The Composition of the Culture Medium Influences the β-1,3-Glucan Metabolism of Aspergillus fumigatus and the Antifungal Activity of Inhibitors of β-1,3-Glucan Synthesis

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The Composition of the Culture Medium Influences the β-1,3-Glucan Metabolism of *Aspergillus fumigatus* and the Antifungal Activity of Inhibitors of β-1,3-Glucan Synthesis

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***In vitro* testing of *Aspergillus fumigatus* susceptibility to echinocandins has always been a challenge. Using a simple and quick colorimetric method to analyze the activity of inhibitors of β-1,3-glucan synthesis, we found that the composition of the culture medium significantly influences glucan synthesis and consequently the antifungal properties of inhibitors of β-1,3-glucan synthesis when they are tested alone or in combination with chitin synthase inhibitors.**

In vitro susceptibility testing of fungal pathogens is commonly used to evaluate the efficacy of new antifungals but also to test the emergence of antifungal resistance of clinical isolates. *In vitro* susceptibility of fungal pathogens to chemicals is usually determined by measuring the MIC by using 2-fold microdilution in 96-well plates. The MIC is usually defined as the concentration resulting in the absence of visible growth after 48 h in the presence of the antifungals. This method is based on the recommendation of the CLSI document M38-A (13) or the EUCAST-AFST recommendations (8). However, MIC determination is difficult when one has to test the effect of echinocandins (cell wall β-1,3-glucan synthesis inhibitors) on the growth of filamentous fungi such as *Aspergillus fumigatus*. *In vitro*, caspofungin does not cause complete inhibition of the mycelial growth but induces only morphological changes that result in short and branched hyphae (3, 10). The concentration resulting in this type of growth is commonly defined as the minimal effective concentration (MEC). Determination of the MEC is not easy to perform in routine tests, since it has to be validated under a light microscope (3). In addition, the unexplained paradoxical effect seen with echinocandins (increase of drug concentration does not better inhibit mycelial growth) complicates this determination (1, 11).

Better methods are required for estimating the impact of echinocandins on filamentous fungi. Among these, colorimetric methods are an attractive alternative to CLSI or EUCAST-AFST methods. Two main colorimetric methods based on metabolic activity have been proposed to measure MICs of antifungals against *A. fumigatus* isolates. MTT and XTT methods use tetrazolium salts and menadione as electron coupling agents followed by optical density (OD) measurement (1, 15). In the alamarBlue method, the resazurin colorimetric reagent changes color from deep blue (absorbance maximum at 600 nm) to bright pink (due to the production of resofurin resulting from the reduction of resazurin by electron transfer reactions associated with respiration with an absorbance maximum at 569 nm) when the fungus grows. Resazurin has been used in the past to test the *in vitro* susceptibility of *A. fumigatus* to antifungals (7, 17).

Although RPMI medium is recommended by the CLSI for *in vitro* susceptibility testing, RPMI medium is more suitable for yeast and dimorphic fungi than for *A. fumigatus*, since the growth of filamentous fungi in RPMI is extremely poor (see Fig. S1 in the supplemental material). The medium that is recommended by the EUCAST-AFST is very similar (RPMI supplemented with 2% glucose) (8) and did not induce better growth (data not shown). *A. fumigatus* grew better on protein hydrolysate-based media such as peptone-glucose, peptone-yeast extract-glucose, and 1% yeast extract (YE) than on RPMI-based media (see Fig. S1 in the supplemental material; also data not shown). Such protein hydrolysate-based media have been used successfully to test the sensitivity of mutants of *A. fumigatus* to antifungal drugs but are not used routinely to test drug efficacy (12). Here we present an easy and rapid colorimetric resazurin-based method to calculate MECs of inhibitors of β-1,3-glucan synthesis tested alone and MICs of combinations of drugs inhibiting glucan and chitin synthesis. Changes in the composition of the culture medium induced modifications of the glucan synthase activity and cell wall composition, resulting in changes in the sensitivity of the fungus to the inhibitors of β-1,3-glucan synthesis tested alone and in combination.

