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Role of FKS Mutations in *Candida glabrata*: MIC Values, Echinocandin Resistance, and Multidrug Resistance

Cau D. Pham, Naureen Iqbal, Carol B. Bolden, Randall J. Kuykendall, Lee H. Harrison, Monica M. Farley, William Schaffner, Zintars G. Beldavs, Tom M. Chiller, Benjamin J. Park, Angela A. Cleveland, Shawn R. Lockhart

*Candida glabrata* is the second leading cause of candidemia in U.S. hospitals. Current guidelines suggest that an echinocandin be used as the primary therapy for the treatment of *C. glabrata* disease due to the high rate of resistance to fluconazole. Recent case reports indicate that *C. glabrata* resistance to echinocandins may be increasing. We performed susceptibility testing on 1,380 isolates of *C. glabrata* collected between 2008 and 2013 from four U.S. cities, Atlanta, Baltimore, Knoxville, and Portland. Our analysis showed that 3.1%, 3.3%, and 3.6% of the isolates were resistant to anidulafungin, caspofungin, and micafungin, respectively. We screened 1,032 of these isolates, including all 77 that had either a resistant or intermediate MIC value with respect to at least one echinocandin, for mutations in the hot spot regions of *FKS1* and *FKS2*, the major mechanism of echinocandin resistance. Fifty-one isolates were identified with hot spot mutations, 16 in *FKS1* and 35 in *FKS2*. All of the isolates with an *FKS* mutation except one were resistant to at least one echinocandin by susceptibility testing. Of the isolates resistant to at least one echinocandin, 36% were also resistant to fluconazole. Echinocandin resistance among U.S. *C. glabrata* isolates is a concern, especially in light of the fact that one-third of those isolates may be multidrug resistant. Further monitoring of U.S. *C. glabrata* isolates for echinocandin resistance is warranted.

*Candida* species continue to be a leading cause of bloodstream infection in U.S. hospitals, especially in intensive care units (1, 2). Although the antifungal amphotericin is limited, there are good options for the treatment of *Candida* species, especially with the arrival of the newest antifungal agents, the echinocandins (3, 4). The echinocandins are intravenously administered agents with a favorable safety profile. As inhibitors of 1,3-β-D glucan synthase in the cell wall, they have a mechanism of action different from that of the older azole antifungals, which act to disrupt ergosterol (cell membrane) synthesis. This alternate mechanism of action allows the echinocandins to be effective against *Candida* isolates that are azole resistant. Early studies of *in vitro* susceptibility showed resistance to echinocandins to be extremely low for all *Candida* species (5, 6).

*Candida glabrata* has recently become the second-most-frequent cause of candidemia in the United States, surpassing *C. parapsilosis* and *C. tropicalis* (6–8). While the ultimate cause for this increase in the prevalence of *C. glabrata* is unknown, the increase might be related to *C. glabrata*’s higher incidence of resistance to fluconazole in comparison to most other *Candida* species (6–9). Because of the increased probability of fluconazole resistance, echinocandins are recommended as first-line therapy against *C. glabrata* (4). Alarming, *C. glabrata* is the first species of *Candida* for which measurable resistance to echinocandins has been detected (6, 10). Case reports of echinocandin-resistant *C. glabrata* following echinocandin therapy are becoming more common (11–17). The majority of these resistant isolates have specific mutations in one of two “hot spot” regions of the *FKS1* or *FKS2* genes, which encode a subunit of the 1,3-β-D glucan synthase protein, target of the echinocandins (11, 18–20).

The Clinical and Laboratory Standards Institute (CLSI) developed species-specific MIC breakpoints for the echinocandins against *C. glabrata* (21). These breakpoints were based upon pharmacokinetic/pharmacodynamic data, outcome data, MIC distribution, and the presence or absence of *FKS* mutations in the isolates (22). One of the primary concerns when the breakpoints were set was that the breakpoint for resistance should encompass the MIC values for as many of the isolates with *FKS* mutations as possible, thus identifying all non-wild-type isolates. This consideration lowered the breakpoints for *C. glabrata* to 1 to 2 dilutions lower than those for *C. albicans* and *C. tropicalis*. The drawback when setting the breakpoints was the paucity of outcome data for patients with non-wild-type isolates. During the clinical trials of the echinocandins, though a great deal of outcome data were generated for patients with susceptible isolates, there were almost no patients with resistant (as defined after the fact) isolates.

