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# In Vitro Activity of Anidulafungin and Voriconazole and Comparators against *Aspergillus* spp. as Determined by CLSI and EUCAST Broth Microdilution Methods


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## ABSTRACT


**Background:** Resistance (R) of *Aspergillus* spp. (ASP) to itraconazole (ITR) and other azoles is a growing concern that merits monitoring by standardized susceptibility testing. We used Clinical and Laboratory Standards Institute (CLSI) broth microdilution (BMD) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) method to test ASP, including amphotericin B (AMB)- and azole-R strains.

**Methods:** CLSI and EUCAST methods were used to test 78 ASP (20 *A. flavus* [AFL], 22 *A. fumigatus* [AFM], 13 *A. niger* [ANG] and 23 *A. terreus* [ATR]), including 15 AMB-R (MIC, ≥2 µg/ml) ATR strains and 10 ITR-R (MIC, ≥4 µg/ml) AFM (7), ANG (2) and ATR (1). Comparators included caspofungin (CSF), ITR, and posaconazole (PSC). All isolates were read visually after 48-h incubation. For ANF and CSF the minimum effective concentration (MEC) was determined as described by CLSI (M38-A2). For ITR, PSC, and VRC an MIC endpoint of complete growth inhibition was used for both methods.

**Results:** CLSI and EUCAST methods were highly concordant for ANF, VRC and all comparators. The essential agreement (EA; ± 2 $\log_2$ dilutions) was 100% for all comparisons with the exception of PSC and ATR (EA = 91.3%). Both ANF and VRC were highly active against all isolates. The MEC/$MIC_{90}$ values (µg/ml) for ANF, CSF, ITR, PSC, and VRC, respectively, were as follows for each species: AFL (≤0.008, 0.12, 1, 1, 1), AFM (0.015, 0.12, >8, 1, 4), ANG (0.03, 0.12, 4, 1, 2), ATR (0.015, 0.12, 1, 0.5, 1). ANF was very active against AMB-R strains of ATR (MEC range, 0.008-0.015 µg/ml) and ITR-R strains of AFM (MEC range, 0.008-0.015 µg/ml), ANG (MEC, 0.008 µg/ml) and ATR (MEC, 0.008 µg/ml). VRC was active against AMB-R strains of ATR (MIC range of 0.25- >8 µg/ml) but showed cross-resistance with ITR against ITR-R strains of AFM (MIC range, of 0.25-8 µg/ml).

**Conclusions:** ANF showed excellent activity against AMB- and ITR-R strains of ASP. VRC was active against AMB-R ATR, but demonstrated cross-resistance with ITR for ITR-R AFM. In vitro susceptibility testing of ANF, VRC and other mould-active antifungal agents may be accomplished by either CLSI or EUCAST BMD methods with comparable results.


## INTRODUCTION

The introduction of mould-active antifungal agents of the triazole (itraconazole, posaconazole, voriconazole) and echinocandin (anidulafungin, caspofungin, micafungin) classes has dramatically increased the options for prophylaxis, empirical and directed therapy for the prevention and treatment of invasive aspergillosis (IA). Although resistance to these agents among clinical isolates of *Aspergillus* is considered to be uncommon, both emergence of resistance and breakthrough infections have been reported increasingly among patients with long-term exposure to these agents. These observations have prompted a call for an expanded search for new antifungal agents with novel mechanisms of action as well as an expanded role for antifungal susceptibility testing of *Aspergillus* spp.

Currently, there are two independent reference standards for broth microdilution (BMD) susceptibility testing of antifungal agents against *Aspergillus* spp.: the Clinical and Laboratory Standards Institute (CLSI) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) methods. Given the important role that both methods currently play in antifungal resistance surveillance and regulatory approval, it is important to demonstrate the comparability of the results in the pre-clinical development of new antifungal agents. In the present study, we have used a panel of *Aspergillus* spp. isolates selected to represent both polyene- and triazole-resistant strains to examine the activity of anidulafungin, voriconazole and comparator agents as determined by both CLSI and EUCAST BMD methods.

