Anticandidal effects of voriconazole and caspofungin, singly and in combination, against *Candida glabrata*, extracellularly and intracellularly in granulocyte-macrophage colony stimulating factor (GM-CSF)-activated human monocytes

Aldona L. Baltch1,2*, Lawrence H. Bopp1, Raymond P. Smith1,2, William J. Ritz1,2 and Phyllis B. Michelsen1,2

1Stratton Veterans Affairs Medical Center, Albany, NY 12208, USA; 2Albany Medical College, Albany, NY 12208, USA

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Objectives: The antifungal effects of voriconazole and caspofungin, singly and in combination, were determined against *Candida glabrata* in time–kill curves in broth, in human monocyte-derived macrophages (MDMs) and in MDMs activated by granulocyte-macrophage colony-stimulating factor (GM-CSF).

Methods: Three strains of fluconazole-resistant *C. glabrata* were evaluated. For intracellular studies, MDM monolayers, with or without GM-CSF activation, were infected with *C. glabrata* and treated with voriconazole and caspofungin at 2.5× and 5× MIC, respectively, or at 1× MIC. Extracellular studies in broth were performed using drug concentrations from 0.1 to 10× MIC. Viable yeast were enumerated at 0, 24 and 48 h.

Results: Significantly greater killing of *C. glabrata* occurred with the drug combination than with either single drug, both intracellularly and extracellularly (*P* < 0.01). For voriconazole, the antifungal activity in MDM activated by GM-CSF was greater than that in unactivated MDM, regardless of antibiotic concentration or time of exposure. However, for caspofungin and for the two-drug combination, enhanced activity in GM-CSF-activated MDM depended on the drug concentration and time of exposure.

Conclusions: Our data suggest that combinations of voriconazole and caspofungin may be efficacious for the treatment of serious *C. glabrata* infections. With single-drug therapy, especially voriconazole, GM-CSF activation of monocytes could be considered.

Keywords: phagocytes, drug combinations, human monocytes, *Candida* spp.

Introduction

*Candida* species have been listed as a cause of 9% of bloodstream infections, with crude mortality rates reaching above 50%.1 Although *Candida albicans* remains the most common *Candida* species associated with bloodstream infections, the occurrence of infections caused by other *Candida* species, especially *Candida glabrata*, is increasing.1–3 *C. glabrata* and *Candida krusei* strains have shown resistance or intermediate resistance to most earlier antifungal agents, and treatment of infections caused by these organisms has been difficult.4–6 With the development of echinocandins and newer triazole drugs, the demonstration of increased susceptibilities to these drugs has become important.7–8

The effectiveness of combinations of antifungal agents, which may be useful in the treatment of severe disseminated and deep-seated candidiasis, including endocarditis, hepatic candidosis or endophthalmitis, has been evaluated mostly under extracellular experimental conditions.7–15 Phagocytic cells, including monocytes and monocyte-derived macrophages (MDMs), are known to be important in clearing fungal infections,16,17 and it is known that antifungal drugs such as fluconazole and voriconazole enter phagocytic cells.18–25 Furthermore, as activation of human monocytes using cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF) is known to increase the antifungal activity of these phagocytes...
against Candida spp., including C. glabrata, the use of cytokines as adjuncts to antifungal therapy merits further investigation.20,21,23,24

The purpose of this study was to evaluate the intracellular antifungal activities of voriconazole and caspofungin, singly and in combination, against C. glabrata in unactivated human monocytes and in monocytes activated with GM-CSF. In addition, in order to provide a frame of reference for the activities of these drugs and combinations in a more conventional setting, their extracellular antifungal activities were also evaluated.

Materials and methods

C. glabrata strains and preparation of yeast inocula

C. glabrata strains 11, 12 and 14 were provided by the Wadsworth Center, New York State Department of Health, Albany, NY, USA. To prepare inocula, C. glabrata strains taken from stock cultures frozen at −80°C in 15% glycerol were streaked on Sabouraud dextrose (SD) agar and incubated overnight at 35°C. Four to five well-isolated colonies from a plate were used to make a suspension in sterile 0.85% (w/v) NaCl. Absorbance at 530 nm was determined and adjusted to yield a final cell density of ~2 × 10⁸ cfu/mL. For opsonization, 2 mL of this suspension was centrifuged at 13,000 g for 30 min at 35°C and the supernatant was discarded, yeast were resuspended in RPMI 1640 medium (Sigma Chemical Co., St Louis, MO, USA) containing 20% pooled, heat-inactivated normal human serum and the suspension was incubated for 30 min at 35°C with gentle shaking. Following opsonization, the yeast were centrifuged and resuspended in sterile saline. They were then diluted 100-fold to 2 × 10⁶ cfu/mL in RPMI 1640 medium (Sigma) supplemented with 15% fetal bovine serum (Sigma, cat. no. F6178; RPMI+). The cell density of each inoculum was verified using the standard plate count method.

