity at 24 h. There were slight differences at 48 h. For strains ATCC 64550 and ATCC 56882, the most effective azoles were fluconazole and itraconazole, respectively, and voriconazole was most effective against strain 8336-2. Voriconazole was least effective against strain ATCC 56882, and itraconazole was least effective.
against strain ATCC 64550. Significant effects of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor \( \alpha \) occurred only with voriconazole and were strain-dependent. Activity of voriconazole was enhanced, inhibited, or unaffected by these cytokines.

**Conclusions:** This study clearly indicates that in the presence of different azoles and cytokines, the intracellular antifungal activity of human MDM cannot be predicted and may differ for individual *C. albicans* strains.

**INTRODUCTION**

Disseminated candidiasis continues to be a serious fungal infection, especially in debilitated and immunocompromised hosts.\(^1\)\(^-\)\(^2\) Furthermore, fluconazole-resistant (flu\(^R\)) *Candida albicans* strains have been found in such patients with increased frequency.\(^2\)\(^-\)\(^3\) Newer azoles, voriconazole and itraconazole, have increased activity against flu\(^R\) *C. albicans* as well as other *Candida* species.\(^4\)\(^-\)\(^9\)

Azoles are also known to enter phagocytic cells.\(^10\)\(^-\)\(^11\) Although antimicrobial agents are important in the treatment of infection caused by *Candida* spp., host factors, including phagocytic cells, are also important in patient survival.\(^12\) Cytokine-activated phagocytic cells have been shown to have increased inhibitory or fungicidal activity, largely related to oxygen burst mechanisms.\(^13\)\(^-\)\(^19\)

Furthermore, the cytokines granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor \( \alpha \) (TNF-\( \alpha \)) have been shown to enhance human monocyte antifungal activity.\(^13\)\(^-\)\(^15\)\(^-\)\(^17\)\(^-\)\(^19\) The addition of cytokines to appropriate antifungal agents in the therapy of immunocompromised patients with disseminated candidiasis and hepatosplenic candidiasis has been reported to increase patient survival.\(^20\)\(^-\)\(^21\) In addition, TNF-\( \alpha \) has a protective role in a murine model of systemic candidiasis.\(^22\)

Azoles inhibit or kill *C. albicans* by inhibiting the cytochrome P-450-dependent enzyme 14-\( \alpha \) sterol demethylase, blocking the sterol biosynthesis pathway essential for the production of a functional membrane.\(^23\)\(^-\)\(^28\) Newer azoles, voriconazole and itraconazole, are more active than the earlier azole, fluconazole, against *C. albicans*.

The purpose of this study was to evaluate the intracellular antifungal activity of three azoles (fluconazole, itraconazole, and voriconazole) against flu\(^R\) and fluconazole-susceptible (flu\(^S\)) *C. albicans* strains in human monocyte-derived macrophages (MDM), and to determine if these activities differed in unactivated MDM and MDM activated with the cytokines GM-CSF and TNF-\( \alpha \). Production of GM-CSF and TNF-\( \alpha \) by MDM exposed to voriconazole, fluconazole, and itraconazole was also evaluated.

**MATERIALS AND METHODS**

**Candida Strains**

*C. albicans* strain T=6, originally isolated from the blood of a patient with candidemia, was provided by Wadsworth Laboratories, New York State Department of Health, Albany, NY. *C. albicans* strain 8336-2 is a clinical isolate obtained from the throat of a patient with AIDS at the Stratton Veterans Affairs Medical Center, Albany, NY. Three other *C. albicans* strains, ATCC 64550, ATCC 90028, and ATCC 56882, were obtained from the American Type Culture Collection, Manassas, VA.

**Antimicrobial Agents**

Voriconazole and fluconazole were provided by Pfizer Laboratories (Sandwich, Kent, England). Itraconazole was purchased from Research Diagnostics Inc. (Flanders, NJ). Antibiotic solutions were made fresh for each experiment in accordance with the suppliers’ instruc-
tions, filter-sterilized, and used immediately. Minimal inhibitory concentrations (MICs) for fluconazole, voriconazole, and itraconazole, respectively, determined according to the National Committee for Clinical Laboratory Standards method M27-A2,29,30 were as follows: ATCC 64550: 32, 0.5, 0.25 μg/mL; 8336-2: 64, 2, 0.5 μg/L; T=6: 1, 0.06, 0.25 μg/mL; ATCC 90028: 0.5, 0.03, 0.125 μg/mL; and ATCC 56882: 0.25, 0.03, 0.03 μg/mL.

