Activities and Ultrastructural Effects of Antifungal Combinations against Simulated Candida Endocardial Vegetations

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In vitro pharmacodynamic model (PDM) simulation of serum antifungal concentrations may predict the value of combination antifungal regimens against Candida sp. endocarditis. We investigated the effects of combinations of flucytosine (5FC), micafungin (Mica), and voriconazole (Vor) against Candida-infected human platelet-fibrin clots, used as simulated endocardial vegetations (SEVs). Single clinical bloodstream isolates of Candida albicans, Candida glabrata, Candida parapsilosis, and Candida tropicalis were used. All four isolates were susceptible to 5FC, while C. glabrata was resistant to Vor and C. tropicalis had a paradoxical resistance phenotype to Mica. The SEVs were prepared with an initial inoculum of 1 × 10^6 CFU/g of SEV and added to a PDM, which utilized yeast nitrogen broth-2% glucose and incubation at 35°C and simulated antifungal pharmacokinetic profiles. Fungal densities in the SEVs were determined in quadruplicate over 72 h. Scanning electron microscopy (SEM) was used to evaluate treatment and control SEVs. Vor was the least active single agent against all Candida spp. except for C. parapsilosis, where it was comparable to Mica. In contrast, 5FC was the most active against all Candida spp. except for C. tropicalis, where it was comparable to Mica. The combination of 5FC plus Vor was superior to either agent alone against C. parapsilosis. The combination of Vor plus Mica was inferior to the use of Mica alone against C. tropicalis. The triple combination of 5FC plus Vor plus Mica was no better than single or dual agents against any of the Candida spp. The ultrastructural features of infected SEVs were unique for each Candida sp., with C. parapsilosis in particular manifesting friable biofilm clusters. In general, 5FC and Mica were superior in their rates and extents of fungal burden reduction compared to Vor against Candida-infected SEVs. Evaluation of 5FC and Mica in animal models of Candida endocarditis is warranted.

Candida species are the fourth most common cause of bloodstream and cardiovascular infections in the United States (27). Candida can adhere to cell surfaces and form a three-dimensional community of microorganisms encased in an exopolymeric matrix, known as a biofilm (9). Biofilm can form on native and prosthetic heart valves to cause infective endocarditis. Fungal endocarditis is associated with a mortality rate in excess of 50% (10). Current medical management includes combination use of amphotericin B and flucytosine followed by prolonged suppression with fluconazole (4, 27). However, multidrug resistance to long-term suppressive therapy can develop (20). Candida albicans cells within biofilms have a decreased cell membrane ergosterol content, have reduced expression of ergosterol biosynthetic genes, express higher levels of genes involved in amino acid and nucleotide metabolism, and up regulate efflux pumps (9, 21). These alterations may explain the poor activities of antifungals that target ergosterol, such as fluconazole and amphotericin. Unlike these agents, the echinocandins, such as caspofungin and micafungin, target the cell walls of Candida species through inhibition of β-1,3-glucan synthase (32). Caspofungin has been shown to be more active than amphotericin B and fluconazole against Candida albicans biofilm (3, 13). However, cell wall integrity pathways and glucan-associated changes can also occur, which could limit echinocandin activity against Candida biofilm (9, 23). Flucytosine, which inhibits DNA synthesis, has also been shown to be more active than fluconazole against Candida albicans biofilm (1). As a result, the use of combination antifungals may improve the management of endocarditis and prevent emergence of resistance associated with monotherapy.

The effectiveness of combination antifungal therapy for Candida endocarditis is difficult to assess clinically. To date, no clinical trial has been performed with patients with Candida endocarditis. The most recent infective endocarditis guidelines highlight no new developments in the management of fungal endocarditis over the past 2 decades (4). Animal models provide valuable data but are not always necessary for initial assessment. Replication of antifungal pharmacokinetics to mimic the human profile cannot be accomplished easily with animal models. Also, evaluation of the interaction of antifungals requires large sample sizes and can lead to unnecessary animal testing with limited information gain. In contrast, in vitro models provide an alternative, more rapid, and controlled environment for the assessment of antifungal combinations. Identification of an optimal antifungal combination in an in vitro model of endocarditis can support more focused animal experiments. This approach permits conformance with the ethical framework of reducing, refining, and replacing animal use (11).

We determined the activities of flucytosine, micafungin, and voriconazole as single agents and in combinations against Candida species, using an in vitro model of infective endocarditis.
This in vitro model simulated the serum pharmacokinetics of these antifungals against human platelet-fibrin clots that mimicked endocardial vegetations infected with one of four clinical Candida species isolates, namely, Candida albicans, Candida glabrata, Candida parapsilosis, and Candida tropicalis. These isolates had various susceptibility profiles to voriconazole and micafungin and included a C. tropicalis isolate with a paradoxical resistance profile to micafungin (25). The purpose of this study was to determine the optimal antifungal regimen associated with a reduction in fungal burden. We also determined the effects of these antifungal agents on the ultrastructural features of these simulated endocardial vegetations (SEVs).

