ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Sept. 2010, p. 3630–3634 Vol. 54, No. 9
0066-4804/10/$12.00 doi:10.1128/AAC.00355-10

T-2307 Shows Efficacy in a Murine Model of *Candida glabrata* Infection despite *In Vitro* Trailing Growth Phenomena[▽]

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Received 15 March 2010/Returned for modification 6 April 2010/Accepted 4 June 2010

T-2307, a novel arylamidine, has been shown to exhibit broad-spectrum *in vitro* and *in vivo* antifungal activities against clinically significant pathogens. In our preliminary studies, *Candida glabrata* exhibited significant trailing growth (partial inhibition of growth over an extended range of antifungal concentrations) in the presence of T-2307 when it was tested using the Clinical and Laboratory Standards Institute (CLSI) guidelines with 0.2% glucose and 48 h of incubation, making reading of the MIC difficult. In the present study, we attempted to attenuate trailing growth to avoid misreading of the MIC. On the basis of the hypothesis that T-2307 may inhibit the mitochondrial functions of cells, the carbon source or the glucose concentration in the medium was changed. The trailing growth of *C. glabrata* ATCC 90030 in the presence of T-2307 was attenuated as the concentration of glucose in the medium decreased to 0.1% or lower, and trailing growth was completely inhibited when glycerol was used. A susceptibility test using Alamar blue was performed to facilitate reading of the MIC without changing the composition of the medium and provided a clear MIC endpoint at 24 h. To investigate if T-2307 shows efficacy against trailing isolates *in vivo*, we evaluated the efficacy of T-2307 in a murine model of disseminated candidiasis caused by *C. glabrata*. T-2307 at 0.05 mg/kg of body weight/day significantly decreased the viable count in the kidneys compared to that for the control group ($P < 0.05$). It would be better to test the susceptibility of *C. glabrata* to T-2307 using modified media or Alamar blue to avoid misreading of the MIC due to the significant trailing growth.

Invasive mycoses are serious life-threatening infections for immunocompromised patients. Fungal infections are primarily treated with azole derivatives, candin derivatives, or amphotericin B. Azole antifungal agents, such as fluconazole, itraconazole, and voriconazole, are now widely used for the treatment of fungal infections due to their broad-spectrum activities and improved safety profiles. However, the increased prevalence of *Candida glabrata* isolates that exhibit reduced susceptibility to triazole antifungals and *Candida krusei* isolates that have developed intrinsic resistance to fluconazole and itraconazole has heightened concerns regarding the empirical use of triazole-based drugs, especially in the case of patients at risk of systemic invasion (1, 8, 14, 22, 23, 24).

We have previously shown that T-2307 (Fig. 1), a novel arylamidine, exhibits broad-spectrum *in vitro* and *in vivo* activities against clinically significant pathogens, including *Candida* species, *Cryptococcus neoformans*, and *Aspergillus* species (13). In our preliminary studies, *C. glabrata* exhibited significant trailing growth (partial inhibition of growth over an extended range of antifungal concentrations) in the presence of T-2307 when it was tested according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI; formerly the National Committee for Clinical Laboratory Standards) (4), making reading of the MIC difficult. Isolates with trailing growth in the presence of azoles appear to be susceptible after 24 h but resistant at 48 h; and testing by other methods (including changes in pH or temperature), animal models, and clinical experience also revealed trailing isolates to be susceptible (3, 12, 16, 17). The reference method recommends reading of the MIC at 24 h for trailing isolates (4). In the present study, we attempted to attenuate trailing growth to avoid misreading of the MIC, and we investigated *in vivo* activity of T-2307 against *C. glabrata*.

(This study was presented at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 27 to 30 September 2006.)

MATERIALS AND METHODS

Antifungal agents. T-2307 was synthesized by Toyama Chemical Co., Ltd. (Toyama, Japan). Fluconazole solution for injection (2 mg/ml) was provided by Toyama Chemical Co., Ltd. Micafungin and amphotericin B were commercially obtained from Astellas Pharma Inc. (Tokyo, Japan) and Bristol-Myers KK (Tokyo, Japan), respectively.

Organisms. *C. glabrata* ATCC 90030 was obtained from the American Type Culture Collection (ATCC).