**Preparation of Human Monocytes**

Monocytes were prepared from heparinized blood of healthy human donors who had signed the informed consent form approved by the Institutional Review Board of the Albany Medical College and Stratton Veterans Affairs Medical Center, Albany, NY. Mononuclear cells were separated from whole blood by using Histopaque-1077 (Sigma Chemical Co., St. Louis, MO). The resulting mononuclear preparation was ≥ 98% pure. The separated cells were resuspended at a concentration of 2 x 10⁶ cells/mL in RPMI+ (RPMI 1640 medium [Sigma] containing 10% fetal bovine serum [Sigma, cat. # F-2442]). Cell viability, determined by using the trypan blue exclusion test, was ≤ 98%.

**Cytokines**

Cytokines and enzyme-linked immunosorbent assay (ELISA) kits for cytokine determinations were obtained from R & D Systems, Inc. (Minneapolis, MN). MDM were activated with GM-CSF and TNF-α at concentrations of 100 U/mL.

**Anticandidal Activities of Voriconazole, Fluconazole, and Itraconazole in MDM**

Human mononuclear cells (2 x 10⁶/mL) were delivered to the wells of 24-well plates (Corning/Costar Corp., Cambridge, MA) at a volume of 1 mL/well and allowed to adhere for 72 h. Medium and nonadherent cells, including lymphocytes, were aspirated from the wells. The adherent, confluent monocyte layers, from this point referred to as MDM, were washed once with RPMI+. RPMI+, with or without GM-CSF and TNF-α (100 U/mL), was added gently to the surfaces of monolayers in duplicate wells. Plates were incubated at 37°C for 24 h in an atmosphere containing 5% CO₂. The medium was removed from the wells by aspiration and a 1-mL aliquot of *C. albicans* containing 2 x 10⁴ cells suspended in RPMI+ was then added to each well. After allowing 1 h for phagocytosis, nonphagocytosed yeast were removed by aspiration and the monolayers were washed once with RPMI+. One mL of RPMI+, with or without cytokines as appropriate, was then added to each monolayer.

Antifungal drugs (1 x MIC for each particular *C. albicans* strain) were then added to appropriate wells. Following incubation of the plates at 37°C for 0, 24, or 48 h in an atmosphere containing 5% CO₂, the medium was removed, the MDM were lysed with sterile distilled water, and the number of viable yeast in the lysates was determined by using the standard plate count method (24 h incubation at 37°C) and Sabouraud dextrose agar (Sigma).

**Effects of Voriconazole, Fluconazole, and Itraconazole on Cytokine Production by MDM**

MDM monolayers prepared as described above were studied for the production of GM-CSF and TNF-α following exposure to serum-attainable concentrations of voriconazole, fluconazole, and itraconazole (2.5, 8.0, and 0.4 μg/mL, respectively). Controls included *Escherichia coli* serotype 055:B5 lipopolysaccharide (LPS, 1 μg/mL) and MDM monolayers alone. Cytokine concentrations in the culture media overlaying the monolayers were determined.
following incubation of the plates at 37°C for 24 h in an atmosphere containing 5% CO₂.

**Statistical Methods**

Each experiment described above was repeated at least three times. The analy-
sis of variance methodology\textsuperscript{31} with the log\textsubscript{10} \(\log_{10}\) transformation was used to analyze the data. For final presentation, average \(\log_{10}\) values were converted to geometric mean values in the original units. Viable counts are expressed as percentages of the cfu/mL at zero hour. The standard errors of the geometric means (SEM)

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**Figure 2.** Anticandidal activity of voriconazole, itraconazole, and fluconazole at 1 x MIC against Candida albicans in human monocyte-derived macrophages. (A) flu\textsuperscript{a} strain ATCC 90028; (B) flu\textsuperscript{b} strain ATCC 56882.
are depicted as error bars in Figures 1 through 3. Multiple contrasts were tested under the Bonferroni criteria.\textsuperscript{32} Null hypotheses were specified a priori. The level of significance is 0.01.

