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New Insight into Amphotericin B Resistance in *Aspergillus terreus*

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Amphotericin B (AMB) is the predominant antifungal drug, but the mechanism of resistance is not well understood. We compared the *in vivo* virulence of an AMB-resistant *Aspergillus terreus* (ATR) isolate with that of an AMB-susceptible *A. terreus* isolate (ATS) using a murine model for disseminated aspergillosis. Furthermore, we analyzed the molecular basis of intrinsic AMB resistance *in vitro* by comparing the ergosterol content, cell-associated AMB levels, AMB-induced intracellular efflux, and prooxidant effects between ATR and ATS. Infection of immunosuppressed mice with ATS or ATR showed that the ATS strain was more lethal than the ATR strain. However, AMB treatment improved the outcome in ATS-infected mice while having no positive effect on the animals infected with ATR. The *in vitro* data demonstrated that ergosterol content is not the molecular basis for AMB resistance. ATR absorbed less AMB, discharged more intracellular compounds, and had better protection against oxidative damage than the susceptible strain. Our experiments showed that ergosterol content plays a minor role in intrinsic AMB resistance and is not directly associated with intracellular cell-associated AMB content. AMB might exert its antifungal activity by oxidative injury rather than by an increase in membrane permeation.

Invasive mold infections (IMI) are a significant determinant of morbidity and mortality in patients undergoing cancer chemotherapy, hematopoietic stem cell transplantation, or solid organ transplantation (1–3). These infections remain difficult to manage with therapeutic treatments because of a usually late diagnosis and complication of the treatment procedure by toxicity or interactions of drugs (4). The majority of IMI are caused by *Aspergillus* spp., and the most pathogenic species are *Aspergillus fumigatus*, *Aspergillus terreus*, and *Aspergillus flavus* (5). In particular, *A. terreus*, a widespread soil saprophyte and producer of several secondary metabolites, is a common cause of infection at the University Hospital of Innsbruck (UHI) in Austria (6–9). *In vivo* and *in vitro* data indicate that almost all *A. terreus* isolates are intrinsically resistant to amphotericin B (AMB), a fungicidal heptaene macrolide antimycotic, and a high mortality rate in patients is associated with this particular mold (10–12).

Previous work has shown that AMB binds to ergosterol, the principal sterol in the fungal cell membrane, and forms aqueous pores in the lipid bilayers. Subsequently, proteins and amino acids leak out, which in turn leads to disrupted membrane proton gradients (13–16). AMB resistance is rare, and it has been suggested that for *A. flavus* and *Candida albicans*, the ergosterol content (17), the composition of the fungal cell wall (18), and the ability to produce catalase might play a role in AMB resistance (19). Sokol-Anderson et al. speculated that AMB causes cell death in *C. albicans* by oxidative damage (19). Despite intensive research for over 50 years, the exact mechanism of action of AMB is still incompletely understood, and the principles of resistance need to be elucidated in more detail. Based on the conventional hypothesis of the AMB mode of action, we compared the responses of AMB-resistant *A. terreus* (ATR) and AMB-susceptible *A. terreus* (ATS) to AMB. The role of fungal ergosterol, cellular AMB uptake (cell-associated AMB content), efflux of intracellular compounds (e.g., potassium), and the presence of oxidative intracellular damages were analyzed in detail. A murine model for disseminated aspergillosis gives insight into the clinical relevance of *in vitro* AMB resistance and its correlation with fitness and virulence *in vivo*.

MATERIALS AND METHODS

***Aspergillus terreus* strains.** All *A. terreus* isolates used in this study were derived from clinical specimens, and the AMB MICs, tested according to EUCAST and CLSI guidelines (20, 21), were 0.5 μg/ml for ATS ($n = 1$) and 4 μg/ml for ATR ($n = 4$). Sublethal (0.1 μg/ml) and lethal (10 μg/ml) AMB deoxycholate (Bristol Meyer Squibb, Austria) concentrations were used for all experiments. AMB preparation was done according to the manufacturer's recommendations. Sublethal and lethal AMB concentrations were chosen according to the strain susceptibility patterns and MIC distribution. Strains with AMB MICs of >2 μg/ml were resistant (22). ATS and ATR were susceptible to other antifungal agents (i.e., echinocandins and azoles). To validate species identification, *A. terreus* strains (ATS and ATR) were analyzed by sequencing the internal transcribed spacer (the ITS3 to ITS4 region), calmodulin, β-tubulin, enolase, and cytochrome B (23–25).