A general theme that is becoming clear through ongoing susceptibility testing of *C. glabrata* and the echinocandins is that not all *FKS* mutations are created equal (23–26). While some mutations such as *FKS2* S663P are associated with very high drug MIC values, others such as *FKS2* F559Y are associated with much lower drug MIC values (24, 25). In 2010, we published data on echinocandin resistance in population-based surveillance isolates from Atlanta, GA, and Baltimore, MD, prior to the official publication of the CLSI species-specific echinocandin breakpoint (24). Here, we expand on that report with 5 years of surveillance data and the addition of two surveillance sites, the tricounty Portland, OR,
metropolitan area and Knox County (Knoxville), TN, and surrounding counties. In this report, we identify many additional isolates with FKS mutations, compare the changes in MIC values caused by those mutations, and discuss the emergence of multi-drug-resistant (MDR) C. glabrata.

MATERIALS AND METHODS

Case and isolate definitions. Isolates were obtained from individuals with an incident episode of candidemia (defined below) identified between 1 March 2008 and 9 June 2013 for residents of metropolitan Atlanta, GA, between 1 June 2008 and 9 June 2013 for residents of Baltimore City or Baltimore County, MD, between 1 January 2011 and 9 June 2013 for residents of Knoxville (Knox County), TN, and surrounding counties, and between 1 January 2011 and 9 June 2013 for residents of the tricounty metropolitan area that includes Portland, OR. When available, isolates from non-catchment-area residents hospitalized in the catchment area were included. An incident episode was defined as in previous reports (6, 27). Institutional review board (IRB) approval or a waiver was obtained for all participating institutions.

Isolate storage and DNA extraction. All isolates were initially stored in glycerol at −70°C. Isolates were identified using a Luminex assay or DNA sequencing of the D1/D2 subunit of the 28S ribosomal DNA (rDNA) at the CDC as previously described (28). DNA was extracted using a MoBio microbial DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA) according to the manufacturer’s instructions.

Antifungal susceptibility testing. Antifungal susceptibility testing was performed with broth microdilution with anidulafungin, caspofungin, and micafungin as described in the Clinical and Laboratory Standards Institute (CLSI) M27-A3 document guidelines (42) using frozen RPMI microbroth trays custom manufactured with no indicator dye (Trek Diagnostics, Clevelad, OH). Results were read visually after 24 h, and the MIC was identified as the lowest concentration of drug that caused a significant decrease in growth compared to the control well results. Recently published CLSI M27-S4 (21) breakpoints were used. Isolates of C. glabrata with a drug MIC of 0.25 μg/ml were considered intermediate to caspofungin and anidulafungin, while those with a drug MIC of 0.12 μg/ml were considered intermediate to micafungin. Isolates of C. glabrata with a drug MIC of ≥0.5 μg/ml were considered resistant to caspofungin and anidulafungin, while those with a drug MIC of ≥0.25 μg/ml were considered resistant to micafungin. For fluconazole, a MIC of ≥64 μg/ml was considered to represent resistance.

PCR amplification and sequencing. Amplification of FKS1 and FKS2 HS1 and HS2 has been described previously (24). Hotspot mutations were detected using either a newly developed Luminex assay (29) or sequencing. Susceptible isolates were screened only for mutations in FKS1 HS1 and FKS2 HS1. All isolates with at least one drug MIC value in the intermediate or resistant range were sequenced at all four hot spot regions. Sequencing of the PCR products was performed using a BigDye Terminator kit (Applied Biosystems, Foster City, CA). Sequences were analyzed using Sequencher 5.1 software (Gene Codes Corporation, Ann Arbor, MI).

