ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 2010, p. 2738–2740 Vol. 54, No. 6
0066-4804/10/$12.00 doi:10.1128/AAC.01546-09

Madurella mycetomatis Is Not Susceptible to the Echinocandin Class of Antifungal Agents▽

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Received 2 November 2009/Returned for modification 28 February 2010/Accepted 21 March 2010

Eumycetoma caused by *Madurella mycetomatis* is treated surgically and with high doses of ketoconazole. Therapeutic responses are poor, and recurrent infections are common. In search of therapeutic alternatives in the treatment of mycetoma, we determined the *in vitro* susceptibilities of *M. mycetomatis* isolates against caspofungin, anidulafungin, and micafungin. As a comparator fungus, *Aspergillus fumigatus* was used. Minimal effective concentrations (MECs) and MICs were assessed and compared to those of ketoconazole. *M. mycetomatis* isolates were not susceptible to the echinocandins.

Eumycetoma is a subcutaneous disease caused by a variety of microorganisms, both bacteria and fungi. The most common causative fungus is *Madurella mycetomatis*. After surgical debridement, eumycetoma is usually treated for extended periods of time with high doses of either itraconazole (ITZ) or ketoconazole (KTZ), which can result in hepatoxicity. In order to identify alternative antifungal therapies, the susceptibilities of *M. mycetomatis* to other antifungal agents (amphotericin B, 5-flucytosine, fluconazole, and voriconazole) have been determined before and compared to the obtained susceptibilities to ITZ and KTZ. *M. mycetomatis* remains most susceptible toward the azoles and amphotericin B; no activity was seen with 5-flucytosine (10).

The echinocandins are a relatively new class of antifungal agents, with caspofungin (CAS), anidulafungin (ANI), and micafungin (MICA) as its licensed representatives. Echinocandins inhibit the synthesis of 1,3-β-glucan, the main component of the fungal cell wall. In *Candida* spp., the echinocandins are fungicidal, but in molds such as *Aspergillus* species, the echinocandins show fungistatic activity. Limited activity has been noted against zygomycetes, basidiomycetes, and some *Scedosporium* species (12). Only one study addressed the susceptibility of *M. mycetomatis* to the echinocandins. In that study, the susceptibilities of only 3 isolates of *M. mycetomatis* against ANI were determined (6). No data are available for the other echinocandins.

We determined the *in vitro* susceptibilities of 17 clinical *M. mycetomatis* isolates to CAS, ANI, and MICA in comparison to the *in vitro* susceptibility of *A. fumigatus* ATCC 204305. All *M. mycetomatis* isolates were identified by internal transcribed spacer (ITS) sequencing. For *M. mycetomatis*, as a comparator, MICs were also determined for KTZ (Janssen Pharmaceuticals, Beerse, Belgium).

MICs were determined independently in triplicate in RPMI medium by using the previously reported 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide (XTT) assays for *M. mycetomatis* and *A. fumigatus* as described elsewhere (1, 10, 11). For *A. fumigatus*, conidia were exposed to the antifungal agents, while for *M. mycetomatis*, a hyphal inoculum was used, since this fungus does not usually sporulate on agar plates. In the past, hyphal inocula were also prepared for *A. fumigatus*, and it appeared that hyphal fragments show antifungal agent susceptibilities similar to those of conidia (11). The MIC endpoints for each antifungal agent were defined as the first concentrations resulting in a spectrophotometric reduction of more than 80%. The minimal effective concentration (MEC) endpoint was determined as the first concentration in which altered

TABLE 1. Susceptibilities of *M. mycetomatis* and *A. fumigatus* to ketoconazole, caspofungin, anidulafungin, and micafungin

Species	Strain	MIC (MEC) (mg/liter)[a]			
		KTZ	CAS	ANI	MICA
M. mycetomatis	Mm31	0.063	64	>128	>128
	Mm35	1	128	>128	>128
	Mm36	0.063	128	>128	>128
	Mm39	0.031	64	>128	>128
	Mm41	0.125	16	0.5	8
	Mm43	0.063	128	>128	128
	Mm45	0.25	64	>128	>128
	Mm46	<0.016	64	>128	>128
	Mm49	0.031	32	>128	>128
	Mm50	0.063	128	>128	>128
	Mm52	0.25	>128	>128	>128
	Mm54	0.031	64	>128	>128
	Mm55	0.25	128	>128	>128
	Mm64	0.063	64	>128	>128
	Mm68	0.125	64	>128	>128
	Mm73	0.063	64	>128	>128
	Mm83	0.125	128	>128	128
A. fumigatus	ATCC 204305	ND	128 (0.125)	>128 (<0.007)	>128 (<0.007)

[a] The *in vitro* antifungal susceptibilities of *M. mycetomatis* and *A. fumigatus* to ketoconazole (KTZ), caspofungin (CAS), anidulafungin (ANI), and micafungin (MICA) are shown. For all 17 *M. mycetomatis* isolates, the MICs are given; for the quality control *A. fumigatus* ATCC 204305 strain, both the MIC and the MEC (in parentheses) are given. ND, not done.

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▽ Published ahead of print on 29 March 2010.