Susceptibility testing was undertaken in a 96-well microtiter format with three clinical isolates of *A. fumigatus*, CBS14489, Af293, and ATCC 46645, the echinocandin-resistant mutant EMFR-S678P (14) (a kind gift from D. Perlin), and its parental strain CBS14489 *akuBΔKu80*. The method is described in the supplemental material. Antifungal agents tested were micafungin (kindly provided by Astellas), caspofungin, the enfumafungin analog MK3118 (kindly provided by Merck), anidulafungin (Lilly 303366; kindly provided by D. Denning), and nikkomycin (Sigma-Aldrich). Amphotericin B (bioMérieux, Paris, France) was used as a control. Two culture media were used: 1% YE (BD) and RPMI 1640 (Sigma-Aldrich) supplemented with 0.3 g/liter glutamine (buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid [MOPS] [Sigma-Aldrich]). Both media were supplemented with 0.1% Tween 20. MECs measured using resazurin

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TABLE 1 Inhibitory effect of β-1,3-glucan synthase inhibitors on the mycelial growth of clinical isolates and mutant strains of *A. fumigatus* grown in RPMI and 1% yeast extract

Strain	Echinocandin	MEC (μg/ml)[a]		MIC (μg/ml) by Etest (1% YE)[d]
		RPMI	1% YE	
CBS14489	Caspofungin	0.24–0.47[b]	0.038–0.077	0.016–0.032
	Micafungin	0.15–0.29[b]	0.23–0.26	0.032
	Anidulafungin	0.48[b]	0.03–0.06	0.012
	Enfumafungin	0.24–0.47	0.038–0.077	ND[e]
ATCC 46645	Caspofungin	0.24–0.47	0.077–0.1	ND
Af293	Caspofungin	0.24–0.47	0.077	ND
CBS14489 *akuB*Δ*Ku80*	Caspofungin	0.12–0.24[c]	0.008–0.06	0.016–0.047
	Micafungin	0.12–0.24[c]	0.03–0.06	0.012–0.032
	Anidulafungin	0.12–0.24[c]	0.06	0.016
	Enfumafungin	0.015–0.12	0.002–0.015	ND
EMFR-S678P	Caspofungin	>3.84[c]	>3.84	>32
	Micafungin	>3.84[c]	>3.84	>32
	Anidulafungin	>3.84[c]	>3.84	>32
	Enfumafungin	>3.84	0.077	ND[e]

[a] MECs were defined for all antifungals as the lowest drug concentration giving the maximal growth inhibition in the resazurin test after 48 h of growth in RPMI and 24 h of growth in 1% yeast extract; the range of concentrations obtained with three biological replicates is shown.
[b] MEC values reported for clinical *A fumigatus* strains grown in RPMI were equal to 0.25-0.5 μg/ml for caspofungin (15), 0.06 (0.06-012) μg/ml for Micafungin (2), and 0.03 (0.015-0.03) μg/ml for Anidulafungin (2).
[c] For CBS144-89*Ku80* MEC values reported were equal to 0.25 μg/ml for caspofungin, 0.015 μg/ml for Micafungin and 0.015 μg/ml for Anidulafungin (14) and for EMFR-S678P MEC values were >16 μg/ml for the three antifungals.
[d] Defined as the concentration responsible for total growth inhibition.
[e] ND, not determined.

conversion were defined for all echinocandins as the lowest drug concentration giving the maximal fungistatic transient growth inhibition, whereas MICs were based exclusively on fungicidal effect. For the Etest, strips with concentration gradients from 0.004 to 32 mg/liter of caspofungin, micafungin, anidulafungin, and amphotericin B were used in 1% yeast extract agar plates as recommended by the manufacturer (bioMérieux, Lyon, France). Glucan synthase activity was measured in membrane extracts by the quantification of the incorporation of radiolabeled UDP-[U-^{14}C]glucose into β-1,3-glucan as described in reference 4. Carbohydrate composition of the cell wall was determined as described in reference 12. A two-dimensional colorimetric checkerboard assay using resazurin is described in the supplemental material. Often used to estimate the combinatorial effects of drugs, fractional inhibitory concentration (FIC), defined as the MIC of drug A in the presence of drug B divided by the MIC of drug A alone (6) cannot be calculated properly in the case of echinocandins because only MECs can be identified when echinocandins are tested alone, whereas true MICs are seen when the combinations are tested.