Antimicrobial agents and MIC testing

Voriconazole was provided by Pfizer Laboratories (Groton, CT, USA). Caspofungin was provided by Merck Pharmaceuticals (Rahway, NJ, USA). Antibiotic solutions were prepared according to the directions provided by the supplier, filter sterilized (0.2 μm pore-size) and used immediately. MICs of voriconazole were determined in triplicate by using the NCCLS-approved macrodilution method M27-A2.26 Caspofungin MICs were determined in the laboratory of Dr M. Pfaller, University of Iowa, by using the NCCLS-approved microdilution method M27-A2 (2002).26 MICs (mg/L) of voriconazole/caspofungin for C. glabrata strains 11, 12 and 14 were 1/0.06, 0.5/0.03 and 2/0.25, respectively. MICs (mg/L) of amphotericin B/fluconazole were 0.125/32, 0.125/16 and 0.125/64, respectively.

Human monocytes

Monocytes were obtained from heparinized blood of normal donors who had signed an informed consent form approved by the Institutional Review Board of the Stratton Veterans Affairs Medical Center, Albany, NY, USA. Mononuclear cells were separated from whole blood by using Histopaque 1077 (Sigma). Cell viability, determined using the Trypan Blue exclusion assay, was >98%.

Intracellular time–kill studies

Preliminary intracellular time–kill experiments were carried out with voriconazole and caspofungin alone at 0.1, 0.25, 0.5, 1, 2.5, 5, 10 and 50× MIC. Based on the results of these experiments, optimal concentrations of both drugs were chosen for intracellular experiments (1 × MIC of voriconazole and caspofungin). At these concentrations, there was antifungal activity, but it was not high enough to cause significant killing of the yeast. For comparison, higher concentrations, at which antifungal activity was more pronounced (2.5 × MIC for voriconazole and 5 × MIC for caspofungin), were also tested. Monocytes were prepared as described earlier, suspended at 2 × 10⁶ cells/mL in RPMI 1640+, added to the wells of 48-well culture plates (1 × 10⁶ cells/well) and allowed to adhere for 2 h. Following this incubation, medium containing unadhered cells was removed by aspiration. After adhesion, monocytes begin the transformation to MDM. Therefore, from this point on, they are called MDM. GM-CSF (R&D Systems, Inc., Minneapolis, MN, USA) in RPMI+ was added to appropriate wells at a final concentration of 100 U/mL. All MDM monolayers (with or without GM-CSF) were then incubated for 24 h at 37°C in an atmosphere containing 5% CO₂ prior to the addition of yeast and antibiotics. Following removal of the overlying medium by aspiration, 500 μL of a suspension of opsonized C. glabrata cells in RPMI+ (2 × 10⁶ cfu/mL) was added to the surface of each monolayer. Cell monolayers were then incubated for 1 h at 37°C in an atmosphere containing 5% CO₂ in order to allow phagocytosis of the yeast to occur. Following phagocytosis, the medium was removed by aspiration and the MDM monolayers were washed once with RPMI+. After washing, RPMI+, with or without GM-CSF, was added. Voriconazole and caspofungin, singly or in combination, were then added to the wells. Concentrations of 1 × MIC of each drug and 2.5 × MIC of voriconazole plus 5 × MIC of caspofungin were tested. Following incubation at 37°C in an atmosphere containing 5% CO₂ for 0, 24 or 48 h, the medium was removed from each well by aspiration, and the MDMs were lysed using sterile H₂O in order to release the yeast. The number of viable yeast in each sample was determined using SD agar and the standard plate count method.