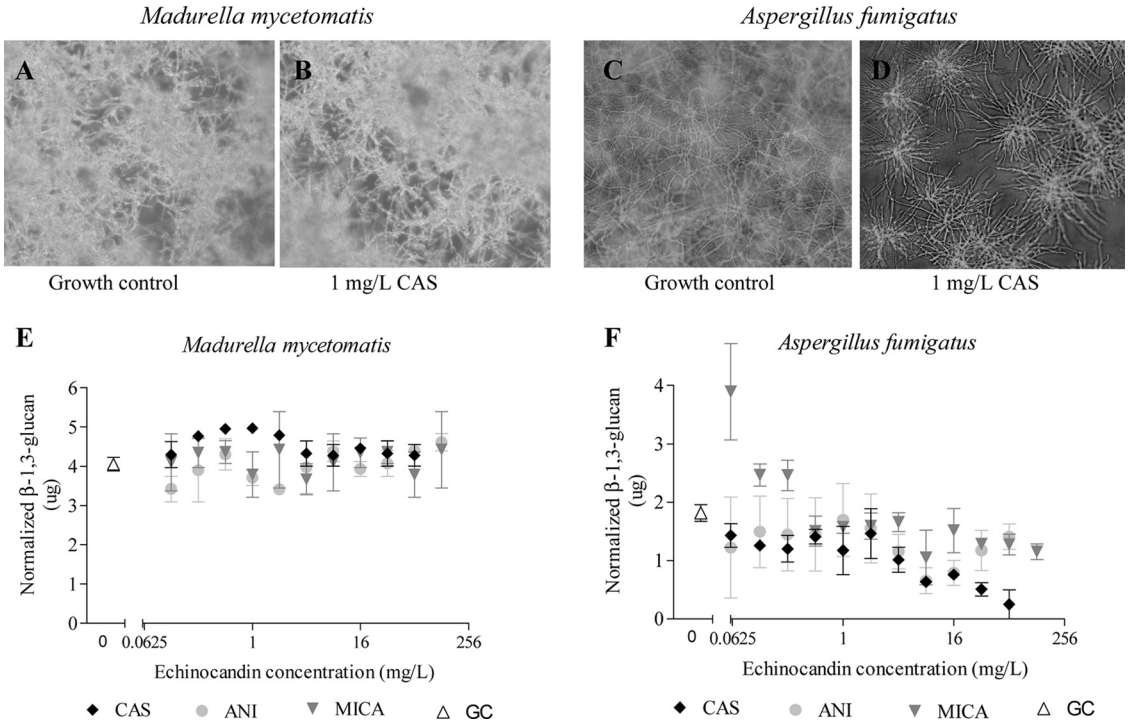

FIG. 1. Effect of echinocandins on *M. mycetomatis* and *A. fumigatus*. (A) *M. mycetomatis* growth control. (B) *M. mycetomatis* exposed to 1 mg/liter CAS. (C) *A. fumigatus* growth control. (D) *A. fumigatus* exposed to 1 mg/liter CAS. (E) *M. mycetomatis* β-1,3-D-glucan concentration of strain Mm55 as determined by the aniline blue assay. β-1,3-D-Glucan concentrations were corrected to the number of viable cells with the XTT assay by the following formula: (amount of beta-glucan measured) × (number of viable cells in tested well/number of viable cells in growth control). Each point represents the mean β-1,3-D-glucan concentration with the standard deviation. (F) *A. fumigatus* β-1,3-D-glucan concentration as determined by the aniline blue assay. β-1,3-D-Glucan concentrations were corrected to the number of viable cells with the XTT assay. Each point represents the mean β-1,3-D-glucan concentration with the standard deviation. CAS, caspofungin; MICA, micafungin; ANI, anidulafungin; GC, growth control.

growth was noticed. Twofold-increasing drug concentrations were used, and they ranged from 0.016 mg/liter to 16 mg/liter for KTZ and 0.007 mg/liter to 128 mg/liter for CAS (Merck and Company, Rahway, NJ), ANI (Pfizer BV, Capelle aan de Ijsel, Netherlands), and MICA (Astellas Pharma, Leiderdorp, Netherlands). KTZ, CAS, and ANI were diluted in dimethyl sulfoxide (DMSO), and MICA was diluted in normal saline. The final concentration of DMSO per inoculum was as stated by the CLSI (2).

To determine the β-1,3-glucan concentration, microcentrifuge tubes were inoculated with 100 μl of an *M. mycetomatis* hyphal suspension in RPMI or an *A. fumigatus* conidial suspension in RPMI as described above. After incubation with the antifungal agents (7 days at 37°C for *M. mycetomatis* or 48 h for *A. fumigatus*), the mycelium was freeze-dried, and 250 μl of 1 M NaOH was added. This mixture was sonicated with a microprobe for 15 s at 26 μm and incubated at 52°C for 30 min. Glucan levels were determined by aniline blue fluorescence as described elsewhere, by using curdlan (Sigma) as a positive control (3, 8).

