Echinocandins are a preferred therapy for invasive candidiasis due to their potency and broad spectrum. Resistance, especially in Candida glabrata, is an emerging threat to their use. Pharmacodynamic (PD) studies examining reduced susceptibility secondary to fks mutations in C. glabrata are lacking. The current study explored PD targets for anidulafungin, caspofungin, and micafungin in an in vivo invasive candidiasis model against 11 C. glabrata isolates with known or putative fks mutations. The PD targets were compared to those of 8 wild-type (WT) isolates. The MIC ranges in the WT group were 0.03 to 0.25 mg/liter for anidulafungin, 0.03 to 0.25 mg/liter for caspofungin, and 0.01 to 0.06 mg/liter for micafungin. The MIC ranges for mutants were 0.06 to 4, 0.25 to 16, and 0.13 to 8 mg/liter for the same compounds, respectively. The mean free drug 24-h area under the concentration-time curve (AUCf)/MIC ratio associated with a stasis endpoint for the WT group was 13.2 for anidulafungin, 2.04 for caspofungin, and 6.78 for micafungin. Comparative values for mutants were 3.43, 2.67, and 0.90, respectively. Pharmacokinetic data from patients suggest that the C. glabrata PD targets needed for success in this model could be achieved based on MIC values of 0.25 mg/liter for anidulafungin, 2 mg/liter for caspofungin, and 0.5 mg/liter for micafungin. These values are higher than recently identified epidemiology cutoff values (ECVs). The results suggest that drug-specific MIC breakpoints could be increased for caspofungin and micafungin against C. glabrata and could include organisms with mutations in fks-1 and fks-2. While identification of genetic mutants is epidemiologically important, the phenotype (MIC) provides a better predictor of therapeutic efficacy.

Invasive fungal infections due to Candida glabrata are increasing in many parts of the world, including the United States (42). Intrinsic and acquired resistance to triazoles and polyenes limits therapeutic options (28, 45, 51). The advent of the echinocandin class was a major step in treatment of invasive candidiasis and is currently the first-line therapy, especially in situations for which C. glabrata is the infecting agent (12, 36). However, resistance to this class is an emerging problem for this Candida species, as detailed in recent case reports and surveillance studies (30, 40, 48, 52, 54, 56). Phenotypic and genetic analyses of these resistant strains have revealed that reduced susceptibilities to the echinocandin class are associated with mutations in fks-1 and/or fks-2 genes, which encode the active subunit of glucan synthase (24, 25, 37, 38).

Susceptibility breakpoints are continually reevaluated and adjusted as more in vitro susceptibility surveillance, in vivo animal model pharmacodynamics (PD) and, most importantly, patient outcome data become available. These advances and the availability of molecular techniques, such as the ability to genotype mutants with reduced susceptibility, have increased our ability to identify and study these isolates. Epidemiologically, the identification of these isolates is of importance for monitoring resistance development, which commonly occurs over the life span of an antimicrobial agent. However, clinical treatment decisions based on the presence or absence of a genetic mutation alone may lead to underutilization of an anti-infective agent in situations where the infection could still be successfully treated with adequate drug exposure. In this study, our aim was to compare PD targets in C. glabrata isolates with sequenced or putative fks-1 or fks-2 mutations to those with wild-type phenotypes. It is hoped that the results of these studies will be useful in the continued evaluation of susceptibility breakpoints and dosing regimen optimization for use of echinocandins in the treatment of invasive C. glabrata infections.

MATERIALS AND METHODS
Organisms. Nineteen clinical Candida glabrata isolates were used for the in vivo treatment studies. These isolates included 8 wild-type clinical isolates, 9 clinical isolates harboring fks-1 or fks-2 hot spot mutations, and 2 clinical isolates that had presumed fks mutations. These latter two organisms were included in the resistant group, based on isolation from patients with breakthrough infections while on echinocandin therapy and elevated MICs (see Table 1, below). The organisms were chosen to include isolates with relatively similar degrees of fitness in the animal model, as determined by the amount of growth in the kidneys of untreated animals over 96 h.