Extracellular (cell-free) time–kill studies

Extracellular time–kill studies were conducted for C. glabrata strains 11, 12 and 14. Inocula (2 × 10⁶ cfu/mL) were prepared as above. Tests were performed in 5 mL tubes using 4 mL of RPMI 1640 medium containing MOPS buffer. Voriconazole and caspofungin were tested individually in duplicate tubes at 0.1, 0.25, 1, 2.5, 5 and 10 × MIC and in combinations at the same concentrations. Following incubation (0, 24 and 48 h) at 35°C, a homogeneous yeast suspension was created in each tube by vigorous vortexing. The number of viable yeast in each suspension was then determined in duplicate using SD agar and the standard plate count method.

In each intracellular and extracellular experiment, each experimental condition was run in duplicate (two wells or tubes), and the number of surviving yeast in each well or tube was determined in duplicate. Each assay was repeated three times. All experiments with GM-CSF were performed with 24 h activation of MDM and the reaplication of GM-CSF.

For time–kill curves, inhibition is defined as the number of viable yeast being statistically significantly lower than that in the untreated control but greater than or equal to the number at 0 h. Killing is defined as the number of viable yeast being statistically significantly lower than the number at 0 h.

Statistical analysis

Numbers of cfu/mL were converted to logarithms, base 10, for statistical analysis. Comparisons among log counts, both intracellularly and extracellularly, were made using the analysis of variance.
**Voriconazole, caspofungin, GM-CSF and C. glabrata**

*Post hoc* comparisons were made under the Dunn procedure. The level of significance was 0.01. For presentation of results, the mean logarithm values were converted into geometric mean numbers of cfu/mL and expressed as a percentage of the geometric mean numbers of cfu/mL at zero assay hour.

**Results**

**Intracellular activity of voriconazole and caspofungin against C. glabrata in human MDM not activated by GM-CSF**

MDM exposed to voriconazole and caspofungin, singly and in combination, had greater antifungal activity than MDM alone (*P < 0.01*). This activity was concentration-dependent for single drugs and the two-drug combination. At 1 × MIC over 48 h, each single drug caused inhibition compared with the untreated control, while there was killing (72%) with the drug combination (Figure 1a). For single drugs at the higher concentrations (2.5 × MIC for voriconazole and 5 × MIC for caspofungin, Figure 1b), killing occurred at 48 h with caspofungin alone (89%) and with the drug combination (98%). At these higher concentrations, the two-drug combination had greater antifungal activity than either single drug at both 24 and 48 h (*P < 0.01*).

**Intracellular activity of voriconazole and caspofungin against C. glabrata in human MDM activated by GM-CSF**

Compared with unactivated MDM, MDM activated with GM-CSF but without antibiotics significantly inhibited the intracellular growth of *C. glabrata* (*P < 0.01*). The addition of voriconazole or caspofungin, singly or in combination, further enhanced this antifungal activity (*P < 0.01*). With GM-CSF-activated MDM at 24 h, there was significant intracellular killing at 1 × MIC (28%, Figure 1a) and at 2.5 × MIC (80%, Figure 1b; *P < 0.01*) of voriconazole. In contrast, with unactivated MDM, there was inhibition of intracellular growth at 1 × MIC and significantly less killing at 2.5 × MIC of voriconazole than in activated MDM (*P < 0.01*). Similar results were observed with 1 × MIC of caspofungin at 24 h (Figure 1a). However, the activation of MDM with GM-CSF did not increase killing by 5 × MIC of caspofungin at 24 and 48 h (89% killing in unactivated MDM and 88% killing in activated MDM, Figure 1b). The activation of MDM by GM-CSF slightly increased intracellular killing of *C. glabrata* in the presence of the drug combination at 1 × MIC only at 48 h (82% killing in activated MDM and 72% in unactivated MDM, Figure 1a; *P < 0.01*). In contrast, at both the lower and higher concentrations of the two-drug combination at 24 h, intracellular killing was slightly but significantly greater in the absence of GM-CSF (94% versus 89%, Figure 1a; 96% versus 89%, Figure 1b; *P < 0.01*).

**Extracellular activities of voriconazole and caspofungin against C. glabrata in broth**

Antifungal activities against *C. glabrata* were determined over a wide range of concentrations. It is clear that inhibition and killing were concentration-dependent for both single drugs and the two-drug combination at concentrations from 0.1 × to 10 × MIC. The combinations of voriconazole and caspofungin at 5 and 10 × MIC resulted in >97% killing at 24 h and >99% killing at 48 h (Figure 2c). At 2.5 × MIC, there was 83% killing at 24 h, but regrowth occurred at 48 h (*P < 0.01*). At concentrations below 2.5 × MIC, there was significant (*P < 0.01*) inhibition but no killing.