Experimental animal model of ATS and ATR. The pathogenicities of ATR ($n = 1$) and ATS ($n = 1$) with and without AMB (Bristol Meyer Squibb, Austria) treatment were compared in a murine model of invasive disseminated *Aspergillus* infection. Inbred BALB/c mice were used for our experiments to guarantee that the immunological backgrounds of the animals were the same, thus enabling optimal comparability. Twenty-four mice (average weight, 20 to 25 g; age, 14 to 16 weeks) were randomly divided in 4 groups and intravenously (i.v.) injected with 200 mg of cyclophosphamide/kg of body weight on day −3 and every 5th day to produce prolonged immunosuppression. All the mice were inoculated with ATR or ATS on day 0 by intravenous injection of 1×10^6 conidia (100 μl of a 10^7-conidia/ml stock solution in 0.9% NaCl) into the tail vein. To control the correct number and viability of the conidia, the final inoculum was checked by plating 100 μl onto Sabouraud glucose (SAB) agar plates for 48 h at 36 ± 1°C. Groups 1 and 3 received ATR, and groups 2 and 4

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received ATS. The mice in groups 1 and 2 were intraperitoneally treated from days 1 to 21 with 5 mg/kg amphotericin B deoxycholate (26). Groups 3 and 4 served as untreated control groups and received daily 0.9% NaCl. The animals were observed daily for clinical symptoms (i.e., lack of food and water uptake, apathy, stiff movement, and forced ventilation). Clinical endpoints were set for moribund mice, which were sacrificed by cervical dislocation. The surviving mice were sacrificed on day 21 by cervical dislocation. Survival was the main focus; therefore, we did not perform CFU counts in this particular study. This animal study was performed in compliance with the Austrian Law for Experimental Animals and in accordance with the Austrian guidelines on care and use of animals for scientific purposes. The protocol was approved by the Austrian Ministry of Science and Research.

Cultivation of *A. terreus* strains. ATR ($n = 4$) and ATS ($n = 1$) cultures (inoculum, 1×10^6 to 4×10^6 CFU/ml; the number and viability control were checked via plating on SAB agar plates and incubating for 48 h at 36 ± 1°C) were grown in RPMI 1640 medium (Sigma-Aldrich, Austria) containing 20 g/liter glucose at 37°C and gently shaken (200 rpm) for 24 h. Cell pellets were harvested, weighed to equivalent amounts, treated, and analyzed according to the following protocols.

Ergosterol quantification. Ergosterol was extracted and saponified from a lyophilized biomass in hot methanol-ethanol-KOH (10 ml/2.5 ml/1 g) solution and extracted into hexane. After the evaporation of hexane, the residue was dissolved in methanol and centrifuged, and the ergosterol content was quantified by high-performance liquid chromatography (HPLC) on a reversed-phase C_{18} (5-cm) column with 95% (vol/vol) methanol (flow rate, 1.0 ml/min), detected at 282 nm. Ergosterol of the same structure (Sigma-Aldrich, Austria) was used as the standard (27).

Cell-associated AMB content. The fungal biomass was weighed and treated in sterile ultrapure water with lethal and sublethal AMB (Bristol Meyer Squibb, Austria) concentrations, respectively, for 4 and 24 h. Hyphae were then filtered (2-μm filter), washed thrice with sterile ultrapure water, weighed, and finely ground in liquid nitrogen. After the addition of 10 ml methanol and 1 h of incubation at room temperature, AMB was quantified via HPLC as described previously (28).