**MATERIALS AND METHODS**

Organisms. Four Candida sp. bloodstream isolates collected from the University of New Mexico Hospital were used for these experiments. These isolates, one each of C. albicans, C. glabrata, C. parapsilosis, and C. tropicalis, were chosen based on their susceptibility profiles for voriconazole and micafungin. Antifungal susceptibility testing was performed using the CLSI M27-A2 broth microdilution methodology (22). Stock solutions of micafungin (10 mg/ml) and fluconazole (10 mg/ml) were prepared using sterile saline, while voriconazole (1 mg/ml) was prepared using dimethyl sulfoxide. All four isolates were susceptible to fluconazole, with a MIC of 0.125 μg/ml. The C. albicans and C. glabrata isolates had a micafungin MIC of 0.125 μg/ml, while the C. parapsilosis isolate had a MIC of >4.0 μg/ml and C. tropicalis was inhibited at 0.125 μg/ml with regrowth at 1.0 μg/ml. All Candida spp. were susceptible to voriconazole (MIC = 0.015 μg/ml), with the exception of C. glabrata, which had a MIC of >4.0 μg/ml.

Antimicrobial agents and susceptibility testing. Fluconazole was purchased from Sigma Chemicals (St. Louis, MO). Micafungin (Mycamine) was purchased through the University of New Mexico Hospital Pharmacy. Voriconazole was kindly donated by Pfizer Inc. (New York, NY). Susceptibility testing was performed in duplicate (broth microdilution), using the CLSI-approved standard reference methodology for broth dilution antifungal susceptibility testing of yeasts (22).

Preparation of SEVs. A single colony of Candida obtained from a 24-h culture on Sabouraud dextrose agar (Colestarch, Vernon Hills, IL) was grown in yeast nitrogen base (YNB) medium (Difco Laboratories, Detroit, MI) supplemented with 2% dextrose at 27°C for 24 h. Fibrin clots were prepared from sterile siliconized 1.5-ml tubes by combining 0.9 ml of human cryoprecipitate from volunteer donors (United Blood Services, Albuquerque, NM), 0.05 ml of aprotinin solution (Sigma, St. Louis, MO), and 0.05 ml of washed platelet-rich plasma suspension (~10^9 platelets/ml diluted in 0.9% NaCl to provide approximately 250,000 to 300,000 platelets per g of vegetation mass). A fungal suspension was added to the preparation to achieve a starting inoculum of ~10^7 CFU/g, which corresponded to fungal densities recovered initially from previous rabbit endocarditis models (6, 14, 33). A sterile monofilament line (Pacifica Inc., Taipei, Taiwan) was inserted, and 0.1 ml of bovine thrombin (Johnson Farms Inc., Bristol, VA) was added. The bovine thrombin (5,000 units) was reconstituted with 5 ml of sterile CaCl_2 (50 mmol) prior to addition of the 0.1-ml volume to the mixture. The resultant gelatinous mixture was removed from the Eppendorf tube by use of a sterilized 21-gauge needle.

In vitro endocarditis pharmacodynamic model. A one-compartment infection model (250 ml) was utilized in duplicate (19). The models were filled with YNB-2% dextrose, and both reservoirs contained YNB-2% dextrose to supplement the models. SEVs were suspended from four sampling ports sealed with a rubber stopper. The entire model was placed in a water bath and maintained at 37°C. At least two SEVs were hung in the model for each pharmacodynamic time point. This equated to hanging a total of 10 to 12 SEVs (three per port) to permit removal of two SEVs for each of the five time points. Each model was run without antifungal drug added to verify the proliferation potential of the Candida isolates. This procedure was repeated for each of the strains of Candida described in this study. A peristaltic pump supplied sterile fresh medium to and from the model at a half-life equal to 6 h. At this rate, the loss of Candida from the model was negligible, since the doubling time far exceeded the loss attributed to the pump settings. A magnetic stir bar was utilized to continuously mix the central compartment's medium. The experiments were conducted at 72 h. Fungal burdens were determined at six time points, i.e., at 0, 8, 24, 32, 48, and 72 h. At every time point, two vegetations were removed from each model, weighed, and placed in a 10-ml sterile test tube prefilled with normal saline. To homogenize the vegetation, a PowerGen 35 homogenizer (Fisher Scientific, Pittsburgh, PA) was used for 30 seconds. Cold 0.9% saline was used to dilute the homogenized vegetations, and 20 μl of each was plated in triplicate onto Sabouraud dextrose agar (Cole-Parmer, Vernon Hills, IL) and incubated for 24 h at 35°C, after which the colonies were counted visually.