Antifungal susceptibility testing. The *in vitro* susceptibility of *C. glabrata* ATCC 90030 was determined by the broth microdilution method in accordance with CLSI guideline M27-A3 (4). RPMI 1640 medium (Sigma-Aldrich Co., St. Louis, MO) was buffered to pH 7.0 with 0.165 M 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (Dojindo Laboratories, Kumamoto, Japan). A stock inoculum suspension was obtained from a 24-h-old culture grown on a Sabouraud dextrose agar (SDA; Eiken Chemical Co., Ltd., Tokyo, Japan) plate at 35°C. The final inoculum concentration was 1×10^3 cells/ml. The MICs for T-2307, fluconazole, and micafungin were defined as the lowest concentration in which a prominent decrease in turbidity was observed; and the MIC for amphotericin B was defined as the lowest concentration in which no visible growth was observed. Percent inhibition was calculated on the basis of the growth in the control well after the optical density at 600 nm was measured using a microplate spectrophotometer to yield an inhibition curve. In an experiment where the carbon source was modified, RPMI 1640 medium without glucose was used; and it was supplemented with glucose or glycerol to yield final concentrations of 2%, 0.2% (equivalent to the concentration in the CLSI guidelines), 0.1%, 0.05%, and 0.02% glucose or 0.2% glycerol. The MICs were read after 48 h for all agents except micafungin; the MICs of micafungin were read after 24 h. The MIC of

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▽ Published ahead of print on 14 June 2010.

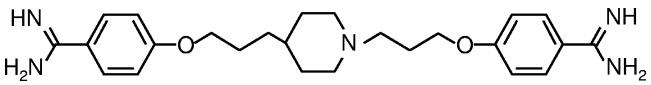

FIG. 1. Chemical structure of T-2307.

micafungin in the presence of 0.2% glycerol was read after 48 h because the level of growth was not enough at 24 h. In a colorimetric assay, 10% Alamar blue (Trek Diagnostic Systems, Inc., Westlake, OH) was added to RPMI 1640 medium (with 0.2% glucose and without phenol red). The MICs were read after 24 h and 48 h of incubation. The colorimetric MICs for T-2307, fluconazole, and micafungin were defined as the lowest concentration in which the color either changed from blue to purple or remained blue; the MIC for amphotericin B was defined as the lowest concentration in which the color remained blue (6).

***In vivo* study.** Four-week-old male specific-pathogen-free (SPF) ICR-strain mice (Japan SLC Inc., Shizuoka, Japan) were used for the *in vivo* study. The mice were provided with food and water *ad libitum*. All the experimental procedures with animals were conducted at Toyama Chemical Co., Ltd., in accordance with the guidelines for the care and use of laboratory animals. *C. glabrata* ATCC 90030 cells obtained from cultures grown overnight on SDA plates at 35°C were suspended in sterile saline. Disseminated candidiasis was induced in neutropenic mice by the intravenous inoculation of 0.2 ml of the cell suspension via the lateral tail vein. The immunosuppressive conditions were in accordance with those used in the method described by Ikeda et al., with slight modifications (7). Briefly, transient immunosuppression was induced by intraperitoneal treatment with 200 mg/kg of body weight cyclophosphamide (CY; Shionogi & Co., Ltd., Osaka, Japan) 3 days before the infection and with 100 mg/kg CY 1 day after the infection. The challenge dose was 7.68×10^6 CFU/mouse. Each group comprised five mice, and sterile saline was administered to the control mice. T-2307 and the reference agents were administered subcutaneously once a day for 8 days, beginning at 2 h after the infection. On day 8 postinfection, the kidneys of the euthanized mice were extirpated and homogenized in 2 ml saline using a 5-ml glass homogenizer. The homogenate was diluted 100- and 10,000-fold with saline, and 100 μl of each homogenate were spread on SDA plates containing 0.1 mg/ml of imipenem (Banyu Pharmaceutical Co., Ltd., Tokyo, Japan). After incubation at 35°C for 5 days, the colonies were counted, and the viable count in the kidneys (log number of CFU/kidneys) was calculated. The viable counts in the kidneys of the drug-treated groups and that of the control group were compared by the parametric Dunnett multiple-comparison test using SAS analytical software, release 8.2 (SAS Institute Japan Ltd., Tokyo, Japan). *P* values of <0.05 (two-tailed) were considered significant.