Release of intracellular protein, amino acids, and potassium. Cultures were filtered (2-μm filter), washed thrice with sterile ultrapure water, weighed, and resuspended in 50 ml ultrapure water. After treatment with lethal and sublethal AMB (Bristol Meyer Squibb, Austria) concentrations for 1 to 4 h, the biomass was separated by filtration, and the concentrations of the released intracellular compounds were analyzed. To highlight the AMB effect, the background efflux values were normalized to 0 for all strains. The measurement of each compound release was done as follows.

Protein was quantified by the Bradford method (29).

Amino acids were determined by the ninhydrin colorimetric method and expressed in terms of aspartic acid (Sigma-Aldrich) and glutamic acid (Sigma-Aldrich), which were used as standards. A ninhydrin (Sigma-Aldrich) solution (200 μl; stock, 0.35 g in 100 ml ethanol) was added to each sample (1 ml) and heated to 95°C for 4 min. After cooling to room temperature in an ice bath, the absorbance at 570 nm was recorded on a spectrophotometer (Beckman) (15).

Potassium release was detected with an ion chromatography (30) system (C_4 100/4.0 column, IC conductivity detector; Metrohm). The eluent flow rate (1.7 mmol/liter nitric acid and 0.75 mmol/liter dipicolinic acid) was 0.9 ml/min.

Catalase activity. The cultures were filtered (2-μm filter), washed thrice with sterile ultrapure water, weighed, and resuspended in 50 ml ultrapure water. H_2O_2 degradation by catalase was measured as described previously (31).

GAPDH activity. From the hyphal pellets, 0.2 g each were weighed and incubated in 2 ml of AMB (Bristol Meyer Squibb, Austria) at lethal and sublethal concentrations for 10 to 30 min under continuous agitation at 37°C. Subsequently, fungi were pelleted by centrifugation at 1,800 × *g* for 5 min, and the supernatant was discarded. The pellet was homogenized for 5 min in a Mixer Mill homogenizer (Qiagen) after the addition of 1 ml cooled PBS and centrifuged at 18,000 × *g*. In the supernatant, conversion of NADH to NAD^+ (glyceraldehyde-3-phosphate dehydrogenase [GAPDH] activity) was measured spectrophotometrically by the decrease of the peak at 340 nm, as described previously (32).

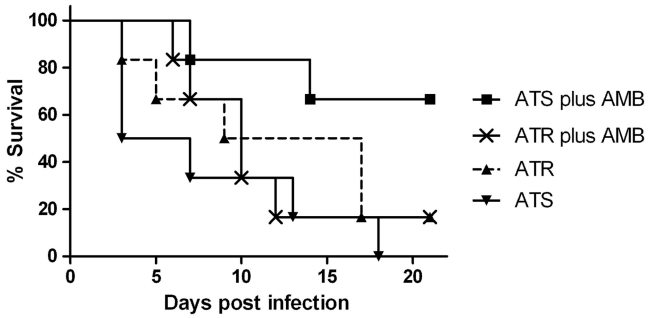

FIG 1 Survival of amphotericin B (AMB)-treated and untreated *Aspergillus terreus* infection. Mice were infected with 1×10^6 conidia. Shown is the improved survival rate of AMB-susceptible *A. terreus* (ATS) plus AMB treatment compared to that of untreated ATS and AMB-resistant *A. terreus* (ATR) with and without treatment ($P < 0.05$, analyzed by the log-rank test).

Lipid peroxidation. Cultures were filtered (2-μm filter), washed thrice with sterile ultrapure water, weighed, and resuspended in 50 ml ultrapure water. After treatment with lethal and sublethal AMB concentrations for 4 and 24 h, hyphal lipid peroxidation was measured with some modifications (33). Mycelia were finely ground in fluid nitrogen, and 50 mM phosphate buffer (pH 7) was added. Sixty-seven microliters of this suspension was mixed with 933 μl 20% trichloroacetic acid (TCA) plus 0.5% thiobarbituric acid (TBA) and incubated at 95°C for 30 min. The tubes were cooled (to ~5°C) in an ice bath and centrifuged for 10 min at 10,000 × *g* and 5°C. Following centrifugation, the specific and nonspecific absorbances were read at 532 and 600 nm, respectively. Twenty percent TCA plus 0.5% TBA was used as the reference standard. After subtraction of the nonspecific absorbance from the specific absorbance, the net absorbance at 532 nm was expressed in terms of the amount of protein in the solutions. The protein amount in the solution used for measurement was quantified by the Bradford method (29).