RESULTS

A total of 1,380 C. glabrata bloodstream isolates (679 from GA, 580 from MD, 89 from TN, and 32 from OR) were collected between 2008 and 2013 (Table 1). Although the proportion remained relatively constant, the number of C. glabrata isolates collected yearly decreased over the study period, as did the overall number of Candida isolates. Susceptibility testing of anidulafungin, caspofungin, and micafungin was performed on all isolates. The majority of isolates were susceptible to all three echinocandins, but a substantial minority was in the intermediate (n = 19, 1.4%) or resistant (n = 58, 4.2%) range for at least one echinocandin (Table 2). The distribution of the 58 resistant isolates was reasonably consistent across the three echinocandins, with 43 (3.1%), 45 (3.3%), and 50 (3.6%) isolates being resistant to anidulafungin, caspofungin, and micafungin, respectively.

Identification of mutations in FKS genes. A total of 1,032 isolates were screened for mutations in FKS1 HS1 and FKS2 HS1: 77 isolates that had drug MIC values in the intermediate or resistant range (Table 3) and 955 isolates with drug MIC values in the susceptible range. In addition, all 77 isolates with drug MIC values in the intermediate or resistant range were screened for mutations in FKS1 HS2 and FKS2 HS2.

Fifteen isolates had mutations in FKS1 HS1: five unique mutations were detected, including 7 isolates with an S629P mutation, 6 isolates with an R631G mutation, 1 isolate with a D632V mutation, 1 isolate with a D632Y mutation, and 1 isolate with a mutation just outside the hot spot region at I634V. No mutations were detected in FKS1 HS2.

Thirty-five isolates had mutations in FKS2 HS1; seven unique mutations were detected, including 21 isolates with an S663P mutation, 5 isolates with an F659Y mutation, 3 isolates with D666V mutations, 2 isolates with a D667Y mutation, 3 isolates with a D667V mutation, 1 isolate with a D668G mutation, and 1 isolate with a D669G mutation.

TABLE 1 C. glabrata isolate collection

<table>
<thead>
<tr>
<th>City of origin</th>
<th>No. of isolates (percent resistant to an echinocandin, if any) in yr of isolation:</th>
<th>% resistant</th>
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<tr>
<td></td>
<td>2008</td>
<td>2009</td>
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<tr>
<td>Atlanta</td>
<td>128 (3.9)</td>
<td>140 (6.4)</td>
</tr>
<tr>
<td>Baltimore</td>
<td>99 (3.0)</td>
<td>144 (2.1)</td>
</tr>
<tr>
<td>Knoxville</td>
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<td>n/a</td>
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<tr>
<td>Portland</td>
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</table>

* Data represent total numbers of isolates and percent resistant to an echinocandin by year of isolation. n/a, not assessed.

TABLE 2 Distribution of MICs for 1,380 bloodstream isolates of C. glabrata

<table>
<thead>
<tr>
<th>Antifungal</th>
<th>0.008</th>
<th>0.015</th>
<th>0.03</th>
<th>0.06</th>
<th>0.12</th>
<th>0.25</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
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<tbody>
<tr>
<td>Anidulafungin</td>
<td>2</td>
<td>77</td>
<td>636 (1)</td>
<td>534 (2)</td>
<td>83 (1)</td>
<td>5 (3)</td>
<td>8 (7)</td>
<td>11 (10)</td>
<td>15 (14)</td>
<td>9 (9)</td>
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<tr>
<td>Caspofungin</td>
<td>0</td>
<td>7</td>
<td>398</td>
<td>772 (1)</td>
<td>141 (5)</td>
<td>17 (3)</td>
<td>9 (4)</td>
<td>8 (7)</td>
<td>6 (5)</td>
<td>3 (3)</td>
<td>3 (3)</td>
<td>16 (16)</td>
</tr>
<tr>
<td>Micafungin</td>
<td>110</td>
<td>939</td>
<td>260</td>
<td>13 (1)</td>
<td>8 (3)</td>
<td>16 (12)</td>
<td>7 (5)</td>
<td>7 (6)</td>
<td>13 (13)</td>
<td>7 (7)</td>
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<td>0</td>
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</tbody>
</table>