RESULTS

Antifungal susceptibility testing. Figure 2 shows the inhibitory activity, which was determined spectrophotometrically, of T-2307 against *C. glabrata* ATCC 90030 at 24 h and 48 h of incubation. At 48 h, the standard incubation time in the CLSI guidelines (4), *C. glabrata* exhibited significant trailing growth in the presence of T-2307 at concentrations between 0.0039 and 0.125 μg/ml. The trailing growth of *C. glabrata* in the presence of T-2307 at 24 h of incubation was similar to that at 48 h.

Figure 3 shows the effects of the glucose concentration and carbon source in the medium on the MIC determination, and Table 1 summarizes the MICs. The trailing growth of *C. glabrata* in the presence of T-2307 was attenuated as the concentration of glucose in the medium decreased to 0.1% or lower, and trailing growth was completely inhibited when glycerol was used. Similar behavior was observed for fluconazole. The inhibitory behavior of micafungin and amphotericin B was not affected by the glucose concentration or the carbon source. The optical densities at 600 nm of the growth control wells, measured using a microplate spectrophotometer, at 24 h/48 h were 0.65/0.73, 0.29/0.30, 0.17/0.19, 0.11/0.16, and 0.07/0.15 in the presence of 2%, 0.2%, 0.1%, 0.05%, and 0.02% glucose, respectively. The optical density of the growth control wells in the presence of 0.2% glycerol was 0.11 at 48 h. These values indicate that the turbidity in the growth control wells was enough for visual reading of the MIC.

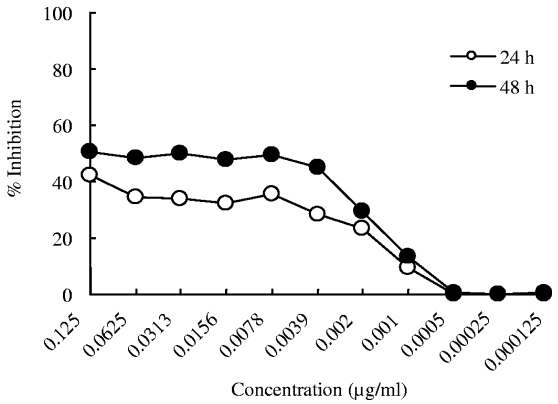

FIG. 2. Inhibitory effect of T-2307 against *C. glabrata* ATCC 90030 at 24 h and 48 h of incubation. The susceptibility of *C. glabrata* to T-2307 was tested in RPMI 1640 medium containing 0.2% glucose. Percent inhibition was calculated on the basis of the growth in the control well after the optical density at 600 nm was measured using a microplate spectrophotometer.

In a colorimetric assay, the susceptibility test using Alamar blue provided a clear MIC endpoint for T-2307 at 24 h; however, the MIC of T-2307 was above 0.125 μg/ml at 48 h (Fig. 4).

Similar results were obtained for 25 clinical isolates of *C. glabrata* under the same conditions described above (data not shown).

***In vivo* efficacy.** The therapeutic effect of T-2307 on disseminated candidiasis caused by *C. glabrata* is shown in Table 2. T-2307 at 0.05 mg/kg/day and micafungin and amphotericin B at 0.5 mg/kg/day each significantly reduced the viable count in the kidneys compared to that for the control group, whereas fluconazole at 10 mg/kg/day did not.

DISCUSSION

T-2307 is an aromatic diamidine derivative related to pentamidine, which is used to treat pneumocystosis, leishmaniasis, and trypanosomiasis. Recently, pafuramidine (DB289), an aromatic diamidine derivative and a prodrug of furamidine (DB75), has been demonstrated to have efficacy against African trypanosomiasis, *Pneumocystis jirovecii* pneumonia, and malaria (25). It also has been reported that *Saccharomyces cerevisiae* cells grown in a medium containing glycerol as the nonfermentative carbon source were more sensitive to the growth-inhibitory effects of DB75 or pentamidine than cells grown in a medium containing glucose, thus suggesting that DB75 and pentamidine inhibit the mitochondrial functions of cells (9, 11). This finding led us to believe that T-2307 may also inhibit the mitochondrial functions of cells and that the trailing growth of *C. glabrata* in the presence of T-2307 may be influenced by the carbon source or glucose concentration in the medium. The current study shows that the trailing growth of *C. glabrata* in the presence of T-2307 was attenuated as the concentration of glucose in the medium decreased to 0.1% or lower and that the trailing growth was completely inhibited when glycerol was used (Fig. 3).