The same model apparatus, using YNB-2% dextrose as the reservoir medium and SEVs as described above, was used to compare the single, dual, and triple combination activity of the aforementioned agents. All antifungal agents were administered as bolus doses. Fluconazole, voriconazole, and micafungin were administered to simulate (for a 70-kg patient) doses of 37.5 mg/kg orally every 12 h (q12h), 4 mg/kg orally q12h, and 150 mg intravenously (i.v.) q24h, respectively. This equated to maximum concentrations (half-lives) of 30 μg/ml (6 h), 4 μg/ml (6 h), and 15 μg/ml (24 h) for fluconazole, voriconazole, and micafungin, respectively. This experiment included assessment of the total concentrations, not free antifungal concentrations. This decision was based on the need to first determine the maximum potential activities of these agents. Peristaltic pumps were activated to mimic an elimination half-life of 6 h for fluconazole and voriconazole and of 12 h for micafungin. In models where micafungin was administered in combination with fluconazole and/or voriconazole, the models were supplemented with micafungin to account for the loss due to the shorter half-life. The supplemental amount of micafungin was ~23 μg/hour, achieved by adding the total 24-h supplemental amount into the input flow (0.56 mg). Two fibrin-platelet clots were removed from each model at each time point (0, 8, 24, 32, 48, and 72 h) and handled as described above. The log_{10} CFU/g over time were also compared between the different regimens tested and growth controls.

**Antifungal carryover.** The effects of antifungals on enumeration of Candida colony counts were studied for each of the isolates with all three antifungal agents. The SEV was prepared and homogenized as described above. The homogenization occurred in saline containing the expected peak or trough concentration of the respective antifungal. One hundred microliters of the suspension was added to 900 μl of saline, and 5 μl, 10 μl, or 20 μl was plated on Sabouraud dextrose agar in triplicate. A reduction in the mean CFU/ml of >25% compared to the control was defined as significant antifungal carryover.

**Antifungal pharmacokinetic validation.** Validation of the concentration-time profile of each individual antifungal agent was performed against each Candida species SEV model in duplicate, using bioassay methodologies. A 2-ml aliquot was aspirated from the model at time zero and 0.5, 2.0, 4.0, 8.0, and 24 h after the last dose of the agent. The samples were passed through a 0.2-μm syringe filter device and stored frozen at −80°C for batch analysis. The analyses of fluconazole

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**TABLE 1. Comparison of time-kill curve parameters for fluconazole, voriconazole, and micafungin combinations against C. albicans**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ABTKC (log_{10} CFU · h/g)</th>
<th>AURKC (unitless)</th>
<th>K_{max} (h⁻¹)</th>
<th>T_{max} (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFC</td>
<td>322.9 ± 41.1*</td>
<td>−5.73 ± 1.41</td>
<td>−0.46 ± 0.098</td>
<td>8 ± 0</td>
</tr>
<tr>
<td>Vor</td>
<td>23.1 ± 31.4**</td>
<td>1.67 ± 0.53**</td>
<td>−0.04 ± 0.01**</td>
<td>16 ± 9</td>
</tr>
<tr>
<td>Mica</td>
<td>252.3 ± 37.6†</td>
<td>−5.49 ± 1.04</td>
<td>−0.23 ± 0.1</td>
<td>28 ± 5†</td>
</tr>
<tr>
<td>SFC + Vor</td>
<td>338.5 ± 10.6</td>
<td>−5.04 ± 1.02</td>
<td>−0.27 ± 0.02</td>
<td>20 ± 8</td>
</tr>
<tr>
<td>Vor + Mica</td>
<td>257.7 ± 29.0</td>
<td>−4.16 ± 0.89</td>
<td>−0.17 ± 0.02</td>
<td>24 ± 11</td>
</tr>
<tr>
<td>SFC + Mica</td>
<td>372.5 ± 2.3</td>
<td>−6.54 ± 0.18</td>
<td>−0.44 ± 0.01</td>
<td>8 ± 0</td>
</tr>
<tr>
<td>SFC + Vor + Mica</td>
<td>352.1 ± 17.63</td>
<td>−5.83 ± 0.94</td>
<td>−0.41 ± 0.01</td>
<td>8 ± 0</td>
</tr>
</tbody>
</table>

* Data are means ± SD (n = 4). SFC, fluconazole; Vor, voriconazole; and Mica, micafungin. Significant comparisons (P < 0.007) are marked as follows: *, SFC > Vor or Mica alone; **, Vor < all treatments; †, Mica < SFC + Vor, SFC + Mica, or SFC + Mica + Vor; ††, SFC + Mica + Vor > Vor + Mica; ‡, SFC > Vor, Mica, SFC + Vor, or Vor + Mica; §, Mica > 5FC; SFC + Mica, or SFC + Mica + Vor.