Antifungal agents. Anidulafungin, caspofungin, and micafungin were obtained from Pfizer, Merck, and Astellas, respectively, for in vitro susceptibility testing. Stock solutions were prepared for susceptibility testing as described in the Clinical and Laboratory Standards Institute (CLSI) standard M27-A3 (21). The same drugs were obtained from the University of Wisconsin Hospital and Clinics Pharmacy for in vivo treatment studies and prepared according to the manufacturers’ instructions.

In vitro susceptibility testing. All isolates were tested in accordance with the standards in CLSI document M27-A3 (21). Studies were per-
formed on three separate occasions in duplicate. Final results were expressed as the means of these replicates. Quality control was performed on each day of testing by using CLSI-recommended reference strains Candida krusei ATCC 6258 and Candida parapsilosis ATCC 22019.

**Animals.** Six-week-old ICR Swiss specific-pathogen-free female mice weighing 23 to 27 g were used for all studies. Animals were maintained in accordance with the American Association for Accreditation of Laboratory Care criteria. Animal studies were approved by the University of Wisconsin Animal Care Committee.

**Infection model.** A neutropenic, murine, disseminated candidiasis model was used for the treatment studies (2). Mice were rendered neutropenic (polymorphonuclear cell counts of <100 mm$^3$) by injecting cyclophosphamide subcutaneously 4 days before infection (150 mg/kg of body weight), 1 day before infection (100 mg/kg), and 2 days after infection (100 mg/kg). Previous investigations had demonstrated that this regimen produces neutropenia throughout a 96-h study period (2). Organisms produced neutropenia throughout a 96-h study period (2). Organisms that varied 4-fold from 0.02 to 80 mg/kg were administered in a 0.2-ml volume by intraperitoneal injection every 24 h for a 4-day period. Groups of three mice were used for each dosing regimen. At the end of the treatment period, mice were euthanized, and the kidneys were immediately processed for determination of the number of CFU as described above.

Dosing regimens were chosen to vary the magnitude of the 24-h area under the concentration-time curve (AUC)/MIC index and to attempt to produce a range of efficacies from no effect to maximal effect. Dose levels that varied 4-fold from 0.02 to 80 mg/kg were administered in a 0.2-ml volume by intraperitoneal injection every 24 h for a 4-day period. Groups of three mice were used for each dosing regimen. At the end of the treatment period, mice were euthanized, and the kidneys were immediately processed for determination of the number of CFU as described above.

The dose-response results were analyzed using a sigmoid dose-effect model. To compare the in vivo potencies of each drug against various C. glabrata strains, we determined the dose level required to produce a net static effect (no change in organism burden compared to the start of therapy) and a 1-log kill (relative to the burden at the start of therapy), as previously described (5). The echinocandin exposure associated with each treatment endpoint was calculated from the following formula: $\log_{10} D = \log_{10} (E/ED_{50}) - \log_{10} (ED_{50})/N$. Where D is the drug dose, E is the growth in untreated control mice, ED$_{50}$ is the maximal effect, N is the slope of the dose-response relationship, and the ED$_{50}$ is the dose needed to achieve 50% of the maximal effect. ED$_{50}$ was constrained when the measured ED$_{max}$ was lower than that predicted. The significance of differences in PD target endpoints was determined by using analysis of variance (ANOVA) on ranks for comparison within each organism group and among the three drugs, and the Mann-Whitney U test was used for comparisons between targets for mutant organisms versus the wild type for each drug.

**RESULTS**

**In vitro susceptibility testing.** The MICs of anidulafungin, caspofungin, and micafungin for the 19 strains of C. glabrata are shown in Table 1. The MICs were determined using the CLSI broth microdilution method (2, 13). The MICs were expressed as the minimum concentration of drug required to inhibit visible growth of the organism. The MICs were determined in triplicate.

