MADURELLA MYCETOMATIS IS NOT SUSCEPTIBLE TO THE ECHINOCANDIN CLASS OF ANTIFUNGAL AGENTS

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Abstract

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. MEC and MIC values were assessed, and compared to ketoconazole. M. mycetomatis isolates were not susceptible to the echinocandins.
Introduction

Eumycetoma is a subcutaneous disease caused by a variety of micro-organisms, 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 hepatotoxicity. 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 susceptibilities obtained for ITZ and KTZ. M. mycetomatis remains most susceptible towards the azoles and amphotericin B; no activity was seen with 5-flucytosine.

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 moulds such as Aspergillus species the echinocandins show fungistatic activity. Limited activity is noticed against zygomycetes, basidiomycetes and some species of Scedosporium (12). Only one study addressed the susceptibility of M. mycetomatis to the echinocandins. In that study the susceptibility of only 3 isolates of M. mycetomatis was determined against ANI (6). No data are available for the other echinocandins.

We determined the in vitro susceptibilities of 17 clinical M. mycetomatis isolates against CAS, ANI and MICA in comparison to the in vitro susceptibility of A. fumigatus ATCC 204305. All M. mycetomatis isolates were identified by 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 using the previously reported 2,3-Bis(2-Methoxy-4-Nitro-5-Sulfophenyl)-5-[(Phenylamino)Carbonyl]-2H-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.
and it appeared that hyphal fragments show similar susceptibility for the antifungal agents compared to conidia (11). The MIC endpoints for each antifungal agent were defined as the first concentration resulting in a spectrophotometric reduction of more than 80%. The MEC endpoint was determined as the first concentration in which altered growth was noticed. Two-fold increasing drug concentrations were used ranging from 0.016 mg/L to 16 mg/L for KTZ and 0.007 mg/L to 128 mg/L for CAS (Merck and Company, Rahway, NJ, USA), ANI (Pfizer BV, Capelle aan de IJssel, The Netherlands) and MICA (Astellas Pharma, Leiderdorp, The Netherlands). KTZ and ANI were diluted in DMSO, CAS and MICA in normal saline. The final concentration 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 a 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 48h for A. fumigatus), the mycelium was freeze-dried, and 250 µl of 1M NaOH was added. This was sonicated with a microprobe for 15 seconds at 26 micron and incubated at 52°C for 30 minutes. Glucan levels were determined by aniline blue fluorescence as described elsewhere, by using curdlan (Sigma) as 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/L to 1 mg/L. A concentration of 0.25 mg/L 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/L while the MICs of ANI and MICA were above 128 mg/L (Table 1). As is seen in Table 1 only for isolate mm41 lower MICs were obtained, namely 16 mg/L for CAS, 0.5 mg/L for ANI and 8 mg/L 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/L were obtained (6). The species M. mycetomatis is not well-characterised 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. 

fumigatus
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 (6) which could explain the discrepancy in results. Another explanation could be that the three isolates of Odabasi resembled isolate mm41, which in our study also appeared to be susceptible to anidulafungin. Since the isolates of Odabasi were not used in our study we cannot exclude this later possibility.

In the present study Mm41 behaves different from the other M. mycetomatis isolates with regard to echinocandin susceptibility, it is the only isolate which shows some susceptibility towards 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 typing this isolate by selective amplification of restriction fragments (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 lack of growth was noticed (CAS (MIC of 128 mg/L), ANI (MIC of 128 mg/L) and MICA (MIC of >128 mg/L)). At much lower concentrations, growth alteration was noted (MEC of 0.125 mg/L for CAS and MECs of <0.03 mg/L for ANI and MICA) (Figure 1C and 1D). Therefore, it was investigated if alteration of growth also occurred in M. mycetomatis after being exposed to the echinocandins. As is shown in figures 1A and 1B, no growth alteration was observed under the tested conditions when M. mycetomatis was exposed to CAS, ANI or MICA (latter two 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 is seen in Figure 1, under the experimental conditions all three echinocandins were unable to inhibit β-1,3-D-glucan synthesis in M. mycetomatis. Similar β-1,3-D-glucan concentrations were documented for M. mycetomatis isolates not exposed to an echinocandin as for M. mycetomatis isolates exposed to various echinocandin concentrations, even in the 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 Figure 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 aminoacid substitutions in 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 melanised, it was hypothesised that this melanisation 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*. Neither inhibition in growth, growth alteration or reduction in β-1,3-glucan biosynthesis were noted for *M. mycetomatis* isolates after exposure to these antifungal agents in the assays used. Therefore, therapeutic potential of the echinocandins in the treatment of mycetoma infections caused by *M. mycetomatis* remains doubtful.

**Transparency declarations**

None to declare
References


Table 1: Susceptibility of *M. mycetomatis* and *A. fumigatus* to Ketoconazole, Caspofungin, Anidulafungin and Micafungin

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<th>Species</th>
<th>Strain</th>
<th>KTZ (mg/L)</th>
<th>CAS (mg/L)</th>
<th>ANI (mg/L)</th>
<th>MICA (mg/L)</th>
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<td>0.5</td>
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<td><em>A. fumigatus</em></td>
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<td>&gt;128 (&lt;0.007)</td>
<td>&gt;128 (&lt;0.007)</td>
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Table 1: The *in vitro* antifungal susceptibilities of *M. mycetomatis* and *A. fumigatus* to ketoconazole (KTZ), caspofungin (CAS), anidulafungin (ANI) and micafungin (MICA). For all 17 *M. mycetomatis* isolates the MICs were given, for the quality control *A. fumigatus* ATCC 204305 both the MIC and the MEC are given. The latter is shown between brackets. ND = not done.
Figure 1: Effect of echinocandins on *M. mycetomatis* and *A. fumigatus*. A. *M. mycetomatis* growth control, B. *M. mycetomatis* exposed to 1 mg/L CAS, C. *A. fumigatus* growth control, D. *A. fumigatus* exposed to 1 mg/L 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 betaglucan 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. 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.