RESULTS
Figures 1 through 3 show the antifungal activity of MDM with voriconazole in the presence and absence of GM-CSF or TNF[-], and the antifungal activity of MDM with fluconazole and itraconazole in the absence of cytokines.

Statistical analysis indicated that neither GM-CSF nor TNF [-] affected the intracellular antifungal activity of MDM with itraconazole or fluconazole, and that the antifungal activities of MDM activated only with GM-CSF or TNF[-] were similar to those of inactivated controls. Therefore, these data are not shown in the figure.

Figure 1A shows the antifungal effects of MDM with voriconazole, itraconazole, and fluconazole at 1 x MIC, with and without cytokines (voriconazole only), against flu\textsuperscript{R} C\textit{albicans} 8336-2 in MDM. All azoles showed significant antifungal activity compared with the control at 24 and 48 h (P<0.01). At 48 h voriconazole was more effective than itraconazole or fluconazole (P<0.01). However, at 24 h the effects of voriconazole and itraconazole were similar and greater than that of fluconazole (P<0.01). GM-CSF reduced the antifungal effect of voriconazole at 24 h, while GM-CSF and TNF[-] reduced this effect at 48 h (P<0.01).

Figure 1B depicts the antifungal effects of MDM with the three azoles at 1 x MIC, with and without cytokines (voriconazole only), against flu\textsuperscript{R} C\textit{albicans} ATCC 64550 in MDM. At both 24 and 48 h, fluconazole was most effective (P<0.01), and voriconazole was more effective than itraconazole (P<0.01). Although both fluconazole and voriconazole were more effective than the control (P<0.01), itraconazole was similar to the control. GM-CSF and TNF[-] did not affect the MDM antifungal activity.

Figure 3. Antifungal activity of voriconazole, itraconazole, and fluconazole at 1 x MIC against \textit{Candida albicans} in human monocyte-derived macrophages: flu\textsuperscript{R} strain T=6.
didal activity with voriconazole.

Figure 2A shows the antifungal activities of MDM with voriconazole, itraconazole, and fluconazole at 1 x MIC, with and without cytokines (voriconazole only), against flu³ C albi - cans ATCC 90028 in MDM. At both 24 and 48 h, all azoles had similar antifungal activity, which was significantly greater than that of the control (P<0.01). While GM-CSF had no effect on the antifungal activity of voriconazole, antifungal activity was decreased by TNF-[] at 24 h but not at 48 h (P<0.01).

Figure 2B depicts the antifungal activities of MDM with all three azoles at 1 x MIC, with and without cytokines (voriconazole only), against flu³ C albi - cans ATCC 56882 in MDM. Although itraconazole was more effective than fluconazole at 24 h, their activities were similar at 48 h (P<0.01). Both itraconazole and fluconazole were more effective than the control (P<0.01), and voriconazole was similar to the control at 48 h but not at 24 h (P<0.01). At 24 h, voriconazole was the least effective antibiotic (P<0.01). However, both GM-CSF and TNF-[] significantly increased the activity of voriconazole at 48 h (P<0.01). The increase was similar for both cytokines.

Figure 3 shows the MDM antifungal activities of MDM with voriconazole, itraconazole, and fluconazole at 1 x MIC, with and without cytokines (voriconazole only), against flu³ C albi - cans ATCC 6 in MDM. At 24 h, the activities of voriconazole, itraconazole, and fluconazole were similar and were significantly greater than the control (P<0.01). However, at 48 h the activity of itraconazole was similar to that of fluconazole and was significantly greater than that of voriconazole (P<0.01). GM-CSF and TNF-[] did not affect the antifungal activity of MDM with voriconazole.

The production of GM-CSF and TNF-[] by MDM following exposure to voriconazole, fluconazole, and itraconazole at serum-attainable levels is shown in Table 1. While LPS stimulated MDM production of TNF-[] and GM-CSF, the effects of the three azoles at serum-attainable concentrations on the production of these two pro-inflammatory cytokines were negligible. Cytokine levels were similar to those of the unstimulated MDM controls.