**Pharmacodynamic target determination.** The 19 C. glabrata strains described above were used for in vivo treatment studies with each of the three echinocandins. Infection in mice was produced as described above. Dosing regimens were chosen to vary the magnitude of the 24-h area under the concentration-time curve (AUC)/MIC index and to attempt to produce a range of efficacies from no effect to maximal effect. Dose levels that varied 4-fold from 0.02 to 80 mg/kg were administered in a 0.2-ml volume by intraperitoneal injection every 24 h for a 4-day period. Groups of three mice were used for each dosing regimen. At the end of the treatment period, mice were euthanized, and the kidneys were immediately processed for determination of the number of CFU as described above.

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in Table 1. The MIC ranges for the wild-type organisms against anidulafungin, caspofungin, and micafungin were 0.03 to 0.25, 0.03 to 0.25, and 0.01 to 0.06 mg/liter, respectively. The median MICs against each of the three drugs for this organism group were 0.06, 0.03, and 0.02 mg/liter, respectively. The MIC ranges for the fks
mutant group against anidulafungin, caspofungin, and micafungin were 0.06 to 4, 0.25 to 16, and 0.13 to 8 mg/liter, respectively. The median MICs for the mutant group with the three drugs were 1, 1, and 0.25 mg/liter, respectively. Interestingly, mutations at the same location, and even those that led to the same amino acid substitution (e.g., fks2_HS1_S663P), produced different MIC values.

Pharmacokinetics. The pharmacokinetics of anidulafungin, caspofungin, and micafungin in this in vivo model have been previously described (5, 6, 11). Briefly, after administration of single intraperitoneal doses of 5, 20, or 80 mg/kg, the AUC from time zero to the end (AUC0-∞) ranged from 96 to 1,975 µg · h/ml for anidulafungin, 164 to 667 µg · h/ml for caspofungin, and 135 to 1,400 µg · h/ml for micafungin. The AUC values were linear (R² = 0.98 to 0.99) over the dose range studied.

Pharmacodynamic target determinations. At the start of therapy, mice had 3.95 ± 0.35 log10 CFU/kidney. In untreated controls, the burden increased by 2.30 ± 0.60 log10 CFU/kidney. There were no significant differences in the burdens at the start of therapy or for growth in control mice between the two organism groups. The dose-response curves on a milligram per kilogram basis for the two groups of study organisms and each echinocandin are shown in Fig. 1, 2, and 3. In general, higher doses of a drug were required to achieve a similar effect for the strains with fks mutations than for wild-type strains.

The 24-h AUC/MIC was used as the pharmacodynamic index for further exploration of the exposure-response relationships for each drug against the two groups of organisms (Fig. 4; Tables 2 and 3). Both total and free drug concentrations were considered, and free drug values are reported in the tables and figures. The AUC/MIC exposure-response relationships were similar for the reference and mutant strain groups.

The amounts of drugs required to achieve a static effect and 1-log kill are reported for wild-type and fks mutant isolates in Tables 2 and 3, respectively. A stasis endpoint was achieved for each drug and organism combination in the wild-type group. However, this level of treatment efficacy was not observed for all organisms in the mutant group. Organisms with a MIC of ≥1 mg/liter for anidulafungin, ≥4 mg/liter for caspofungin, or ≥2 mg/liter for micafungin did not achieve a stasis endpoint. In the wild-type group, the mean 24-h static doses were 3.96, 0.09, and

FIG 1 Dose-response relationships for anidulafungin against multiple C. glabrata isolates, including the wild type and fks mutants. Each point represents the mean burden of infection in the kidneys of three neutropenic mice. The dashed line represents the burden of organisms at the start of therapy. Points above the line represent organism growth, whereas points below the line represent organism death (i.e., fungicidal activity).

FIG 2 Dose-response relationships for caspofungin against multiple C. glabrata isolates, including the wild type and fks mutants. Each point represents the mean burden of infection in the kidneys of three neutropenic mice. The dashed line represents the burden of organisms at the start of therapy. Points above the line represent organism growth, whereas points below the line represent organism death (i.e., fungicidal activity).