MECs of *A fumigatus* strains obtained with echinocandins were lower in 1% YE than in RPMI (Table 1). This result was verified using different strains as well as different batches of yeast extract (Table 1; also data not shown). Although MECs were lower in 1% YE, the residual growth was higher in this medium than in RPMI (Fig. 1). This increased sensitivity in the YE medium also occurred with enfumafungin (Table 1; also, see Fig. S2 in the supplemental material). Light microscopy confirmed that the concentration of echinocandin inducing a change of slope in OD values in Fig. 1 truly represented the MEC, as confirmed by the hyperbranching phenotype of the mycelium. Strain EMRF-S678P, the *fks1*(*S678P*) mutant strain of Ku80, was resistant to echinocandin in both media. It was sensitive to amphotericin B (MIC = 1.0 to 7.7 μg/ml obtained by Etest) and, interestingly, to enfumafungin (which also targets β-1,3-glucan synthase inhibition) in 1% YE but not in RPMI. Echinocandin MICs estimated by Etest assays were similar to the MECs calculated from the liquid resazurin test in a 96-well

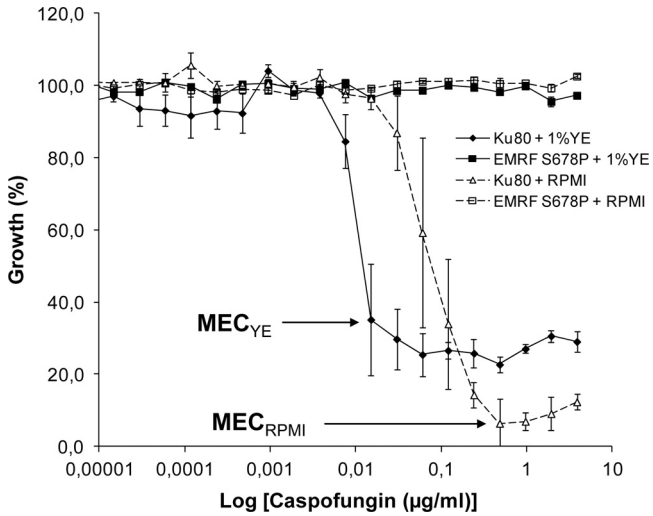

FIG 1 Growth of *A. fumigatus* CBS14489 *akuB*Δ*Ku80* and EMFR-S678P in the presence of increasing concentrations of caspofungin. Mycelial growth is estimated from OD readings with the resazurin method after 24 h of growth in 1% yeast extract or after 48 h of growth in RPMI.