* 43 (3.1%), 45 (3.3%), and 50 (3.6%) isolates fell into the resistant range for anidulafungin, caspofungin, and micafungin, respectively.
<table>
<thead>
<tr>
<th>Anidula MIC (µg/ml)</th>
<th>Caspo MIC (µg/ml)</th>
<th>Mica MIC (µg/ml)</th>
<th>Flucon MIC (µg/ml)</th>
<th>Gene and hot spot region(s)</th>
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<td>0.125</td>
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(Continued on following page)
mutation, 2 isolates with an S663F mutation, 1 isolate with an F659S mutation, 1 isolate with a P667H mutation, and 2 isolates with a deletion at F658. All three of the isolates with the D666V mutation, 1 isolate with a P667H mutation, and 2 isolates with an S663F mutation, 1 isolate with an R631G mutation in the resistant range. FKS sequencing identified 47 with an FKS1 mutation and 1 with an FKS2 mutation in the resistant range.

No mutations were detected in FKS2 HS2. There was only a single isolate with an FKS mutation (FKS1, D632Y) for which none of the three echinocandins gave an MIC value in the resistant range, although the value for anidulafungin was in the intermediate range.

Using an FKS mutation as a marker of resistance, micafungin MIC values were the strongest predictor of isolates possessing mutations; 43/47 (91%) isolates with an FKS mutation had an MIC value in the resistant range. Anidulafungin (85% of isolates with an FKS mutation in the resistant range) and caspofungin (81% of isolates with an FKS mutation in the resistant range) MIC values were slightly less predictive of resistance due to an FKS mutation. Though there were six isolates with micafungin MIC values in the resistant range that did not have an FKS mutation and six isolates with caspofungin MIC values in the resistant range that did not have an FKS mutation, there were only two isolates with anidulafungin MIC values in the resistant range that did not have an FKS mutation. Two isolates with FKS mutations were identified as solely resistant to anidulafungin, and five isolates with FKS mutations (four with the mutations in FKS1 and one with the mutations in both FKS1 and FKS2) were identified as solely resistant to micafungin. Only one isolate resistant to all three echinocandins did not have an FKS mutation. Overall, 81% of isolates showing resistance to at least one echinocandin had an FKS mutation.

Detection of multidrug resistance. Because fluconazole is still frequently used to treat some C. glabrata infections, we also screened the entire collection of isolates for resistance to fluconazole (MIC ≥ 64 μg/ml). Of the 1,380 isolates, 142 (10.3%) were resistant to fluconazole. Of the fluconazole-resistant isolates, 21 (14.8%) were also resistant to at least one echinocandin (Table 3).

Conversely, 36.2% of the isolates that were resistant to an echinocandin were also resistant to fluconazole.

Treatment and outcome. Antifungal treatment and 30-day mortality data were available for 804 patients with C. glabrata candidemia. All-cause mortality for these patients was 42%. For those patients who were treated with an echinocandin only (n = 169), the all-cause mortality was slightly lower at 40%. For patients who did not receive any echinocandin as part of their therapy (n = 232), the all-cause mortality was 49%. The all-cause mortality for patients who received an echinocandin either exclusively or in addition to other antifungal therapy was lower at 33%. The difference in 30-day outcomes between those patients with a C. glabrata isolate who received an echinocandin either exclusively or in addition to other antifungal therapy and those who received no echinocandin was statistically significant (Fisher’s exact test, P < 0.001). The presence of an FKS mutation did not appear to affect patient survival; 22 (76%) patients with C. glabrata isolates with FKS mutations survived, and seven (24%) patients died within 30 days of the initial culture. Three of the seven who died were treated with an echinocandin only. Six of the 22 patients who survived their infections were treated with an echinocandin only, including two patients infected with isolates harboring the FKS2 S663P mutation and anidulafungin, caspofungin, and micafungin MIC values of 2.0, 2.0, and 0.5 μg/ml and of 2.0, 16, and 1.0 μg/ml, respectively. The remaining patients who survived C. glabrata candidemia and had an isolate with an FKS mutation were treated either with something other than an echinocandin (7 patients) or with an echinocandin and another antifungal.