FIG 3 Dose-response relationships for micafungin against multiple C. glabrata isolates, including the wild type and fks mutants. Each point represents the mean burden of infection in the kidneys of three neutropenic mice. The dashed line represents burden of organisms at the start of therapy. Points above the line represent organism growth, whereas points below the line represent organism death (i.e., fungicidal activity).
1.58 mg/kg for anidulafungin, caspofungin, and micafungin, respectively. The mean 24-h static dose free drug AUC/MIC ratios for this organism group were 13.2, 2.04, and 6.78, respectively. The AUC/MIC difference between anidulafungin and caspofungin reached statistical significance, with a P value of 0.003 by Kruskal-Wallis ANOVA on ranks. In comparison, for the mutant group the mean 24-h static dose for anidulafungin, caspofungin, and micafungin were 11.3, 4.01, and 3.22 mg/kg, respectively. These values correspond to 24-h free drug AUC/MICs of 3.43, 2.67, and 0.90, respectively. These AUC/MIC targets were not statistically different. It is noteworthy that the 24-h free drug AUC/MIC targets for anidulafungin and micafungin were lower in the mutant group than for the wild type. These differences were statistically significant for both drugs (Mann-Whitney U test, $P < 0.05$).

One-log kill endpoints were achieved for all but 3 of the wild-type strains for each antifungal (Table 2). For the fks mutant group, a 1-log kill endpoint was achieved most commonly following therapy with caspofungin (7 of 10 strains) or micafungin (6 of 10), whereas for anidulafungin this effect was only observed against 2 mutant strains. The mean 24-h doses associated with a 1-log reduction in organism burden in the wild-type group were 5.78, 0.35 and 3.40 mg/kg for anidulafungin, caspofungin, and micafungin, respectively. The corresponding 24-h free drug AUC/MIC ratios were 26.6, 4.71, and 14.9 mg · h/liter, respectively. In comparison, the mean 24-h 1-log kill doses in the mutant group for anidulafungin, caspofungin, and micafungin were 14.4, 15.4, and 7.64 mg/kg, respectively (2.25- to 44-fold higher than that needed in the treatment against the wild-type group). These values correspond to 24-h free drug AUC/MIC ratios of 8.87, 8.21, and 1.91 mg · h/liter, respectively. For the two organism groups, there was not a significant difference between the caspofungin and micafungin 24-h 1-log kill free drug AUC/MIC targets. A statistical comparison between caspofungin or micafungin and anidulafungin for this endpoint was not undertaken due to the small number of endpoints achieved in the fks group for anidulafungin ($n = 2$).

In comparing the wild-type and mutant group for each drug, the 1-log kill PD target endpoint was numerically higher in the mutant group for caspofungin, but the difference was not statistically significant ($P = 0.694$). In contrast, the AUC/MIC PD target was statistically lower in the mutant group for micafungin ($P = 0.009$). A statistical comparison between the wild-type and mutant group for this endpoint for anidulafungin was not undertaken due to the small number of endpoints achieved in the fks group for anidulafungin ($n = 2$).