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and voriconazole were performed using previously validated bioassay methods (28, 29). Micafungin was analyzed using a bioassay methodology validated against caspofungin (16). Briefly, fluconosine concentrations were determined using Saccharomyces cerevisiae (ATCC 9763) (29). Voriconazole concentrations were determined using Candida kefyr (ATCC 46764) (28). Quantiﬁcation of micafungin was performed using a clinical isolate (CIMR 93-27; kindly provided by David A. Stevens, San Jose, CA) (16). Briefly, the bioassay method included preparation of a 2.0 McFarland standard of the assay organism to inoculate agar. After the agar containing the organism solidiﬁed, a sterile cork borer was used to bore a 16-well (5-mm diameter) template on each plate. A standard curve was generated for each antifungal, using 20–200 μg/mL concentrations. A standard curve was generated over 72 h was translated into the area under the time-kill curve (AUTKC) by the Microsoft Equation Editor.

The approach of McFarland and colleagues was modiﬁed to assess these changes. Nine infected fibrin clot specimens (time points) were collected, as noted above, and included a growth control (0, 24, and 72 h) and each time point was tested in duplicate. Resistance was deﬁned as a >3-fold higher doubling dilution MIC at the 72-h time point compared to the 0-h MIC.

SEM. We previously demonstrated distinct morphological changes in infected fibrin clots with and without exposure to fluconosine and voriconazole (24). Consequently, ultrastructural characterization of all Candida sp.-infected platelet-fibrin clots was performed by using scanning electron microscopy (SEM) to assess these changes. Nine infected fibrin clot specimens (time points) were collected, as noted above, and included a growth control (0, 24, and 72 h) and fluconosine (24 and 72 h), voriconazole (24 and 72 h), and micafungin (24 and 72 h)-treated specimens for each strain. The specimens were ﬁxed with 4% glutaraldehyde solution, critical point dried after a series of ethanol and acetone dehydration steps, and then sputter coated with gold-palladium. Samples were imaged using a JEOL S900LV SEM and an Oxford Isis analytical system at the University of New Mexico’s Department of Earth and Planetary Sciences Electron Microprobe Facility.

Statistical analysis. We sought to evaluate the rate and extent of fungal burden reduction (17). The approach of McFarland and colleagues was modiﬁed to normalize treatment values to those of the control and to include the assumption that the rate of kill changes with time. The rate of kill (K) was normalized to the control as follows: K = (log10(tf) − log10(tc))/t, where tf and tc are the differences in CFU/mL for treatment (Tn − T0 = Tf − T0, Tn−1 − T0, etc.) and control (Cn − C0, Cn−1 − C0, Cn−2 − C0, etc.) for the speciﬁed time difference (Δt = t1 − t0, t2 − t0, t3 − t0, etc.). The value of K was assumed to be 0 for time zero, and K for each respective Δt value was plotted against the values for 8, 24, 32, 48, and 72 h. The area under the rate-of-kill curve (AURKC) was determined using the linear trapezoidal rule across a zero reference. The maximum rate of kill (Kmax), that is, the time to maximum rate of kill (Tt max) were determined based on graphical inspection. To measure the extent of kill, the change in log10 CFU/mL over 72 h was translated into the area under the time-kill curve (AUTKC) by the linear trapezoidal rule. The treatments were normalized by subtracting the treatment AUTKC from the control AUTKC to generate the area between the treatment and control time-kill curves (ABTKC).

RESULTS

In vitro endocarditis pharmacodynamic model. The data for each individual Candida species are summarized graphically in the ﬁgures and Tables 1 to 4. In general, the interaction of the triple antifungal combination was no better than the use of fluconosine alone. Given the complexity of the interactions that were noted, the following sections outline the effects of these agents based on species. Antifungal carryover was not demonstrated, so a 20-μL deposition volume for colony count enumeration was selected. The measured and expected concentration-time proﬁles were congruent for fluconosine and voriconazole but not for micafungin. The mean (± standard deviation [SD]) Cmax, half-life (t1/2), and AUC0–∞ for fluconosine and voriconazole were determined using the rate of kill (K) was normalized to the control as follows: K = (log10(tf) − log10(tc))/t, where tf and tc are the differences in CFU/mL for treatment (Tn − T0 = Tf − T0, Tn−1 − T0, etc.) and control (Cn − C0, Cn−1 − C0, Cn−2 − C0, etc.) for the speciﬁed time difference (Δt = t1 − t0, t2 − t0, t3 − t0, etc.). The value of K was assumed to be 0 for time zero, and K for each respective Δt value was plotted against the values for 8, 24, 32, 48, and 72 h. The area under the rate-of-kill curve (AURKC) was determined using the linear trapezoidal rule across a zero reference. The maximum rate of kill (Kmax), that is, the time to maximum rate of kill (Tt max) were determined based on graphical inspection. To measure the extent of kill, the change in log10 CFU/mL over 72 h was translated into the area under the time-kill curve (AUTKC) by the linear trapezoidal rule. The treatments were normalized by subtracting the treatment AUTKC from the control AUTKC to generate the area between the treatment and control time-kill curves (ABTKC). Individual clot data parameters were generated for each experimental condition (two replicates in duplicate), equating to four data points that were then averaged and whose standard deviation (SD) was calculated using Stata IC, version 10.0 (StataCorp LP, College Station, TX).