## MATERIALS AND METHODS

Organisms. A total of 78 clinical isolates of *Aspergillus* spp. obtained from centers participating in the 2008-2009 SENTRY and ARTEMIS Antimicrobial Surveillance Programs were tested against anidulafungin, caspofungin, itraconazole, posaconazole and voriconazole. The collection included 20 isolates of *A. flavus*, 22 of *A. fumigatus*, 13 of *A. niger*, and 23 of *A. terreus*. The phenotypically resistant isolates included 15 amphotericin B-resistant (MIC, ≥2 µg/ml) isolates of *A. terreus* and 10 itraconazole-resistant (MIC, ≥4 µg/ml) isolates of *A. fumigatus* (7 isolates), *A. niger* (2 isolates), and *A. terreus* (1 isolate). The isolates were obtained from a variety of sources, including sputum, bronchoscopy, and tissue biopsy specimens; each represented an individual infectious episode. The isolates were collected at individual study sites and sent to JMI Laboratories (North Liberty, Iowa, USA) for identification and susceptibility testing as described previously. All isolates were identified by standard microscopic morphology and were stored as spore suspensions in sterile distilled water at room temperature until used in this investigation. Before testing, each isolate was subcultured at least twice on potato dextrose agar (Remel, Lenexa, Kansas, USA) to ensure viability and purity.

Antifungal agents. Reference powders of anidulafungin, caspofungin, itraconazole, posaconazole and voriconazole were obtained from their respective manufacturers. Stock solutions were prepared in dimethyl sulfoxide and serial two-fold dilutions in RPMI 1640 medium (Sigma, St. Louis, Missouri, USA) buffered to pH7.0 with 0.165 M MOPS (morpholine-propanesulfonic acid) buffer (Sigma) were made.

Antifungal susceptibility testing. All isolates were tested for in vitro susceptibility to anidulafungin, voriconazole and comparators using the CLSI and EUCAST BMD methods. CLSI BMD testing was performed as outlined in document M38-A2 (CLSI, 2008) by using RPMI 1640 medium with 0.2% glucose, round-bottom microdilution trays, inocula of 0.4 X $10^4$ to 5 X $10^4$ CFU/ml, and incubation at 35°C for 48-hours. MIC values for the triazoles were determined visually as the lowest concentration of drug that caused complete inhibition of growth (first clear well) relative to that of the growth control. MEC values for anidulafungin, and caspofungin were determined as described previously and by CLSI (2008).

EUCAST BMD testing was performed exactly as detailed by EUCAST by using RPMI 1640 medium with 2.0% glucose, flat-bottom microdilution trays, inocula of 2 X $10^5$ to 5 X $10^5$ CFU/ml, and incubation at 35°C. MICs (triazoles) and MECs (E1210, echinocandins) were determined after 48-h as described above and by both CLSI and EUCAST.

Quality control was ensured by testing the following strains recommended by CLSI (2008) and by EUCAST: *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258, and *A. flavus* ATCC 204304.

Analysis. The MIC/MEC results for each antifungal agent obtained with the EUCAST method were compared to those of the CLSI BMD method. High off-scale MIC/MEC results were converted to the next highest concentration and low off-scale MIC/MEC results were left unchanged. Discrepancies of more than ± two $\log_2$ dilutions among MIC results were used to calculate the essential agreement (EA) between the two methods.

## RESULTS

- All 78 isolates of *Aspergillus* spp. were inhibited by ≤0.015 µg/ml of anidulafungin as determined by the CLSI and EUCAST BMD methods (Table 1).
- Caspofungin displayed the lowest $MEC_{50}$/$MIC_{50}$ (ranging from 0.06 to 0.12 µg/ml) values among comparator agents, followed by posaconazole ($MIC_{50}$, 0.5 µg/ml for all *Aspergillus* spp.) and voriconazole ($MIC_{50}$, 0.5 to 1 µg/ml for all *Aspergillus* spp.).
- The EA between the CLSI and EUCAST methods was 100.0% for anidulafungin and voriconazole across all four species (Table 1). The MEC values generated by the EUCAST method tended to be one $\log_2$ dilution higher than those obtained by the CLSI method.
- The overall EA between EUCAST and CLSI results was 100.0% for caspofungin and itraconazole and was 97.4% (76 of 78 results) for posaconazole.
- Anidulafungin and caspofungin were the most active agents against amphotericin B-resistant strains of *A. terreus* as determined by the CLSI method (Table 2).
- Anidulafungin was the most potent agent ($MEC_{90}$, 0.015 µg/ml) followed by caspofungin ($MEC_{90}$, 0.25 µg/ml), posaconazole ($MIC_{90}$, 0.5 µg/ml), itraconazole and voriconazole ($MIC_{90}$, 1 µg/ml), and amphotericin B (4 µg/ml).
- A similar rank order was noted with the itraconazole-resistant isolates: anidulafungin ($MEC_{90}$, 0.015 µg/ml) > caspofungin ($MEC_{90}$, 0.12 µg/ml) > amphotericin B ($MIC_{90}$, 1 µg/ml) > posaconazole ($MIC_{90}$, 2 µg/ml) > voriconazole ($MIC_{90}$, 8 µg/ml) > itraconazole ($MIC_{90}$, >8 µg/ml; Table 3).