Statistics. All *in vitro* assays were performed on three independent replicates. The results presented are the means ± standard errors. Statistical analyses were performed using Student's two-tailed *t* test or one-way analysis of variance (ANOVA) with the Bonferroni multiple-comparison test. Kaplan-Meier survival curves were analyzed with the log-rank test. *P* values of <0.05 were considered statistically significant.

RESULTS

The survival rates of mice infected with ATS and those infected with ATR are shown in Fig. 1. ATS infection killed *per se* 50% of the mice by day 3 and was significantly more lethal than ATR ($P < 0.05$). At day 18, all mice infected with the ATS strain had died, whereas 17% of the animals that were infected with ATR and received no treatment survived. Treatment with AMB improved survival in ATS infection by up to 75%, while this was not the case with ATR infection ($P < 0.05$).

Ergosterol and cell-associated AMB in ATS and ATR. The ergosterol content of ATR and ATS showed no significant differences, as the mean ergosterol values were 5.41 ± 1.96 and 5.26 ± 1.34 μg/mg, respectively. ATR and ATS showed significant differences in cell-associated AMB content (Fig. 2). The accumulated AMB concentration was significantly higher in ATS than in ATR

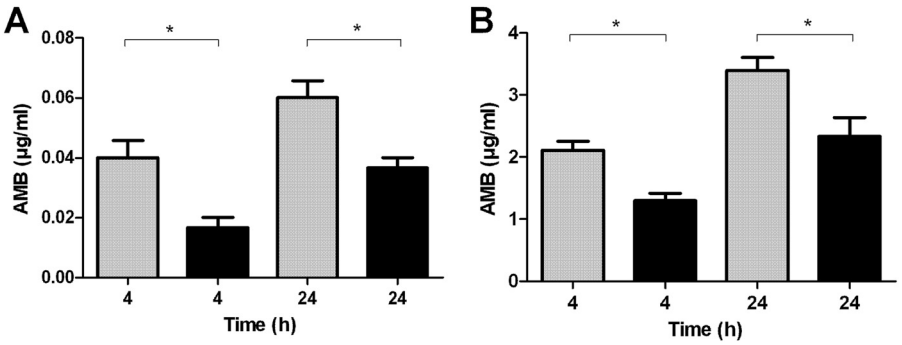

FIG 2 Cell-associated amphotericin B (AMB) content of susceptible (gray columns) and resistant (black columns) *Aspergillus terreus*. Fungal hyphae were treated with different AMB concentrations for 4 and 24 h. (A) Cell-associated AMB content of resistant (ATR) and susceptible (ATS) *A. terreus* incubated with sublethal (0.1 μg/ml) AMB concentrations. (B) Cell-associated AMB content of ATR and ATS incubated with lethal (10 μg/ml) AMB concentrations. *, $P < 0.05$, analyzed with Student's two-tailed *t* test.

after 4 and 24 h of incubation with both concentrations (sublethal and lethal) tested ($P < 0.05$).

Differences in protein, amino acid, and potassium release of ATS versus ATR. In response to AMB, intracellular protein, amino acid, and potassium release differed in ATR and ATS (Fig. 3). ATR showed higher protein ($P < 0.05$) and amino acid release than ATS did at both concentrations and time points tested. The highest amino acid peaks in ATR were detected after 4 h of AMB treatment with sublethal and lethal concentrations (3.29 ± 0.59 and 10.14 ± 1.9 μg/ml, respectively). Similar data were obtained for potassium efflux in ATR (54.41 ± 5.65 mg/liter). In ATS, the highest potassium release (46.81 ± 5.55 mg/liter) was measured after 2 h of incubation with the lethal concentration.