### Table 2. Comparison of time-kill curve parameters for fluconosine, voriconazole, and micafungin combinations against C. glabrata

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ABTKC (log10 CFU · h/g)</th>
<th>AURKC (unitless)</th>
<th>Kmax (h⁻¹)</th>
<th>Tmax (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FC</td>
<td>-8.24 ± 0.55*</td>
<td>-0.57 ± 0.03*</td>
<td>8 ± 0*</td>
<td></td>
</tr>
<tr>
<td>Vor</td>
<td>-2.99 ± 1.93*</td>
<td>-0.16 ± 0.03**</td>
<td>26 ± 4</td>
<td></td>
</tr>
<tr>
<td>Mica</td>
<td>-2.63 ± 2.65†</td>
<td>-0.34 ± 0.02</td>
<td>24 ± 0</td>
<td></td>
</tr>
<tr>
<td>5FC+Vor</td>
<td>-7.19 ± 1.34</td>
<td>-0.46 ± 0.10</td>
<td>8 ± 0</td>
<td></td>
</tr>
<tr>
<td>Vor+Mica</td>
<td>-6.59 ± 0.28</td>
<td>-0.31 ± 0.01</td>
<td>24 ± 0</td>
<td></td>
</tr>
<tr>
<td>5FC+Mica</td>
<td>-7.01 ± 0.55</td>
<td>-0.48 ± 0.10</td>
<td>8 ± 0</td>
<td></td>
</tr>
<tr>
<td>5FC+Mica+Vor</td>
<td>-8.18 ± 0.70</td>
<td>-0.54 ± 0.06†</td>
<td>8 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

* Data are means ± SD (n = 4). 5FC, fluconosine; Vor, voriconazole; and Mica, micafungin. Significant comparisons (P < 0.007) are marked as follows: *, 5FC > Vor or Mica alone; †, Vor or Mica alone < 5FC+Vor or 5FC+Mica, or 5FC+Mica+Vor; ‡, 5FC+Vor or > all treatments alone or in combination; ††, all treatment combinations > Vor or Mica alone; §, 5FC+Vor < all treatments except for 5FC+Mica+Vor.

### Table 3. Comparison of time-kill curve parameters for fluconosine, voriconazole, and micafungin combinations against C. parapsilosisa

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ABTKC (log10 CFU · h/g)</th>
<th>AURKC (unitless)</th>
<th>Kmax (h⁻¹)</th>
<th>Tmax (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5FC</td>
<td>283.2 ± 22.4*</td>
<td>-5.95 ± 0.94*</td>
<td>-0.29 ± 0.11</td>
<td>24 ± 0</td>
</tr>
<tr>
<td>Vor</td>
<td>152.7 ± 15.4**</td>
<td>-2.72 ± 0.24</td>
<td>-0.16 ± 0.01</td>
<td>24 ± 0</td>
</tr>
<tr>
<td>Mica</td>
<td>124.9 ± 17.0**</td>
<td>-2.59 ± 0.75††</td>
<td>-0.11 ± 0.03</td>
<td>30 ± 4</td>
</tr>
<tr>
<td>5FC+Vor</td>
<td>350.5 ± 3.5†</td>
<td>-8.16 ± 0.16†‡</td>
<td>-0.55 ± 0.02‡</td>
<td>8 ± 0£</td>
</tr>
<tr>
<td>Vor+Mica</td>
<td>168.6 ± 4.4</td>
<td>-4.75 ± 0.44</td>
<td>-0.15 ± 0.01</td>
<td>18 ± 12</td>
</tr>
<tr>
<td>5FC+Mica</td>
<td>263.1 ± 66.0</td>
<td>-5.36 ± 0.48</td>
<td>-0.20 ± 0.08</td>
<td>24 ± 0£</td>
</tr>
<tr>
<td>5FC+Mica+Vor</td>
<td>302.9 ± 9.9</td>
<td>-6.58 ± 0.62</td>
<td>-0.32 ± 0.03</td>
<td>12 ± 8</td>
</tr>
</tbody>
</table>