Only exposure to voriconazole as a single drug resulted in significant killing (*P < 0.01*; Figure 2a): at 5 × MIC at 48 h (60%) and at 10 × MIC at 24 h (36%) and 48 h (92%). For caspofungin, inhibition but not killing was observed, and only at the highest concentrations tested (Figure 2b; *P < 0.01*). At both 5 and 10 × MIC, the combination of voriconazole and caspofungin resulted in significantly greater killing (>97%) than voriconazole alone (*P < 0.01*; Figure 2c). At both 24 and 48 h, voriconazole caused inhibition (*P < 0.01*) at lower concentrations than caspofungin (Figure 2a and b).

**Discussion**

In complicated *Candida* infections, elimination of the pathogen as early and as effectively as possible is essential for patients' survival. It has been demonstrated in a neutropenic murine *C. albicans* infection model that the window for effective therapy is short (24 h), following which a rapidly growing fungal burden becomes more difficult or even impossible to treat. Our study indicates that even more effective therapy may be possible using a combination of voriconazole and caspofungin rather than either drug alone. Phagocytic cells are essential not only to clear the pathogen, but also to activate the host immune response. In addition to their antifungal effects, agents such as voriconazole can up-regulate gene expression and release of cytokines, especially tumour necrosis factor-α (TNF-α), by human THP-1 monocyctic cells exposed to *Aspergillus fumigatus*. Caspofungin-induced changes of *A. fumigatus* hyphae causing β-glucan exposure resulted in Dectin-1-mediated inflammatory responses with the release of TNF-α by murine macrophages.

Results of previously published *in vitro* studies indicate that GM-CSF activation of monocytes increases the intracellular antifungal activity of voriconazole against *C. glabrata*. A recent meta-analysis revealed that in patients undergoing chemotherapy or following stem cell transplantation, CSFs may reduce infection-related morbidity and mortality by decreasing febrile neutropenia, resulting in better quality of life and decreased cost. However, overall mortality was not affected. Reversal of immunoparalysis in patients with severe sepsis treated with recombinant GM-CSF has been associated with the up-regulation of HLA-DR receptors on monocytes. Our study confirms that GM-CSF activation of human monocytes increases their antifungal activity, regardless of the antibiotic concentration or time of exposure. However, for caspofungin and the voriconazole–caspofungin combination, enhanced activity of GM-CSF-activated MDM depends on the concentrations of the antifungal drugs and time of exposure. GM-CSF activation of MDM resulted in minimal enhancement and sometimes slight inhibition of drug activity for the voriconazole–caspofungin combination at 2.5 × MIC. In contrast, activities of the single drugs at low concentrations (1 × MIC) were enhanced at 48 h by GM-CSF-activated MDM.

Our data indicate that combinations of voriconazole and caspofungin are more effective than either drug alone, both intracellularly in MDM and extracellularly in broth. These are *in vitro* observations. Further laboratory and clinical studies are required to determine whether the use of voriconazole and caspofungin in combination would be beneficial in the treatment of fungal infections.
caspofungin combinations will be associated with better survival in candidiasis. For infections for which single drugs are used and low intracellular drug concentrations may be anticipated, the activation of monocytic phagocytes with GM-CSF against intracellular \textit{C. glabrata} may increase the intracellular activities of the drugs, especially voriconazole.

**Figure 1.** Intracellular anticandidal effects of voriconazole and caspofungin, singly and in combination, at 1× MIC of voriconazole and caspofungin (a) and 2.5× MIC of voriconazole and 5× MIC of caspofungin (b), against three strains of \textit{C. glabrata} in human MDM, previously activated or not activated by GM-CSF. VRC, voriconazole; CPF, caspofungin.
Voriconazole, caspofungin, GM-CSF and *C. glabrata*

**Figure 2.** Extracellular anticandidal activity of (a) voriconazole, (b) caspofungin and (c) combinations of voriconazole and caspofungin, at 0.1–10 × MIC against three strains of *C. glabrata.*
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Transparency declarations

None to declare.

References