AMB-induced oxidative injury. The basal catalase activity of ATR was significantly ($P = 0.010$) higher than that of ATS (Fig. 4). The mean catalase activity levels of ATR and ATS were 2.54 ± 0.24 and 0.77 ± 0.08 U/mg, respectively.

Conversion of NADH to NAD^+ by the fungal GAPDH was demonstrated by the decrease of absorption at 340 nm (Fig. 5, controls) in both ATR and ATS. Oxidative GAPDH inactivation due to AMB is shown in Fig. 5 by the significantly lower decrease of NADH ($P < 0.01$) in both AMB-treated samples versus the controls. The absorbance values of ATS and ATR were also significantly different ($P < 0.05$).

Lipid peroxidation was significantly higher in ATS than in ATR ($P < 0.05$), independent of AMB concentration and exposure time (Fig. 6). ATS treated with 0.1 μg/ml AMB showed nearly the same effect on peroxidation after 4 and 24 h of incubation. Ten micrograms per milliliter AMB increased lipid peroxidation dramatically, especially after 24 h of incubation. In general, ATR showed less peroxidation than ATS did, and after 24 h of AMB treatment, the lipid peroxidation level decreased.

DISCUSSION

This study investigated the differences between the *in vivo* and *in vitro* responses of AMB-resistant and -susceptible *A. terreus* strains to AMB treatment. Our *in vivo* murine model of disseminated aspergillosis showed ATS (AMB MIC, 0.5 μg/ml) to be highly virulent, indicating that loss of fungal fitness is not associated with the appearance of AMB susceptibility. AMB therapy (5 mg/kg) significantly enhanced the *in vivo* outcome in mice infected with ATS, as 75% of them survived. The survival rates of ATR-infected mice with and without AMB treatment were similar in both groups. These data show the crucial clinical relevance of *in vitro* AMB MICs in *A. terreus*. Dannaoui and colleagues showed comparable survival rates of AMB-treated and untreated mice infected with AMB-resistant *A. terreus* (34).

In the second part of our study, we investigated the *in vitro* mechanisms related to AMB resistance in ATR and ATS. Membrane damage increased membrane permeability and intracellular oxidative damage, thought to be responsible for AMB action (14, 19). Reduced susceptibility to AMB has been associated with the upregulation of several ergosterol biosynthesis genes (ERG5, ERG6, and ERG25) and decreased ergosterol content in the fungal cell membrane (35–37). In contrast, our data suggest that the ergosterol content does not play a major role in AMB resistance in the *A. terreus* strains we tested. The mean ergosterol contents of ATR and ATS were equal. However, major differences were observed in cell-associated AMB content. ATS assimilated more AMB than ATR did. Therefore, we conclude that cell-associated AMB content is not directly related with membrane ergosterol. Reeves et al. showed AMB to induce amino acid efflux in *A. fumigatus* (15), and we also demonstrated an efflux of amino acids, intracellular proteins, and potassium in response to AMB, depending on the incubation time and concentration. In contrast to the expectation from these findings, ATR dispersed higher outflow levels of intracellular compounds in our study. The disruption of the cell membrane by AMB causes intracellular component efflux, but it obviously does not explain the fungicidal activity of AMB. Therefore, pore formation might play a minor role in AMB resistance in *A. terreus*.

Others have speculated that AMB leads to cell death by the formation of oxygen radicals, such as hydrogen peroxide (H_2O_2) (16, 19). Here, AMB induced intracellular oxidation, which in turn led to lipid peroxidation and, subsequently, to cell death. H_2O_2 permeates through biological membranes and causes peroxidation, but it might be neutralized via endogenous catalase (19). We showed significantly ($P = 0.01$) higher catalase activity in ATR than in ATS. Furthermore, ATR exhibited reduced lipid peroxidation at sublethal and lethal AMB concentrations, and ATR was able to reduce lipid peroxidation over time. These findings underline the fact that ATR better manages oxidative damage and possesses improved intracellular recovery systems.