In accordance with previously published MICs for *M. mycetomatis*, all strains were strongly inhibited by KTZ, the drug of choice to treat eumycetoma in Sudan (Table 1). MICs for KTZ ranged from <0.016 mg/liter to 1 mg/liter. A concentration of 0.25 mg/liter was needed to inhibit the growth of 90% of the isolates (Table 1). Most of the *M. mycetomatis* strains were not inhibited in growth by the echinocandins (Table 1). Most MICs for CAS were 128 mg/liter, while the MICs of ANI and MICA were above 128 mg/liter (Table 1). As is seen in Table 1, only for isolate Mm41 were lower MICs obtained, and these were 16 mg/liter for CAS, 0.5 mg/liter for ANI, and 8 mg/liter for MICA. The results shown here are different from previously published susceptibility data for *M. mycetomatis*. In that study, the spores of three sporulating strains of *M. mycetomatis* were used. Conidia were harvested and exposed to ANI, and MICs of 1 mg/liter were obtained (6). The species *M. mycetomatis* is not well characterized, and in the past, misidentifications have occurred. One of the key features of this species is its lack of sporulation on agar plates. To ascertain that only *M. mycetomatis* isolates were used in the present study, all isolates were identified by ITS sequencing. None of our isolates did sporulate, and we therefore used hyphal fragments to determine the *in vitro* susceptibilities against the echinocandins. Our inoculation procedure, therefore, differs from that of Odabasi et al. (6), which could explain the discrepancy in results. Another explanation could be that the three isolates of Odabasi et al. resembled isolate Mm41, which in our study also appeared to be susceptible to anidulafungin. Since the isolates of Odabasi et al. were not used in our study, we cannot exclude this possibility.

In the present study, Mm41 behaved different from the other *M. mycetomatis* isolates with regard to echinocandin suscepti-

bility; it is the only isolate which shows some susceptibility toward the echinocandins, especially against ANI. Mm41 is not morphologically different from the other *M. mycetomatis* isolates and has the same cellular beta-glucan quantity as the other isolates. Furthermore, when this isolate was typed by selective amplification of restriction fragments (amplified fragment length polymorphism [AFLP]), this isolate clustered together with other *M. mycetomatis* isolates isolated from Sudan and used in this study (9).

For *A. fumigatus*, growth was not completely inhibited by high concentrations of the echinocandins. Only at very high concentrations was lack of growth noticed (CAS [MIC of 128 mg/liter], ANI [MIC of 128 mg/liter], and MICA [MIC of >128 mg/liter]). At much lower concentrations, growth alteration was noted (MEC of 0.125 mg/liter for CAS and MECs of <0.03 mg/liter for ANI and MICA) (Fig. 1C and D). Therefore, it was investigated if alteration of growth also occurred in *M. mycetomatis* after exposure to the echinocandins. As shown in Fig. 1A and B, no growth alteration was observed under the tested conditions when *M. mycetomatis* was exposed to CAS, ANI, or MICA (the last two are not shown).

To confirm the lack of echinocandin activity against *M. mycetomatis*, β-1,3-glucan production was determined in *M. mycetomatis* and *A. fumigatus*. As shown in Fig. 1, under the experimental conditions, all three echinocandins were unable to inhibit β-1,3-D-glucan synthesis in *M. mycetomatis*. β-1,3-D-Glucan concentrations were documented for *M. mycetomatis* isolates not exposed to an echinocandin that were similar to those for *M. mycetomatis* isolates exposed to various echinocandin concentrations, even in ANI-inhibited isolate Mm41. In contrast, in *A. fumigatus*, the echinocandins did inhibit β-1,3-D-glucan synthesis as seen by the lowering β-1,3-D-glucan concentrations represented in Fig. 1F and reported by Kahn et al. (3).

From our results, it appears that *M. mycetomatis* is not susceptible to the echinocandin class of antifungal agents. The reason behind this intrinsic resistance was not explored in the present study, but some clues might be obtained from other fungi. Echinocandin agents are also ineffective against *Fusarium* species, *Cryptococcus neoformans*, and agents of zygomycosis. Resistance in *Fusarium solani* is shown to be partly caused by certain amino acid substitutions in the protein encoded by the target gene *fks1* (4). Differences in the *fks1* gene are not the only mechanism underlying echinocandin resistance. In the caspofungin-resistant fungus *C. neoformans*, the FKS enzyme itself was fully inhibited by low concentrations of CAS (5). Since the echinocandins require transport into the cell to their site of action, the surface properties of fungi might contribute to resistance. Since *C. neoformans* is highly melanized, it was hypothesized that this melanization could affect echinocandin susceptibility (7). For *M. mycetomatis*, the *fks1* sequence is not known, but it has been demonstrated that the fungus can produce melanin both *in vitro* and *in vivo*. Further study is needed to determine the mechanism of this resistance.

In conclusion, in our assay, the echinocandins CAS, ANI, and MICA are not active against *M. mycetomatis*. There was no inhibition in growth, growth alteration, or reduction in β-1,3-glucan biosynthesis noted for *M. mycetomatis* isolates after exposure to these antifungal agents in the assays used. Therefore, the therapeutic potential of the echinocandins in the treatment of mycetoma infections caused by *M. mycetomatis* remains doubtful.

We have no transparency declarations to declare.

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