**Table 1.** In vitro susceptibilities of *Aspergillus* spp. to anidulafungin, voriconazole and selected comparators as determined by CLSI and EUCAST broth microdilution methods.

| | | | No. of isolates at MIC/MEC (µg/ml) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Species (no. tested) | Antifungal agent | Test method | 0.008 | 0.015 | 0.03 | 0.06 | 0.12 | 0.25 | 0.5 | 1 | 2 | 4 | ≥8 | %EA |
| *A. flavus* (20) | Anidulafungin | EUCAST | 19 | 1 | | | | | | | | | | 100.0 |
| | | CLSI | 20 | | | | | | | | | | | |
| | Caspofungin | EUCAST | | | | 4 | 16 | | | | | | | 100.0 |
| | | CLSI | | | 1 | 15 | 4 | | | | | | | |
| | Itraconazole | EUCAST | | | | | | 7 | 13 | | | | | 100.0 |
| | | CLSI | | | | | | | 7 | 13 | | | | |
| | Posaconazole | EUCAST | | | | | 2 | 17 | 1 | | | | | 100.0 |
| | | CLSI | | | | | | 1 | 16 | 3 | | | | |
| | Voriconazole | EUCAST | | | | | | | 1 | 12 | 7 | | | 100.0 |
| | | CLSI | | | | | | | 4 | 14 | 1 | 1 | | |
| *A. fumigatus* (22) | Anidulafungin | EUCAST | 17 | 5 | | | | | | | | | | 100.0 |
| | | CLSI | 14 | 8 | | | | | | | | | | |
| | Caspofungin | EUCAST | | | | 3 | 15 | 4 | | | | | | 100.0 |
| | | CLSI | | | | 4 | 17 | 1 | | | | | | |
| | Itraconazole | EUCAST | | | | | | 2 | 13 | | | | 7 | 100.0 |
| | | CLSI | | | | | | | 1 | 14 | | 1 | 6 | |
| | Posaconazole | EUCAST | | | | | 13 | 1 | 2 | 5 | 1 | | | |
| | | CLSI | | | | | | 8 | 7 | 6 | 1 | | | |
| | Voriconazole | EUCAST | | | | | | 1 | 12 | 4 | 4 | 1 | | 100.0 |
| | | CLSI | | | | | | 4 | 10 | 3 | 4 | | 1 | |
| *A. niger* (13) | Anidulafungin | EUCAST | 13 | | | | | | | | | | | 100.0 |
| | | CLSI | 12 | | 1 | | | | | | | | | |
| | Caspofungin | EUCAST | | | | | 13 | | | | | | | 100.0 |
| | | CLSI | | | | 4 | 9 | | | | | | | |
| | Itraconazole | EUCAST | | | | | | 1 | 3 | 6 | 3 | | | 100.0 |
| | | CLSI | | | | | | | | 6 | 5 | 2 | | |
| | Posaconazole | EUCAST | | | | | 2 | 4 | 7 | | | | | 100.0 |
| | | CLSI | | | | | | 1 | 8 | 4 | | | | |
| | Voriconazole | EUCAST | | | | | | | 4 | 5 | 4 | | | 100.0 |
| | | CLSI | | | | | | | 4 | 5 | 4 | | | |
| *A. terreus* (23) | Anidulafungin | EUCAST | 20 | 3 | | | | | | | | | | 100.0 |
| | | CLSI | 15 | 8 | | | | | | | | | | |
| | Caspofungin | EUCAST | | | | 1 | 18 | 4 | | | | | | 100.0 |
| | | CLSI | | | | | 21 | 2 | | | | | | |
| | Itraconazole | EUCAST | | | | | | 15 | 7 | | 2 | | | 100.0 |
| | | CLSI | | | | | | 1 | 15 | 6 | | 1 | | |
| | Posaconazole | EUCAST | | | | 1 | 14 | 7 | | 1 | | | | 91.3 |
| | | CLSI | | | | | | 7 | 13 | 1 | 1 | | | |
| | Voriconazole | EUCAST | | | | | | | 11 | 11 | | | 1 | 100.0 |
| | | CLSI | | | | | | 4 | 13 | 5 | | | 1 | |