DISCUSSION
With the clinical implementation of echinocandins in the decade beginning in 2000, the general belief was that resistance to this antifungal class would be rare. For the most part, that has been the case, with the overall susceptibility of all Candida isolates at
around 99% (6, 8, 30). While occasional case reports of echino-
candin resistance were published, these were initially infrequent.
In the United States, the rate of resistance of *C. glabrata* to the
echinocandins had started to increase into the 2% to 3% range by
2010 (10, 24, 30). Even more alarming, resistance to echinocan-
dins was higher among *C. glabrata* isolates already resistant to
fluconazole (23). This trend was cause for alarm and is an indica-
tion that there needs to be closer scrutiny of echinocandin resis-
tance in *C. glabrata* in the United States. To that end, we screened
almost 1,100 *C. glabrata* isolates for drug MIC values in the resis-
tant range and for FKS mutations that may be causing resistance.

Several important findings have come from this study.

The first is that, despite the fact that the number of *C. glabrata*
candidemia cases at our study sites has decreased, the level of
echinocandin resistance has remained steady or has increased.
The second is that elevated MIC values and echinocandin resis-
tance are caused by an increasing number of distinct mutations in
*C. glabrata*, primarily in the FKS1 and FKS2 genes. The third and
final significant finding is that multidrug resistance (MDR) among
*C. glabrata* isolates was high (1.5%) in our catchment area
and may be an indication of future problems with MDR *C.
glabrata*.

We used our surveillance data set to measure the proportion of
*C. glabrata* isolates resistant to echinocandins over the course of
our surveillance. For this exercise, we used all isolates received
at hospitals in the catchment area, not just isolates from patients
residing in the catchment area. While this does not allow us to
generate a population-based rate of resistance, it does provide a
surveillance profile of resistance detected in the catchment area
hospitals. In the hospitals in Atlanta, GA, echinocandin resistance
in *C. glabrata* increased between 2008 and 2013 (Table 1). While it
dropped considerably between 2009 and 2010, it steadily rose after
2010 to a proportion of over 10% in 2013, even though the number
of isolates received in 2013 dropped sharply. Although there
was a single large hospital with 30% of the resistant isolates, resis-
tant isolates were recovered from 11 different hospitals in Atlanta.
Similarly, in Baltimore, MD, echinocandin resistance in *C.
glabrata* peaked at 9.7% in 2011, fell to 5% in 2012, and remained
steady at 6% in 2013 despite a fairly sharp drop in the number of
isolates received. Two large hospitals accounted for 62% of the
resistant isolates, but resistant isolates were recovered from nine
different hospitals in Baltimore. Despite having fewer than 100
isolates over 3 years of surveillance, the proportion of echinocan-
din resistance in *C. glabrata* isolates from Knoxville, TN, was
3.4%. No echinocandin-resistant *C. glabrata* isolates were re-
ceived from Oregon among the 32 isolates received over 3 years.
These results are similar to those of a single-institution study
where the proportion of *C. glabrata* remained steady but echino-
candin resistance rose steadily over the 10-year period between
2001 and 2010 (26).

Among 47 isolates with FKS mutations, 12 unique mutations
were identified: five in FKS1 and seven in FKS2. All of these mu-
tations were in HS1; we did not detect any HS2 mutations. Among
the limitations of this study were that we did not search for FKS
mutations outside the HS region and that we did not look for HS2
mutations among any isolates that were not intermediate or resis-
tant to at least one echinocandin. Nevertheless, FKS mutations
were detected in 81% of the echinocandin-resistant isolates. In a
recent study by Alexander and coworkers (26), of the 25 patient
isolates with FKS mutations, only two of the mutations were in
HS2 (both I1379V); both isolates had drug MICs in the susceptible
range, and both patients were treated with an echinocandin and
recovered. Similarly, Castanheira and coworkers (25) identified
29 isolates with FKS mutations, and only one had an HS2 mutation.
This isolate (with a P1371S mutation) was susceptible to
micafungin and anidulafungin but had an intermediate caspofun-
gin MIC. It is reasonable to conclude that the majority of mu-
tations that affect *C. glabrata* susceptibility to the echinocandins
would be in the FKS1 and FKS2 HS1 regions, which should be the
primary targets of future assays developed to detect resistance
(29).