Also, ATR seems to be best equipped with the intracellular

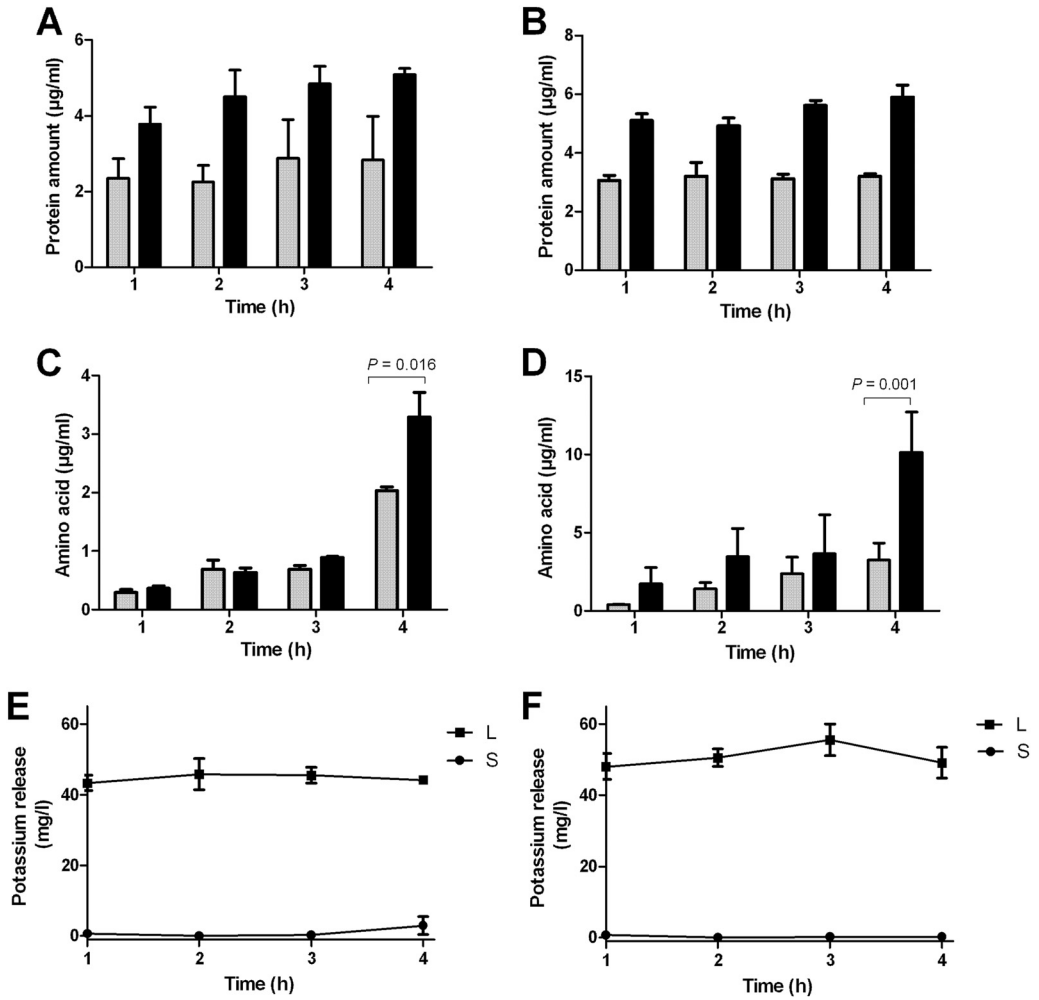

FIG 3 Amphotericin B (AMB)-induced efflux of AMB-susceptible (ATS) (gray columns) and AMB-resistant (ATR) (black columns) *Aspergillus terreus*. (A) Protein efflux at sublethal (0.1 μg/ml) AMB concentrations. (B) Protein efflux at lethal (10 μg/ml) AMB concentrations. In all experiments, the level of protein release of ATR was significantly higher ($P < 0.05$). (C) Amino acid efflux at sublethal (0.1 μg/ml) AMB concentrations. (D) Amino acid efflux at lethal (10 μg/ml) AMB concentrations. (E) Potassium release in susceptible *A. terreus* treated with sublethal (S) and lethal (L) concentrations of AMB. (F) Potassium release in resistant *A. terreus* treated with sublethal and lethal concentrations of AMB. For statistical analyses, Student's two-tailed *t* test was used.