**DISCUSSION**

*Candida* species are the fourth most common nosocomial bloodstream pathogen, the most common cause of systemic fungal infection, and have the highest mortality rate among nosocomial pathogens (42). *Candida glabrata* infections have risen markedly since the 1990s, and in most areas of the United States this species is now responsible for 20 to 40% of cases of invasive candidiasis (42, 43). The addition of the echinocandin class was a major step forward in treating invasive candidiasis and in particular those infections due to *C. glabrata*. However, as with all anti-infective therapies, emergence of resistance has begun to occur. For the echinocandin class, this was noted first for the species *C. glabrata* (16, 18, 20, 22, 24, 25, 29–31, 41, 47, 48, 54, 56). Elegant studies have delineated specific mutations within the fks genes that are associated with this resistance phenotype (24, 25, 37, 38). Inter-
Interestingly, the phenotypes associated with these mutations have varied (13, 15). For example, some mutant strains exhibit an in vivo fitness cost relative to reference strains. All mutants are associated with an elevation in MICs; however, the relative changes in MICs compared to wild-type strains have been shown to differ widely. The development of multidrug resistance, specifically to fluconazole and echinocandin, in C. glabrata has recently been documented and poses a serious threat to antimicrobial therapy options (40, 46). For example, examination of the SENTRY Antimicrobial Surveillance Program data and the CDC Prevention Population-Based Surveillance data found coresistance (to fluconazole and echinocandin) in 10% of fluconazole-resistant C. glabrata clinical isolates from the years 2006 to 2010. While this remains a small percentage of the total C. glabrata population (<2%), this phenotype was not detected in an earlier study period from 2001 to 2004 (40).

Identification of these genetic changes, which can be associated with elevated MICs, is of critical importance as they are potential sentinels of treatment failures for patients. Indeed, in many cases these changes have been linked to echinocandin treatment failures (18, 20, 22, 24, 30, 48, 52, 54). The lowest MIC values associated with these genetic changes have been used recently in the determination of epidemiological cutoff values (ECV) to help laboratories identify strains harboring resistance mutations (39, 44). However, since the change in the MIC associated with fks mutations can vary significantly, it will be important to discern the in vivo treatment and ultimate clinical impact of these mutations and MIC changes.

### Table 2 In vivo activities of anidulafungin, caspofungin, and micafungin against wild-type C. glabrata isolates in a neutropenic murine disseminated candidiasis model

<table>
<thead>
<tr>
<th>Antifungal agent and C. glabrata isolate no.</th>
<th>MIC (µg/ml)</th>
<th>Static dose (mg/kg/24 h)</th>
<th>1-log kill (mg/kg/24 h)</th>
<th>AUCI/MIC based on:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anidulafungin</td>
<td></td>
<td></td>
<td></td>
<td>Static dose 1-log kill</td>
</tr>
<tr>
<td>CG 570</td>
<td>0.03</td>
<td>1.97</td>
<td>3.72</td>
<td>12.6</td>
</tr>
<tr>
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<td>3.57</td>
<td>11.8</td>
<td>11.4</td>
</tr>
<tr>
<td>CG 513</td>
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<td>2.45</td>
<td>4.13</td>
<td>7.83</td>
</tr>
<tr>
<td>CG 33609</td>
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<td>2.00</td>
<td>4.21</td>
<td>12.6</td>
</tr>
<tr>
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<td>1.58</td>
<td>3.31</td>
<td>5.00</td>
</tr>
<tr>
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<td>6.40</td>
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</tr>
<tr>
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<td>7.50</td>
<td>40.4</td>
</tr>
<tr>
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<td>6.90</td>
<td>NA</td>
<td>11.0</td>
</tr>
<tr>
<td>Mean</td>
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<td>3.96</td>
<td>5.78</td>
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</tr>
<tr>
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<td>3.01</td>
<td>4.17</td>
<td>11.2</td>
</tr>
<tr>
<td>SD</td>
<td>0.07</td>
<td>2.35</td>
<td>3.31</td>
<td>11.4</td>
</tr>
<tr>
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<td></td>
<td>Static dose 1-log kill</td>
</tr>
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<td>0.17</td>
<td>0.21</td>
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</tr>
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<td>0.14</td>
<td>2.62</td>
</tr>
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</tr>
<tr>
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<td>0.00</td>
<td>0.02</td>
<td>0.10</td>
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<tr>
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<tr>
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</tr>
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<td>Static dose 1-log kill</td>
</tr>
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<td>0.02</td>
<td>0.95</td>
<td>1.61</td>
<td>7.54</td>
</tr>
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</table>

* a Statistically significant comparisons included the anidulafungin versus caspofungin static dose free drug AUC/MIC and the 1-log kill free drug AUC/MIC P = 0.003 and 0.004, respectively.