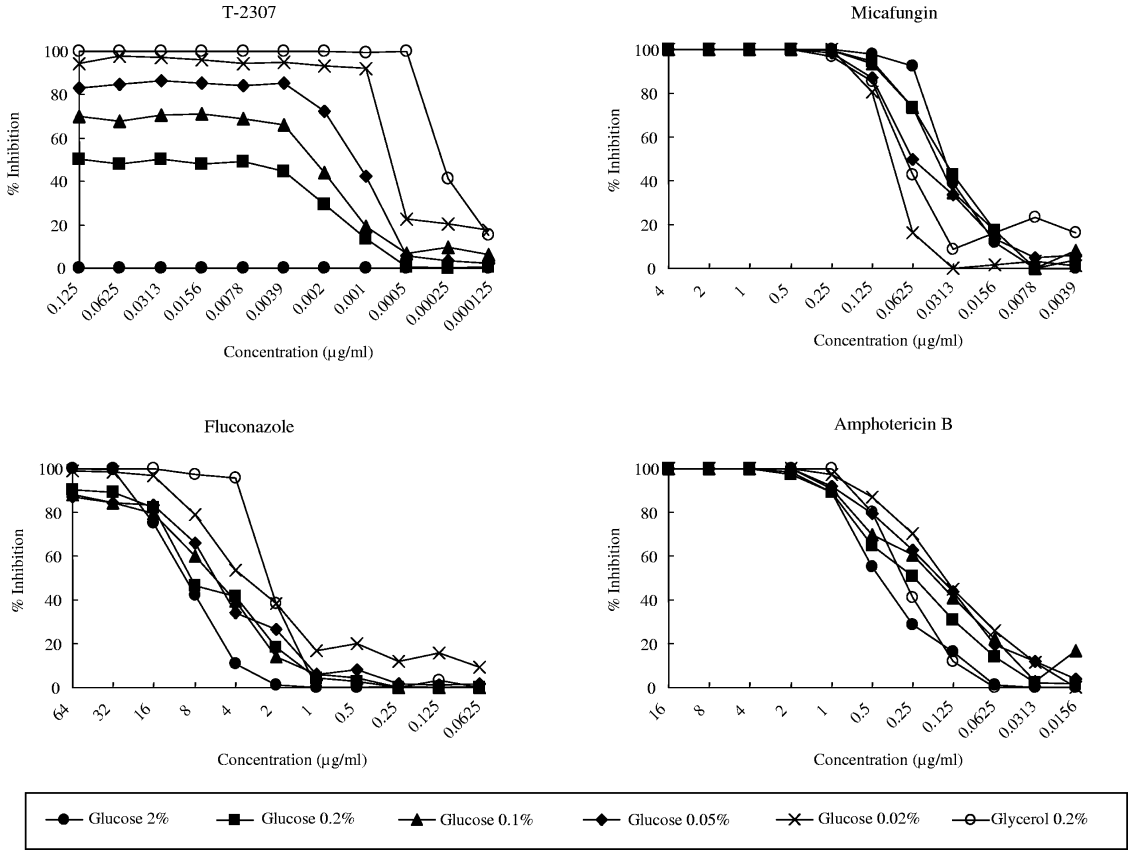

FIG. 3. Inhibitory effects of T-2307 and other agents against *C. glabrata* ATCC 90030 in modified media. The susceptibility of *C. glabrata* to T-2307 and the other agents was tested in RPMI 1640 medium containing 2%, 0.2%, 0.1%, 0.05%, and 0.02% glucose or 0.2% glycerol. Percent inhibition was calculated on the basis of the growth in the control well after the optical density at 600 nm was measured using a microplate spectrophotometer at 48 h of incubation. The percent inhibition by micafungin in the presence of 2%, 0.2%, 0.1%, 0.05%, and 0.02% glucose was calculated at 24 h of incubation.