enzyme activity responsible for survival strategies. This is supported by our data showing intracellular GAPDH activity, which can be used as an intracellular oxidation marker (38). Values of absorbance at 340 nm (λ_{max} of NADH) were lower in ATS than in ATR in response to AMB. Although the shapes of both curves were similar, this might indicate a slightly lower GAPDH activity in AMB-treated ATS than in AMB-treated ATR. Thus, ATR might have an improved ability to prevent oxidative GAPDH inactivation, which might lead to improved survival.

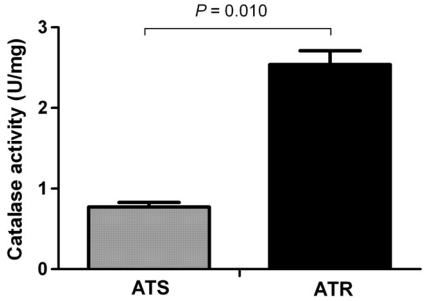

FIG 4 Hyphal catalase activity of amphotericin B (AMB)-susceptible (ATS) and AMB-resistant (ATR) *Aspergillus terreus* species. Significant ($P = 0.010$, analyzed by Student's two-tailed *t* test) differences were seen between the hyphal catalase activity levels of ATR and ATS.

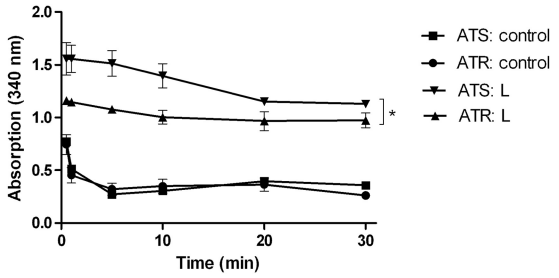

FIG 5 GAPDH activity in amphotericin B (AMB)-susceptible (ATS) and AMB-resistant (ATR) *Aspergillus terreus* with lethal (L) AMB treatment (10 μg/ml) and without (control) AMB treatment. *, $P < 0.05$, analyzed by one-way analysis of variance (ANOVA) with the Bonferroni multiple-comparison test.

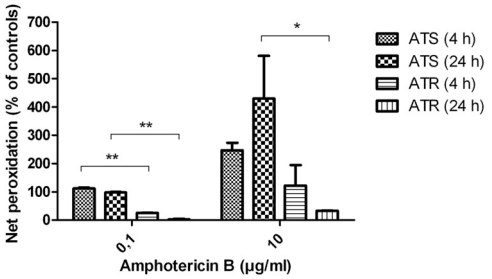

FIG 6 Lipid peroxidation of amphotericin B (AMB)-susceptible (ATS) and AMB-resistant (ATR) *Aspergillus terreus* normalized to that of untreated controls in response to AMB. *, $P < 0.05$, and **, $P < 0.005$, analyzed by Student's two-tailed *t* test. Incubation times are given in parentheses.

In conclusion, AMB susceptibility in *A. terreus* is not necessarily associated with loss of *in vivo* virulence, but the outcome under therapy is improved in infections with AMB-susceptible *A. terreus* compared to those with resistant *A. terreus*. In contrast to current knowledge, our experiments show that ergosterol content plays a minor role in AMB resistance and is not directly associated with intracellular cell-associated AMB content. However, our results indicate that resistant *A. terreus* species absorb less AMB, discharge more intracellular compounds, and have better protection against oxidative damage than susceptible ones. AMB possibly exerts its antifungal activity by oxidative injury rather than by an increase of membrane permeation.

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