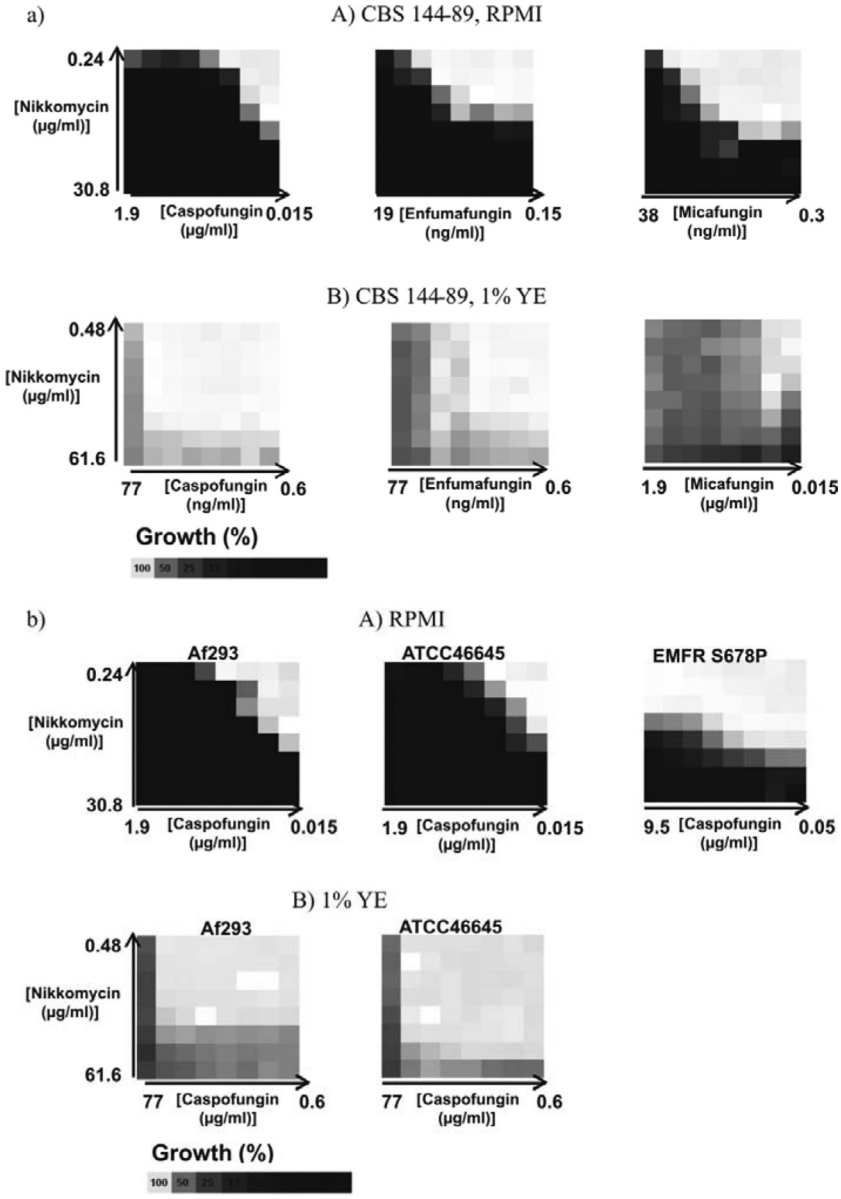

FIG 2 Checkerboard assays. (a) Effect of various concentrations of caspofungin, enfumafungin, and micafungin in combination with various concentrations of nikkomycin Z on the growth of *A. fumigatus* CBS 144-89 in RPMI (A) (48 h growth) and in 1% YE (B) (24 h growth). Note that in the data obtained with RPMI, all the combinations indexed with black squares correspond to drug combinations that kill the fungus, whereas in the data for 1% YE, none of the combinations tested killed the fungus. (b) Effect of various concentrations of caspofungin in combination with various concentrations of nikkomycin Z on the growth of *A. fumigatus* Af293 and ATCC 46645 and the caspofungin-resistant strain EMFRS678P in RPMI (A) and on Af293 and ATCC 46645 in 1% YE (B). Conditions for estimating mycelial growth are the same as for Fig. 1.

format. The effect of the medium composition on the fungistatic and fungicidal activity of polyenes and azoles against *A. fumigatus* had been demonstrated (16), but the effect had not been studied with echinocandins.

The effect of caspofungin and nikkomycin Z combinations was tested under the same culture conditions, since a synergistic effect of these drugs was previously reported (9). The combined effect of caspofungin with nikkomycin Z was measured by the resazurin checkerboard broth microdilution assay (Fig. 2). In spite of a lower sensitivity to drugs of the fungus grown in RPMI, the combinations of caspofungin and nikkomycin were more efficient in this medium than in the YE medium. In addition, when the microtiter plates containing RPMI-based medium were incubated for an additional 24 h at 37°C, there was no change in the OD after 48 h in the wells with a blue color (corresponding to a 100% inhibition [Fig. 2]). In addition, no germination of the conidia (estimated by light microscopy or CFU counts) was seen when the conidia of these wells were inoculated into fresh liquid or agar-based culture medium. These data confirmed that the fungus in the wells showing no change in OD compared to the control wells without fungus was killed by the caspofungin-nikkomycin Z drug combinations, whereas these drugs alone did not kill the fungus in RPMI. In contrast, in 1% yeast extract-based medium, all wells that were negative after the first 24 h (blue color) became positive

(pink color) after an additional 24 h of incubation. These results showed that combinations of nikkomycin and caspofungin were fungicidal in RPMI, while in YE they were only fungistatic.

To investigate the reasons for these differences in drug sensitivity, the cell wall composition and the β-1,3-glucan synthase activity were monitored. First, there was a significant (30%) reduction in the β-1,3-glucan content of the cell wall of wild-type strain CBS14489 cultivated in 1% yeast extract compared to RPMI medium (see Fig. S3 in the supplemental material). This reduction in the β-1,3-glucan content was also concomitant with a 3-fold reduction in α-1,3-glucan and a 4-fold increase in chitin levels. Second, the specific glucan synthase activity was lower ($P < 0.01$, two-way analysis of variance [ANOVA] with Student's *t* test) in 1% yeast extract compared to RPMI (see Fig. S4 in the supplemental material). The reduction in β-1,3-glucan synthase activity and the small amount of β-1,3-glucan in the cell wall logically explained the increased sensitivity of *A. fumigatus* to the echinocandins in the 1% YE medium (5). Similarly, the fungicidal effect of the caspofungin-nikkomycin combination in RPMI may be due to a lower chitin content seen in the cell wall of the mycelium grown in this medium. Alternatively, two mechanisms may govern the echinocandin effect: an immediate fungistatic growth inhibition due to the noncompetitive inhibition of target 1 seen in both culture media and a fungicidal effect due to the inhibition of target 2 that is revealed only after the addition of nikkomycin. The resazurin method that has been used in the past and was modified here is simple, can be used with all antifungals as demonstrated here with echinocandins and amphotericin B (but also azoles [data not shown]), and should be reintroduced for routine testing. Also, microscopic observations to confirm the effect of echinocandins are not required. The method is easy to perform and can be used for high-throughput screening to test libraries of chemical compounds or to monitor the appearance of clinical strains resistant to echinocandins.