The phenomenon of trailing growth for *Candida* spp. was originally reported with azole antifungal agents (18). It has been shown that the *in vivo* response to fluconazole in murine invasive candidiasis caused by *Candida albicans* isolates with the trailing growth phenomenon matched the MIC obtained at 24 h of incubation rather than that obtained at 48 h (3, 17). In addition, the clinical responses of three patients with trailing *C. albicans* isolates matched the MICs obtained at 24 h of incubation rather than those obtained at 48 h (16). Therefore, the reference guidelines suggest that the results obtained at 24 h of incubation may be more appropriate for such isolates (4). Several studies have attempted to resolve the difficulty of trailing growth. In one study, the trailing growth of *Candida* spp. was attenuated in media adjusted to a pH of ≤5.0 (12). In another study, *Candida* spp. lost the trailing phenotype at 25°C and 42°C (2). It has also been reported that RPMI 1640 medium containing 2% glucose facilitates reading of the fluconazole MIC for *C. albicans* (5, 19).

In the current study, several differences between the trailing growth of T-2307 and that reported for fluconazole were ob-

TABLE 1. Comparison of MICs of T-2307 and other antifungal agents against *C. glabrata* ATCC 90030 determined in various media

Antifungal agent	MIC (μg/ml)							
	Glucose[b]					0.2% glycerol[c]	0.2% Glucose + Alamar blue	
	2%	0.2%[a]	0.1%	0.05%	0.02%		24 h	48 h
T-2307	>0.125	0.0039	0.0039	0.001	0.001	0.0005	0.0156	>0.125
Fluconazole	16	8	8	8	4	4	8	16
Micafungin	0.0625	0.0625	0.0625	0.0625	0.125	0.125	0.0625	0.0625
Amphotericin B	2	2	2	2	2	1	1	2

[a] Equivalent to the amount used in the CLSI method.
[b] Incubation for 48 h except with micafungin (incubation for 24 h).
[c] Incubation for 48 h.