**Table 2.** In vitro activity of anidulafungin, voriconazole and selected comparators against amphotericin B-resistant *Aspergillus terreus* as determined by CLSI broth microdilution methods[a].

| | MIC/MEC (µg/ml)[b,c] | | | | | |
|---|---|---|---|---|---|---|
| Isolate # | AMB | ITR | PSC | VRC | ANF | CSF |
| 446 | 4 | 0.5 | 0.5 | 0.5 | 0.008 | 0.12 |
| 618 | 4 | 0.5 | 0.25 | 0.25 | 0.008 | 0.12 |
| 8691 | 2 | 1 | 0.5 | 1 | 0.015 | 0.25 |
| 8693 | 2 | 1 | 0.5 | 0.5 | 0.015 | 0.25 |
| 8694 | 2 | 0.5 | 0.25 | 0.5 | 0.008 | 0.12 |
| 8695 | 2 | 1 | 0.5 | 1 | 0.008 | 0.12 |
| 8696 | 2 | 0.25 | 0.25 | 0.25 | 0.008 | 0.12 |
| 8722 | 2 | 0.5 | 0.5 | 1 | 0.015 | 0.12 |
| 8723 | 2 | 0.5 | 0.25 | 0.5 | 0.008 | 0.12 |
| 8727 | 2 | 0.5 | 0.5 | 1 | 0.008 | 0.12 |
| 8728 | 2 | 0.5 | 0.5 | 0.5 | 0.008 | 0.12 |
| 8729 | 2 | 1 | 0.5 | 1 | 0.008 | 0.12 |
| 8730 | 2 | 0.5 | 0.5 | 1 | 0.008 | 0.12 |
| 8731 | 2 | 4 | 2 | >8 | 0.008 | 0.12 |
| 8733 | 2 | 0.5 | 0.5 | 1 | 0.015 | 0.12 |

a. The minimum inhibitory concentrations (MIC) and minimum effective concentrations (MEC) were determined after 48-h incubation.
b. The MICs for AMB (amphotericin B), ITR (itraconazole), PSC (posaconazole), and VRC (voriconazole) were read at complete (100%) inhibition.
c. The MECs for ANF (anidulafungin) and CSF (caspofungin) were read as described in CLSI document M38-A2 (CLSI, 2008).

**Table 3.** In vitro activity of anidulafungin, voriconazole and selected comparators against itraconazole-resistant *Aspergillus* spp. as determined by CLSI broth microdilution methods[a].

| | | MIC/MEC (µg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| Species | Isolate # | ITR | PSC | VRC | AMB | ANF | CSF |
| *A. fumigatus* | 8686 | >8 | 1 | 2 | 1 | 0.015 | 0.12 |
| | 8687 | >8 | 1 | 2 | 1 | 0.015 | 0.12 |
| | 8688 | >8 | 1 | 1 | 1 | 0.008 | 0.12 |
| | 8689 | >8 | 1 | 2 | 1 | 0.015 | 0.12 |
| | 8690 | >8 | 2 | 8 | 1 | 0.008 | 0.12 |
| | 8737 | 4 | 1 | 2 | 1 | 0.015 | 0.12 |
| | 8737 | >8 | 1 | 0.25 | 1 | 0.008 | 0.12 |
| *A. niger* | 8698 | 4 | 1 | 2 | 1 | 0.008 | 0.12 |
| | 301 | 4 | 1 | 2 | 0.5 | 0.008 | 0.12 |
| *A. terreus* | 8731 | 4 | 2 | >8 | 2 | 0.008 | 0.12 |

a. Definitions for MIC and MEC and all abbreviations as described in Table 2. Resistance to itraconazole defined as MIC ≥4 µg/ml.

## CONCLUSIONS

- These results confirm the high level of concordance between the MIC results produced by both reference BMD methods for the triazoles and *Aspergillus* spp.; and demonstrate for the first time that both CLSI and EUCAST methods generate comparable MEC values for anidulafungin and caspofungin against *Aspergillus* spp.
- Whereas cross-resistance was apparent between itraconazole, posaconazole, and voriconazole, different mechanisms of action represented by amphotericin B and the echinocandins resulted in activity of these antifungal agents against this subset of *Aspergillus* spp. isolates that are of great clinical concern.
- Anidulafungin and caspofungin demonstrated excellent in vitro potency and spectrum of activity against contemporary isolates of *Aspergillus* spp., including both polyene- and triazole-resistant strains. These compounds exhibit impressive activity against both wild-type and antifungal-resistant strains of *Aspergillus*. Further evaluation of this antifungal class against *Aspergillus* is warranted.

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