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REFERENCES

1. **Antachopoulos C, Meletiadis J, Sein T, Roilides E, Walsh TJ.** 2007. Concentration-dependent effects of caspofungin on the metabolic activity of *Aspergillus* species. Antimicrob. Agents Chemother. **51**:881–887.
2. **Antachopoulos C, Meletiadis J, Sein T, Roilides E, Walsh TJ.** 2008. Comparative in vitro pharmacodynamics of caspofungin, micafungin, and anidulafungin against germinated and nongerminated *Aspergillus* conidia. Antimicrob. Agents Chemother. **52**:321–328.
3. **Arikan S, Lozano-Chiu M, Paetznick V, Rex JH.** 2001. In vitro susceptibility testing methods for caspofungin against *Aspergillus* and *Fusarium* isolates. Antimicrob. Agents Chemother. **45**:327–330.
4. **Beauvais A, Drake R, Ng K, Diaquin M, Latge JP.** 1993. Characterization of the 1,3-beta-glucan synthase of Aspergillus fumigatus. J. Gen. Microbiol. **139**:3071–3078.
5. **Beauvais A, et al.** 2005. Two alpha(1–3) glucan synthases with different functions in *Aspergillus fumigatus*. Appl. Environ. Microbiol. **71**:1531–1538.
6. **Ben-Ami R, Lewis RE, Kontoyiannis DP.** 2011. In vitro interactions among echinocandins against Aspergillus fumigatus: lack of concordance among methods. Med. Mycol. **49**:285–288.
7. **Bowman JC, et al.** 2002. The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus* in vitro. Antimicrob. Agents Chemother. **46**:3001–3012.
8. **Chryssanthou EM, Cuenca-Estrella M.** 2006. Comparison of the EUCAST-AFST broth dilution method with the CLSI reference broth dilution method (M38-A) for susceptibility testing of posaconazole and voriconazole against *Aspergillus* spp. Clin. Microbiol. Infect. **12**:901–904.
9. **Fortwendel JR, et al.** 2009. Differential effects of inhibiting chitin and 1,3-β-D-glucan synthesis in Ras and calcineurin mutants of *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **53**:476–482.
10. **Kurtz MB, et al.** 1994. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **38**:1480–1489.
11. **Lewis RE, Albert ND, Kontoyiannis DP.** 2008. Comparison of the dose-dependent activity and paradoxical effect of caspofungin and micafungin in a neutropenic murine model of invasive pulmonary aspergillosis. J. Antimicrob. Chemother. **61**:1140–1144.
12. **Mouyna I, et al.** 2010. Members of protein O-mannosyltransferase family in Aspergillus fumigatus differentially affect growth, morphogenesis and viability. Mol. Microbiol. **76**:1205–1221.
13. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38-A. National Committee for Clinical Laboratory Standards, Wayne, PA.
14. **Rocha EM, Garcia-Effron G, Park S, Perlin DS.** 2007. A Ser678Pro substitution in Fks1p confers resistance to echinocandin drugs in *Aspergillus fumigatus*. Antimicrob. Agents Chemother. **51**:4174–4176.
15. **Te Dorsthorst DT, Zwaaftink RB, Rijs AJ, Meletiadis J, Verweij PE.** 2007. A colorimetric and spectrophotometric method for in vitro susceptibility testing of Aspergillus species against caspofungin. Med. Mycol. **45**:351–355.
16. **Tortorano AM, et al.** 2002. Effect of medium composition on static and cidal activity of amphotericin B, itraconazole, voriconazole, posaconazole and terbinafine against Aspergillus fumigatus: a multicenter study. J. Chemother. **14**:246–252.
17. **Yamaguchi H, Uchida K, Nagino K, Matsunaga T.** 2002. Usefulness of a colorimetric method for testing antifungal drug susceptibilities of Aspergillus species to voriconazole. J. Infect. Chemother. **8**:374–377.