* b NA, pharmacodynamic endpoint was not achieved.
The current investigation was designed to address the in vivo impact of these changes for echinocandin therapy, by using an experimental in vivo model that had been previously shown to correlate with outcomes in patients (1, 10, 14). More specifically, the in vivo murine model and pharmacodynamic analyses of results were used to discern the impacts of echinocandin dose, MIC, and fks-1 or fks-2 mutation on treatment effect. These approaches have recently demonstrated utility in defining the clinical relevance of bacterial resistance mutations, design of susceptibility breakpoints, and optimal treatment strategies (4, 33, 34).

The treatment findings with this large set of organisms demonstrated that the outcome was closely linked to MIC. Infection with isolates exhibiting a higher echinocandin MIC required a larger dose for response or did not respond to the highest doses.
administered. However, the echinocandin AUC/MIC values associated with efficacy were relatively similar across the entire group of isolates. If anything, the AUC/MIC targets were somewhat lower for the strains harboring resistance mutations than for the wild-type strain group. The basis for this relatively small difference is not entirely clear but will be an important focus of future studies. One common hypothesis for explaining outcome differences in mutant strains is the presence of a fitness cost associated with drug resistance. This phenomenon has been described for strains harboring \( fks^{-1} \) mutations (15). However, we prescreened the isolates used in the current investigation for fitness, and thus this was less likely a major factor in the treatment effect differences.

The ability to translate preclinical animal model PD experiment results to patients is an important tool that has proven useful for studies of a number of infections (1, 3, 14, 23). Previous studies with \( \text{Candida} \) and triazoles showed that the PD target associated with successful outcome in animal models was similar to the PD target in patients with esophageal or invasive candidiasis who were successfully treated (7–10, 14, 19, 32, 49, 50, 53). Therefore, we attempted to extrapolate the current PD targets in this study to patients by considering echinocandin population pharmacokinetics, the PD target needed for efficacy in the current animal model study, and MICs in wild-type and mutant \( C. \ glabrata \) strains from surveillance studies. The approved steady-state dosing regimens for treatment of invasive candidiasis with these drugs include 100 mg/day for both anidulafungin and micafungin and 50 mg/day for caspofungin. These regimens produce free drug 24-h AUC values in healthy volunteers of 1.12 \( \text{mg} \) · h/ml for anidulafungin, 2.94 \( \text{mg} \) · h/liter for caspofungin, and 0.38 \( \text{mg} \) · h/ml for micafungin (17, 26, 27, 55). The pharmacokinetics for each echinocandin and the presented mean static dose pharmacodynamic target for \( C. \ glabrata \) isolates with \( fks \) mutations can be used to estimate the highest MIC for which efficacy is expected. On this basis, the highest MICs for which the PD target would be achieved is approximately 0.25 mg/liter for anidulafungin, 1 mg/liter for caspofungin, and 0.5 mg/liter for micafungin. The most recently reported EC\( _V \) values for \( C. \ glabrata \) are lower than these MIC values (caspofungin EC\( _V \), 0.12 mg/liter; micafungin EC\( _V \), 0.03 mg/liter). The current data suggest that the EC\( _V \) breakpoints for \( C. \ glabrata \) include organisms for which PK/PD estimates predict treatment success. Collection and analysis of clinical data will be important for further exploration of these questions.

In summary, the present investigation demonstrated that the relationship between drug exposure (the AUC) and the MIC provides the most sensitive predictor of therapeutic efficacy. More specifically, the PD target, whether it is the static dose or 1-log kill level, is similar in the presence or absence of genetic mutations in \( fks \). The results suggest that drug-specific MIC breakpoints could be increased for caspofungin and micafungin against \( C. \ glabrata \). Furthermore, given the relatively wide therapeutic indices of these drugs, study of higher doses of echinocandins may allow sufficient exposures for successful treatment of \( C. \ glabrata \) infections with even higher MICs.

REFERENCES