* Data are means ± SD (n = 4). 5FC, fluconosine; Vor, voriconazole; and Mica, micafungin. Significant comparisons (P < 0.007) are marked as follows: *, 5FC > Vor, Mica, Vor+Mica; †, Vor or Mica alone < 5FC+Vor, 5FC+Mica, or 5FC+Mica+Vor; ‡, Vor+Mica > all treatments alone or in combination; ††, all treatment combinations > Vor or Mica alone; §, 5FC+Vor < all treatments except for 5FC+Mica+Vor.
tosine were 30.8 ± 2.9 mg/liter, 8.13 ± 2.0 h, and 79.8 ± 35.0 mg · hour/liter, respectively. The mean (± SD) $C_{\text{max}}$, $t_{1/2}$, and $AUC_{\text{0-24}}$ for voriconazole were 2.2 ± 0.6 mg/liter, 9.2 ± 2.3 h, and 19.3 ± 3.7 mg · hour/liter, respectively. The bioassay methodology utilized to evaluate micafungin with isolate CIMR 93-27 could not reliably generate a standard curve, despite six attempts on different days. Consequently, we screened 10 clinical isolates of Candida species from our laboratory organism bank known to be highly susceptible to micafungin (MICs of <0.0075 μg/ml). We identified a C. tropicalis isolate that permitted generation of a standard curve based on zones of inhibition over a range of 0.125 to 8 μg/ml, with an $R^2$ value of >0.90 and intraday and interday coefficients of variation of <1% and <4.3%, respectively. The mean (± SD) $C_{\text{max}}$, $t_{1/2}$, and $AUC_{\text{0-24}}$ for micafungin were 7.3 ± 1.2 mg/liter, 9.6 ± 1.4 h, and 49.5 ± 19.5 mg · hour/liter, respectively. These values were approximately 50% lower than predicted and were likely caused by error during supplementation of the model with micafungin.

### Candida albicans SEVs

The time-kill and rate-of-kill curves for C. albicans are illustrated in Fig. 1 and 2, respectively. Voriconazole was no better than the growth control against C. albicans. Flucytosine demonstrated the greatest rate ($K_{\text{max}}$) and extent of kill compared to either voriconazole or micafungin ($P < 0.007$). The rate-of-kill curve demonstrates a pattern of kill and potential regrowth during the 72-h experimental timeframe that was not seen with micafungin but occurred with flucytosine. As a result, the AURKC for flucytosine did not reach statistical significance relative to those for other treatment groups (Table 1). The combination of voriconazole and micafungin was no better than micafungin alone. However, the triple combination of the agents was better than the combination of voriconazole and micafungin but similar to flucytosine alone. These quantitative results correlated well with the ultrastructural features of SEVs (Fig. 3). As illustrated, a dense biofilm was achieved within 24 h. Voriconazole did not prevent this from developing, while both flucytosine and micafungin arrested this development.

![Graph](http://aac.asm.org/)  
**FIG. 1.** Effects of antifungal combinations on mean ± SD log$_{10}$ CFU/g of Candida albicans SEVs over time. 5FC, flucytosine; Vor, voriconazole; Mica, micafungin.
Candida glabrata SEVs. The time-kill curve for C. glabrata is illustrated in Fig. 4. Voriconazole was least active against C. glabrata relative to all other treatments (Table 2). Flucytosine demonstrated an overall rate (AURKC), $K_{\text{max}}$, and $T_{\text{max}}$ that were superior to those of voriconazole or micafungin alone. Combination regimens were superior to voriconazole and micafungin alone in the rate and extent of fungal burden reduction. Flucytosine also demonstrated an overall rate (AURKC), $K_{\text{max}}$, and $T_{\text{max}}$ that were superior to those of voriconazole or micafungin alone. Combination regimens were superior to voriconazole and micafungin alone in the rate and extent of fungal burden reduction. Flucytosine also demonstrated an overall rate (AURKC), $K_{\text{max}}$, and $T_{\text{max}}$ that were superior to those of voriconazole or micafungin alone. Combination regimens were superior to voriconazole and micafungin alone in the rate and extent of fungal burden reduction.

Candida parapsilosis SEVs. The time-kill curve for C. parapsilosis is illustrated in Fig. 5. Flucytosine was superior (rate and extent) to micafungin and voriconazole alone, but the difference between micafungin and voriconazole did not reach statistical significance. All treatment combinations were superior to micafungin or voriconazole alone in terms of AURKC. The combination of flucytosine and voriconazole demonstrated a superior $K_{\text{max}}$ and $T_{\text{max}}$ to those of essentially all other groups. The ultrastructural features of the C. parapsilosis growth control were remarkable, as shown in a higher-magnification illustration (Fig. 6). As noted with C. glabrata, the surface of the SEV was extensively blistered within 24 h of the experiment. A closer review of the surface demonstrated multiple friable dense biofilm spheres that were 50 to 75 μm in diameter (Fig. 6). This phenomenon was inhibited by flucytosine, voriconazole, and micafungin use alone.