**DISCUSSION**

This study was undertaken in order to compare the intracellular antifungal effects of three azoles in human MDM and to define the effects of GM-CSF and TNF-[] on this antifungal activity. The results clearly indicate that the

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**Table 1.** Production of GM-CSF and TNF-[] by Human MDM Exposed to Azoles at Serum-Attainable Concentrations

<table>
<thead>
<tr>
<th>Azole</th>
<th>Geometric mean cytokine concentration in pg/mL (SEM)</th>
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<tbody>
<tr>
<td></td>
<td>GM-CSF</td>
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<tr>
<td>Voriconazole (2.5 mg/mL)</td>
<td>2 (&lt;1)</td>
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<tr>
<td>Fluconazole (8.0 mg/mL)</td>
<td>3 (&lt;1)</td>
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<tr>
<td>Itraconazole (0.4 mg/mL)</td>
<td>4 (&lt;1)</td>
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<tr>
<td>Controls:</td>
<td></td>
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<tr>
<td>LPS (1 mg/mL)</td>
<td>11 (3)</td>
</tr>
<tr>
<td>Untreated MDM</td>
<td>10 (3)</td>
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</tbody>
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intracellular effects of voriconazole, itraconazole, and fluconazole studied at 1 x MIC in human MDM are variable and strain-dependent. For two of the five C. albicans strains (ATCC 90028 and T=6), all azoles had similar activity at 24 h, although there were slight differences at 48 h. For strains ATCC 64550 and ATCC 56882, the most effective azoles were fluconazole and itraconazole, respectively, while voriconazole was most effective against strain 8336-2 (flu³). Voriconazole was least effective against strain ATCC 56882 and itraconazole was least effective against strain ATCC 64550.

Previous studies have shown that fluconazole and voriconazole enter polymorphonuclear leukocytes. The authors of this study are unaware of any study indicating that azoles also penetrate into human monocytes. Our data indicate that all of the azoles studied have intracellular antifungal activity in human MDM. However, this activity varies with the azole, C. albicans strain, and duration of infection in MDM. An earlier study that used different methodology and single strains of flu³ and flu⁶ C. albicans showed that the antifungal effect of voriconazole was greater than that of fluconazole in both monocytes and neutrophils, and that activation of these phagocytes with GM-CSF increased the intracellular effect of voriconazole. In two other studies that used GM-CSF-activated neutrophils, fluconazole, and C. albicans, the intracellular activity of the phagocytes was shown to be strain-dependent. This study using human monocytes, three different azoles, and five strains of C. albicans showed that although all three azoles increased the intracellular antifungal activity of the MDM, this increase was azole- and strain-dependent. Furthermore, activation of MDM with GM-CSF or TNF-α did not affect the antifungal activity of MDM in the presence of itraconazole or fluconazole when 1 x MIC of these azoles was used. In contrast, in the presence of voriconazole and cytokines, a decrease in MDM activity was observed in two C. albicans strains (GM-CSF and TNF-α for 8336-2 and TNF-α for ATCC 90028), while an increase in the MDM activity was observed on ATCC 56882 with GM-CSF and TNF-α, and no effect was observed on strains ATCC 64550 and T=6.

The effects of azoles on cytokine production by MDM were determined in order to ensure that any effects of added cytokines were attributable to the added cytokines and did not result from cytokine production stimulated by the azoles. These results indicate that the effects of voriconazole, fluconazole, and itraconazole on production of GM-CSF and TNF-α by human MDM were negligible. Cytokine levels were similar to those of unstimulated MDM controls.

In conclusion, by studying five different strains of C. albicans, both flu³ and flu⁶, and three azoles, these data clearly show that the intracellular antifungal effects of human MDM cannot be predicted and that they are C. albicans strain- andazole-dependent. C. albicans strains may need to be evaluated individually in order to select the azole with the greatest intracellular antifungal activity. The antifungal effect of cytokine-activated MDM in the presence of voriconazole was strain-dependent and could be increased, decreased, or remain unchanged. Cytokines did not affect the antifungal activity of MDM in the presence of fluconazole or itraconazole.

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