Invasive aspergillosis has emerged as a leading cause of morbidity and mortality in immunocompromised patients (7). Aspergillus terreus in particular is an amphotericin B (AMB)-resistant mold that has been recognized as a cause of frequently lethal infections (25). The exact mechanism of AMB action is still incompletely understood. Previous work has shown that AMB binds to the ergosterol in the fungal cell membrane; this step introduces aqueous pores into the lipid bilayers, and thus small ions leak out, causing a disruption of the proton gradient (24). Others have demonstrated AMB to cause cell death by oxidative damage (21). AMB is active against a wide range of fungi, yet certain species, such as Aspergillus fumigatus (21). So far, the reason for the in vitro and in vivo AMB resistance in A. terreus is not known. This study examined the basis for AMB resistance in A. terreus.

Clinical isolates of AMB-sensitive A. fumigatus (n = 10) and AMB-resistant A. terreus (n = 10) were used in this study. AMB susceptibility testing was performed according to the CLSI (formerly NCCLS) M38-A technique (16). In parallel, we prepared cell wall-free AMB protoplasts that were AMB sensitive (MIC < 1 μg/ml) and resistant (MIC > 2 μg/ml); protoplast preparation, including the determination of the regeneration rate, was done according to a protocol of Hearn et al. (10). MIC testing with protoplasts was performed in RPMI 1640 containing 0.55 M sorbitol as a stabilizer. Our results provide evidence that AMB resistance in A. terreus is not associated with the cell wall, as MICs for conidia and cell wall-free fungi did not differ. The mean AMB MICs for conidia and protoplasts of A. fumigatus were 0.21 ± 0.06 and 0.19 ± 0.05 μg/ml, and those for conidia and protoplasts of A. terreus were 1.70 ± 0.58 and 1.41 ± 0.38 μg/ml, respectively.

Fungal ergosterol was analyzed by a previously published method with slight modifications (2, 4). Fifty milliliters of Sambouraud medium (Merck, Vienna, Austria) was inoculated with 10⁶ conidia and incubated for 24 h with shaking (200 rpm) at 35°C. Subsequently, fungi were harvested by vacuum filtration, and the wet weight was determined; the pellet was minced with a blade, 6 ml of 25% alcoholic potassium hydroxide solution was added and mixed for 2 min, and the mixture was incubated at 85°C for 3 h. Sterols were extracted by adding 2 ml of sterile water and 6 ml of n-heptane, mixing for 4 min, and allowing the layers to separate for 2 h. The n-heptane layer was used for spectrophotometric analysis between 240 and 300 nm with a Beckmann spectrophotometer, and the ergosterol content was calculated as previously described (2). Overall, the data on fungal ergosterol contents and AMB resistance are contradictory: Sterling and Merz (22) and Dick et al. (9) showed that AMB resistance in yeasts is based on the absence or the reduction of fungal ergosterol; changes in the sterols and phospholipids affect membrane fluidity and, thus, decrease AMB impact (19). By contrast, Joseph-Horne et al. proposed non-sterol-related mechanisms of AMB resistance in Ustilago maydis (11); the ergosterol contents do not vary between sensitive and resistant strains. Based on in vivo and biochemical in vitro studies, Dannaoui et al. (7) and Walsh et al. (25) suggested that the AMB resistance of A. terreus is not related to a lack of fungal ergosterol. Our data also suggested that the ergosterol content does not explain AMB resistance. The mean ergosterol contents for A. fumigatus and A. terreus were 4.4 ± 1.4 and 5.1 ± 2.4 μg/mg, respectively.

It is speculated that AMB leads to cell death by acting as an oxidizing agent (21, 24). Hydrogen peroxide (H₂O₂), which permeates freely through biological membranes, potentially causes cell damage by lipid peroxidation (12). Therefore, hypheal lipid peroxidation and endogenous catalase levels were measured using the thiobarbituric acid test for malonaldehyde (6, 18) and the H₂O₂ degradation test (1); the decrease in absorbance at a wavelength of 240 nm over 30 s was recorded (5), and the protein concentration was determined with the
Bradford method (3). There were no clear differences in lipid peroxidation levels at moderate AMB concentrations between *A. fumigatus* and *A. terreus* (Fig. 1A). However, the addition of 5 and 10 μg of AMB/ml resulted in significantly higher levels of lipid peroxidation in *A. fumigatus* than in *A. terreus*. Catalases are ubiquitous metalloenzymes in aerobic organisms and protect cells from oxidative damage by converting H$_2$O$_2$ into 2H$_2$O and O$_2$. Sokol-Anderson et al. demonstrated oxidative damage to be involved in the AMB-induced killing of *C. albicans* (21). This effect was stopped by the addition of exogenous catalase. Our results show that *A. terreus* produced significantly more catalase than *A. fumigatus* (Fig. 1B), a finding which may play a role in the AMB resistance. *A. terreus* may be able to catch toxic H$_2$O$_2$ and transform it into less toxic substances before H$_2$O$_2$ can harm the fungus. Differences in oxidative-stress-buffering capacity may underlie the differential susceptibilities to AMB.

This study shows that ergosterol content and cell wall composition do not play a crucial role in the AMB resistance of *A. terreus*. In contrast, catalase activity in *A. terreus* was significantly stronger than that in *A. fumigatus* (*P* < 0.05). The level of lipid peroxidation was higher in *A. fumigatus*, yet only at very high AMB concentrations. Catalase may be one of the several reactive-oxidant scavengers playing a role in AMB resistance in *A. terreus*.

REFERENCES


9. Reference deleted.


14. Reference deleted.