It is clear from the MIC data that not all FKS mutations cause
the same level of resistance. Within FKS1, mutations at S629 result
in higher drug MIC values than mutations at R631 or D632. Mu-
tations at R631 in most cases were reflected by higher micafungin
MICs but lower caspofungin and anidulafungin MICs (in the sus-
tceptible to intermediate range). The mutation D632V had resis-
tant MIC values for all three echinocandins, but the mutation
D632Y did not have resistant drug MIC values for any of the
echinocandins. S629P was similar to S663P in that it was associ-
ated with drug MIC values at either the low end or high end of the
resistant category (23, 24). For equivalent mutations (FKS1 S629P
is functionally equivalent to FKS2 S663P), the mutation in the
FKS2 gene was always associated with higher drug MIC values
than the mutation in the FKS1 gene. It is interesting that in our
collection, with the exception of S629P and S663P, FKS1 and
FKS2 equivalents were not seen. There were no FKS2 equiva-

cents for FKS1 R631 mutations, and there were no FKS1 equiv-

cents for FKS2 F659 mutations, even though both of these

mutations have been noted in other studies (25, 26).

As has been reported previously (23, 26), in our study, resist-
ance of *C. glabrata* to fluconazole increased the likelihood that the
isolate would be resistant to an echinocandin and vice versa. *Can-
dida glabrata* is capable of genomic changes, including point mu-
tations as well as changes in chromosome structure that may be
mechanisms of adaptation to changing environments (31, 32).
These genomic changes may be a coping mechanism that allows it
to rapidly become resistant to multiple drugs following limited
exposure (13, 17, 33–35). For MDR *C. glabrata* with resistance
to azoles and echinocandins, the only remaining available antifungal
is amphotericin B. This is especially alarming because *Candida
glabrata* carriage and infections increase as patients age (27, 36–
39) and because amphotericin B is often poorly tolerated in the
elderly.

Prior echinocandin use has been shown to be a risk factor for
failure of echinocandin therapy in *C. glabrata* (12, 16, 40). How-
ever, there is evidence that some patients with isolates that show in
vitro echinocandin resistance, even those harboring FKS mu-
tations, do respond to echinocandin therapy (15, 26). Additionally,
there is evidence from mouse infection models that there may be
differential effects of the echinocandins depending on which FKS
mutation is present (41). This is an indication that, as we see with
other infections, the attributes of the infecting organism are not
the sole determining factor for the successful completion of ther-
apy. In our study, we had three patients who were infected with *C.
glabrata* isolates that harbored FKS mutations (including two with
the S663P mutation) but survived even though their sole antifun-
gal therapy was an echinocandin. It is also important that the
all-cause 30-day mortality of our patients was considerably lower
when an echinocandin was used in a patient. We did not collect
information on whether the echinocandin was used as therapy or as part of surveillance were the incident isolates and additional isolates were not collected; it is not known if any of the isolates went on to develop echinocandin resistance following exposure.

Clearly, there is further work to be done to further our understanding of echinocandin use for the treatment of *C. glabrata*. There is a need for more dosage, duration, and outcome data, especially among patients harboring isolates with FKS mutations. The echinocandin breakpoints for *C. glabrata* were developed largely on the basis of differentiation between isolates with and without FKS mutations (22). Our data indicate that these breakpoints perform that function reasonably well. With limited options for treatment, it is important to determine whether echinocandins can be used for isolates that harbor FKS mutations but have drug MIC values at the lower end of the resistance range. With current advances in molecular detection and characterization of infecting species, direct assessment of FKS mutations should become part of the routine identification protocols for *C. glabrata* isolates in the clinical laboratory. Where it is practical, laboratories should consider routine MIC testing of *C. glabrata* isolates collected from sterile sites. Continued collection of *C. glabrata* and echinocandin treatment outcomes will allow more-informed determinations of the value of these drugs in the anti-fungal armamentarium.

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