Candida tropicalis SEVs. The time-kill curve for C. tropicalis is illustrated in Fig. 7. As noted previously, voriconazole was inferior to all comparative treatments with respect to rate and extent of fungal burden. Flucytosine alone was not significantly better than micafungin by these parameters. In contrast, micafungin alone was superior to the combination of micafungin and voriconazole, suggesting a potential antagonistic interaction of this combination. The $K_{\text{max}}$ was generally lower with C. tropicalis than with the other species. Regardless, the flucytosine-associated $K_{\text{max}}$ was superior to those of all other treatments. The ultrastructural features of the C. tropicalis SEVs were similar to those generated with C. albicans. A distinct difference in the SEM data was that although voriconazole did not alter the fungal burden within SEVs, pseudohyphae and extensive biofilm formation were markedly reduced.

Resistance testing. Susceptibility testing of isolates recovered from the 72-h evaluation time point demonstrated MICs that were not more than one doubling dilution above the baseline value. The employed methodology did not detect emergence of antifungal resistance as a factor that rationalized failure of drug activity.

DISCUSSION

Ellis and colleagues completed the most extensive review of fungal endocarditis, based on an appraisal of over 220 case reports (10). Their analyses revealed that Candida species accounted for 48% of the cases and included isolation of C. albicans 50% of the time. The mortality rate was 72%, with recurrence in 30% of cases. The vast majority of patients were treated with amphotericin B (93%) and flucytosine (22%), with surgical intervention performed in 61% of treated cases. Most of the reviewed cases occurred prior to the introduction of the triazoles and echinocandins. However, a more recent review of the literature comparing treatments of C. parapsilosis and C.
albicans suggests increased use of alternative agents, such as fluconazole, voriconazole, and caspofungin (12).

Despite pharmacologic innovations, treatment guidelines for Candida endocarditis have not changed given the lack of well-controlled trials. The most recent guidelines from the American College of Cardiology and the American Heart Association provide no therapeutic strategy, with the exception of surgical intervention, for fungal endocarditis (5). Surgical removal of the infected valve is clearly the most effective management strategy to prevent embolic complications. However, not all patients qualify for surgery, and time to surgical intervention can be institution dependent. The low incidence and high morbidity and mortality associated with this disease demand alternative research strategies to improve current care. As a result, in vitro and animal models will remain the most relevant approach for studying novel therapeutic agents for fungal endocarditis.

Several antifungal agents with unique mechanisms of action currently exist in our armamentarium. Combination antifungal therapy provides a potential strategy to improve the prognosis of Candida endocarditis. Initial evaluation of antifungal combinations in an in vitro system is a means to eliminate potentially negative-effect combinations and to further study positive-effect ones. Echinocandins as a class have demonstrated the greatest potential activity against Candida species biofilms under in vitro conditions (3, 7, 13). Consequently, the current study tested the role of flucytosine, micafungin, and voriconazole as a combination regimen to manage Candida species-infected fibrin clots that simulated endocarditis.

Complex models of interaction have been tested to define synergy and antagonism of antifungal combinations (18). Assessment of antifungal combination interactions is clearly no simple matter, especially under dynamic concentration conditions. As a result, no standardized definitions for synergy and antagonism exist for in vitro pharmacodynamic model-based systems. The standard definition of synergy has been a change of $\geq 2 \log_{10}$ CFU/ml in the viable count at a specified time point, such as 24 h, in the presence of the combination relative to the count with the most effective single agent (17). This definition relies on the assumption that the concentrations of the agents are constant and restricts evaluation to a single time point. An alternative approach includes evaluation of fungal

FIG. 3. Representative scanning electron micrographs of SEVs of Candida albicans. (A) Control at 72 h with dense biofilm network; (B) markedly reduced number of organisms due to flucytosine at 24 h; (C) limited effect of voriconazole on organism load at 72 h; (D) markedly deformed cells after exposure to micafungin for 48 h.
burden at each time point. This approach leads to an increased probability of finding erroneous differences due to chance from the process of multiple comparisons. Ultimately, synergy and antagonism are in vitro concepts that are difficult to translate clinically. As such, the primary goal of combination antimicrobial testing includes finding a combination that is different (positive or negative) from the single agent. For this reason, we approached this problem by compressing the time-kill data into AUCs and tested to see if differences existed between treatments.

Our data suggest that voriconazole has limited activity against *C. albicans* and *C. tropicalis* SEVs relative to that against *C. parapsilosis* and *C. glabrata* SEVs. The susceptibility profiles of these isolates did not influence the activity of vori-
conazole. Voriconazole was less active than flucytosine against all *Candida* species tested. Similarly, voriconazole was less active than micafungin against all species except for *C. parapsilosis*. This result was consistent with the reduced activity of echinocandins, such as micafungin, against *C. parapsilosis* in both planktonic and biofilm states (7). In contrast, the potentially paradoxical resistance profile of *C. tropicalis* to micafungin did not noticeably influence micafungin activity.