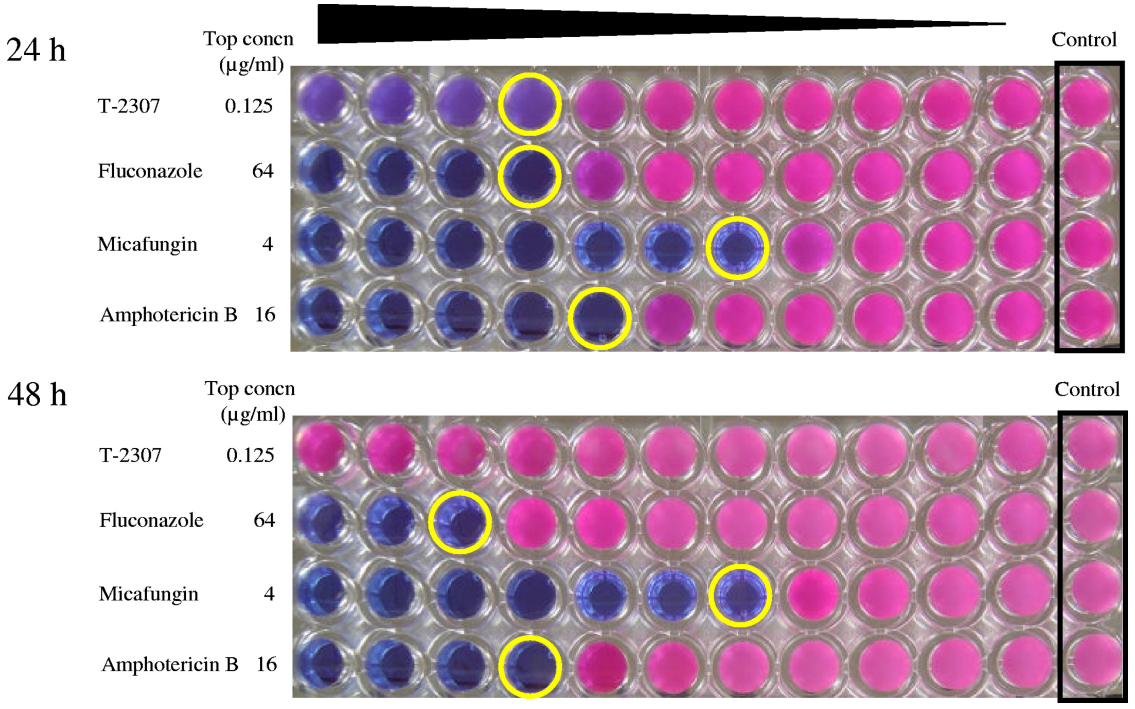

FIG. 4. Colorimetric MIC determination of T-2307 and other agents against *C. glabrata* ATCC 90030 using Alamar blue at 24 h and 48 h of incubation. The susceptibility of *C. glabrata* to T-2307 and the other agents was tested in RPMI 1640 medium containing 0.2% glucose. Yellow circles, MICs.

served. First, the trailing growth of *C. glabrata* in the presence of T-2307 at 24 h of incubation was similar to that at 48 h, determined using standard CLSI methods (Fig. 2). Second, *C. glabrata* exhibited more significant trailing growth in the presence of T-2307 and 2% glucose (Fig. 3). Third, the trailing growth of *C. glabrata* in the presence of T-2307 was not attenuated when the pH of the medium was lowered to 5.4 (data not shown). These differences in behavior may be derived from a difference in the mechanism of trailing growth. The mechanisms proposed to explain the trailing growth for azoles include activation of calcineurin and the altered regulation of genes that mediate resistance, such as *ERG11*, *CDR1*, and *MDR1* (10, 21). On the other hand, T-2307, like pentamidine and DB75, may be considered an inhibitor of the mitochondrial function; therefore, the cause of trailing growth may be fermentation.

TABLE 2. Viable counts in kidneys in disseminated candidiasis caused by *C. glabrata* ATCC 90030

Antifungal agent	Dose (mg/kg/day)	Log CFU/kidneys	SE
Control		5.26	0.06
T-2307	0.025	4.30	0.20
	0.05	4.00[a]	0.26
	0.1	3.86[a]	0.19
Fluconazole	2.5	4.96	0.36
	5	5.33	0.27
	10	4.62	0.20
Micafungin	0.125	5.13	0.35
	0.25	5.21	0.30
	0.5	3.59[b]	0.17
Amphotericin B	0.125	4.61	0.40
	0.25	4.53	0.29
	0.5	2.93[c]	0.45

[a] $P < 0.05$ compared with the results for the control.
[b] $P < 0.01$ compared with the results for the control.
[c] $P < 0.001$ compared with the results for the control.

Another approach to overcoming the difficulty of the MIC reading for azoles in *C. albicans* is the addition of an oxidation-reduction indicator such as Alamar blue (15). In the current study, the colorimetric method using Alamar blue provided a clear MIC endpoint for T-2307 at 24 h of incubation with a standard (0.2%) glucose concentration. However, at 48 h of incubation, all the wells with T-2307 changed to pink, indicating that 24 h of incubation was appropriate with this method. Modifying the glucose concentration or carbon source may be a better option because the MIC is easily read at up to 48 h of incubation.

To investigate if T-2307 shows efficacy against trailing isolates *in vivo*, we evaluated the *in vivo* efficacy of T-2307 in a murine model of disseminated candidiasis caused by *C. glabrata*, and T-2307 had an excellent therapeutic effect (Table 2). This result seems reasonable because the normal blood glucose level of mice is approximately 0.1% (20), at which the trailing growth of *C. glabrata* in the presence of T-2307 is less than that observed using the standard CLSI method. Since the normal blood glucose level in humans is also approximately 0.1% (20), T-2307 may show efficacy against candidiasis caused by *C. glabrata*.

In conclusion, susceptibility testing of *C. glabrata* using modified media or Alamar blue facilitates the MIC endpoint de-

termination for T-2307, as compared to the standard CLSI method. Although *C. glabrata* exhibits trailing growth in the presence of T-2307 with the CLSI method, T-2307 has an excellent therapeutic effect in the murine model of disseminated candidiasis caused by *C. glabrata*. It would be better to test the susceptibility of *C. glabrata* to T-2307 using modified media or Alamar blue to avoid misreading of the MIC endpoint due to the significant trailing growth seen with the standard CLSI method. Further studies on the correlation between the trailing growth of *C. glabrata* in the presence of T-2307 and the clinical response are needed.

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