The superior activity of flucytosine overwhelmed any potential positive-effect combinations that may have resulted through its interaction with voriconazole and micafungin. In select cases, superior and inferior interactions were identified.

**FIG. 6.** Scanning electron micrographs of 72-h-old growth control of *Candida parapsilosis*-infected fibrin clot demonstrating the friability (A) of the large biofilm pods (B).

**FIG. 7.** Effects of antifungal combinations on mean ± SD log_{10} CFU/g of *Candida tropicalis* SEVs over time. 5FC, flucytosine; Vor, voriconazole; Mica, micafungin.
The combination of fluconosine and voriconazole was superior to all other treatments against C. parapsilosis. In contrast, the combination of voriconazole and micafungin was inferior to micafungin alone for this isolate. Our approach of utilizing mean-kill-rate-over-time curves provides an improved perspective of the dynamics of growth and regrowth within in vitro models. Combination antifungals appeared to blunt regrowth, especially compared to the use of fluconosine, for C. albicans and C. glabrata.

As expected, several limitations exist within our model to limit the generalizability of these data. Only voriconazole concentrations were modeled appropriately, with observed concentration profiles that mimicked an expected dosage regimen of 3 to 4 mg/kg i.v. q12h (31). The micafungin concentration-time profile that was achieved mimicked a 50-mg i.v. q24h regimen instead of the proposed target of 150 mg (30). As a result, the effects of micafungin in our study are likely to underestimate true activity. In contrast, the concentration-time profile achieved with fluconosine was consistent with a patient population with chronic renal insufficiency (creatinine clearance = 30 to 50 ml/min) (8). The expected time-dependent pharmacodynamics of fluconosine would imply overestimation of its activity by our model. This is because patients with normal creatinine clearances would theoretically have shorter periods when the concentration would exceed the MIC (2). Despite these modeling limitations, the concentration-time profiles achieved in our pharmacodynamic model still represent data that can be expected in the clinical setting.

This model also utilized a simultaneous bolus input of these antifungals, which does not represent the clinical setting. Future exploration of staggered input may yield interesting results. For example, introduction of micafungin into the model around the expected T max (~8 h) of fluconosine activity may have enhanced the overall K max. Alternatively, the addition of a second agent 24 h after initial drug introduction may have prevented regrowth of resistant subpopulations. Although these ideas were not directly explored in our current model, the presented data support their further study. We did not investigate changes in resistant subpopulations over time in our model. Changes in these populations may have been identified better if we had placed aliquots from the model at specified time points on antifungal-containing agar (for example, agar containing 100 μg/ml fluconosine). We also did not study potential differences in adherence of Candida spp. to the platelet-fibrin clot and effects of turbulence generated by the magnetic stir bar on biofilm formation.

Despite the various limitations of the present model, our pilot work yielded useful information that can be applied by investigators interested in improving the pharmacological management of Candida endocarditis. We determined that the ultrastructural features of infected fibrin-platelet clots are Candida species dependent. Most striking were the biofilm structures produced by C. parapsilosis, which were consistent with clinical data reported 2 decades ago (15). The less dense structure of C. glabrata was entirely consistent with its lack of ability to form pseudohyphae compared to that of C. albicans and C. tropicalis. Importantly, structural features that may easily embolize could potentially be attenuated by antifungals despite not having an appreciable decline in fungal burden. Again, this aspect of analysis was beyond the scope of the present work but highlights potential avenues for understanding antimicrobial interactions beyond the constraints of measuring changes in microbial burden alone. Our work also supports the continued exploration of fluconosine and micafungin as potential therapeutic agents to manage Candida species-related endocarditis. We did not study the effects of amphotericin B in the current model. However, preliminary data from our laboratory suggest that micafungin demonstrates superior activity to liposomal amphotericin B and that the combination of the two agents is no better than micafungin alone against Candida albicans-infected fibrin clots (26; data not shown).

In summary, the present study evaluated the interactions of fluconosine, micafungin, and voriconazole in an in vitro model of Candida species endocarditis. We compared the rates and extents of fungal kill between single, dual, and triple combinations. Our modeled pharmacokinetic profiles mimicked typical doses of voriconazole but atypical doses of fluconosine and micafungin. We demonstrated that the ultrastructural features of Candida-infected fibrin clots varied greatly between species and were most unique for C. parapsilosis. Voriconazole was identified to be the least active agent in our model, while fluconosine had the greatest activity as a single agent. Micafungin was superior to voriconazole for all species except C. parapsilosis. Continued exploration of micafungin and fluconosine as a therapeutic strategy for Candida-related endocarditis is warranted.

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