Aspergillus terreus and the Interplay with Amphotericin B: from Resistance to Tolerance?

Roya Vahedi-Shahandashti,[a] Anna-Maria Dietl,[a] Ulrike Binder,[a] Markus Nagl,[a] Reinhard Würzner,[a] Cornelia Lass-Flörl[a]

[a]Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck, Innsbruck, Austria

ABSTRACT *Aspergillus terreus* is an opportunistic causative agent of invasive aspergillosis and, in most cases, it is refractory to amphotericin B (AMB) therapy. Notably, AMB-susceptible *Aspergillus terreus sensu stricto* (s.s.) representatives exist which are also associated with poor clinical outcomes. Such findings may be attributable to drug tolerance, which is not detectable by antifungal susceptibility testing. Here, we tested *in vitro* antifungal susceptibility (AFST) and the fungicidal activity of AMB against 100 clinical isolates of *A. terreus* species complex in RPMI 1640 and antibiotic medium 3 (AM3). MICs ranged from 0.5 to 16 μg/mL for RPMI 1640 and from 1 to >16 mg/L for AM3. AMB showed medium-dependent activity, with fungicidal effects only in antibiotic medium 3, not in RPMI 1640. Furthermore, the presence of AMB-tolerant phenotypes of *A. terreus* has been examined by assessing the minimum duration for killing 99% of the population (MDK99) and evaluating the data obtained in a *Galleria mellonella* infection model. A time-kill curve analysis revealed that *A. terreus* with AMB MICs of $\leq$1 mg/L (susceptible range) displayed AMB-tolerant phenotypes, exhibiting MDK99s at 18 and 36 h, respectively. Survival rates of infected *G. mellonella* highlighted that AMB was effective against susceptible *A. terreus* isolates, but not against tolerant or resistant isolates. Our analysis reveals that *A. terreus* isolates which are defined as susceptible based on MIC may comprise tolerant phenotypes, which may, in turn, explain the worse outcome of AMB therapy for phenotypically susceptible isolates.

KEYWORDS *Aspergillus terreus*, tolerance, amphotericin B, antifungal susceptibility testing, amphotericin B tolerance, aspergillosis, antifungal resistance, clinical breakpoint

Invasive fungal infections (IFIs) are a leading cause of infectious morbidity and mortality (1, 2). Species belonging to *Aspergillus*, the most common opportunistic mold representatives, cause an array of superficial to deep-seated systemic infections (3, 4). Their immunological status triggers the onset of infection and clinical manifestations (5, 6). Antifungal treatment remains a challenge (7) and, currently, only four classes of antifungal agents are available in clinical routines to treat IFIs; namely, azoles, polyenes, echinocandins, and 5-flucytosine (8). Amphotericin B (AMB) remains the broadest drug available, with only a few fungal pathogens harboring primary or acquired resistance; essentially, the rising number of fungi with reduced azole-susceptibility is a matter of concern (9–12).

Although *A. fumigatus* represents the most prevalent species involved in invasive aspergillosis (13), members of the sections *Terrei* or *Flavi* are the second or third most important species in specific regions (14–16). A current multicenter study surveying the global prevalence of *A. terreus* species complex demonstrated an overall occurrence of 5.2% (among 370 cases of fungal disease) (17). *A. terreus* holds an exceptional clinical state by representing a high propensity for dissemination and mortality (51 to 70%) in invasive aspergillosis (14, 18–21). Most *A. terreus* isolates display high (>2 mg/L) AMB MICs, and hence have been considered intrinsically resistant (22–25). However, some

TABLE 1 Susceptibility profiles of amphotericin B against *Aspergillus* section *Terrei* in RPMI 1640 and AM3[a]

| Species (no. of isolates) | RPMI 1640 | | | | | | AM3 | | | | | |
| | MIC (mg/L)[b] | | | MFC (mg/L) | | | MIC (mg/L) | | | MFC (mg/L) | | |
	Range	MIC_{50}	MIC_{90}	Range	MFC_{50}	MFC_{90}	Range	MIC_{50}	MIC_{90}	Range	MFC_{50}	MFC_{90}
A. section *Terrei* (100)[c]	0.5−16	2	4	>16	>16	>16	1−>16	8	16	4−>16	16	>16
A. terreus s.s. (78)	0.5-4	2	4	>16	>16	>16	1−>16	8	16	4−>16	16	>16
A. hortai (10)	0.5−2	1	2	>16	>16	>16	4−16	8	16	4−>16	16	>16
A. citrinoterreus (11)	1−16	2	8	>16	>16	>16	4−16	8	16	4−>16	16	>16
A. alabamensis (1)	1			>16			8			8		

[a]AM3, antibiotic medium 3; MFC, minimum fungicidal concentration; s.s., *sensu stricto*.
[b]MIC_{50}, MIC_{90}, MFC_{50}, and MFC_{90} are only shown for species with 10 or more isolates.
[c]Isolate S164 (susceptible control) is not included in this table.

studies have reported *A. terreus* isolates showing a wide range of AMB MICs, including low MICs (<0.5 mg/L) (26–28). Interestingly, high virulence potential was observed both in a mouse model and in a *Galleria mellonella* model infected with AMB-susceptible strains (24, 29, 30). The broad ranges of AMB-MIC phenotypes and MIC-independent clinical outcomes (31, 32) led us to investigate the role of tolerance in *A. terreus* under miscellaneous growth conditions (33–36). This study analyzed the efficacy of AMB against isolates of section *Terrei* by (i) evaluating MIC ranges in RPMI 1640 and antibiotic medium 3 (AM3), (ii) determining minimum fungicidal concentrations (MFCs), (iii) examining AMB killing-kinetic patterns to define tolerant phenotypes, (iv) evaluating germination rates, and finally (v) assessing AMB efficacy in the *G. mellonella* infection model.

RESULTS

Medium-dependent shift in AMB MIC distribution. The MIC distributions for AMB against 100 clinical isolates of *Aspergillus* section *Terrei*, which provided a sufficiently large sample size, have been determined for RPMI 1640 and AM3, and range from 0.5 to 16 mg/L and from 1 to >16 mg/L, respectively (Table 1). The MIC_{50} and MIC_{90} were 2 and 4 mg/L for RPMI 1640, shifting to 8 and 16 mg/L for AM3 (Table 1).

AMB showed medium-dependent fungicidal activity. AMB did not exhibit fungicidal activity against tested isolates ($n = 100$) in RPMI 1640; all fungi displayed MFCs of >16 mg/L (Table 1). In contrast, in AM3, AMB showed a different fungicidal profile, with MFCs ranging from 4 to >16 mg/L.

AMB tolerant phenotypes required a longer time to be killed than the susceptible representative. In RPMI 1640, T81, T31, and R134 did not reach killing detection limits at any concentrations over 48 h, indicating a lack of tolerant phenotypes. The susceptible control (S164) reached the 99%-killing detection limit after 36 h exposure to AMB, irrespective of concentration ($P = 0.0005$) (Fig. 1A to C). Due to the fungicidal activity of AMB even at low concentrations (1× MIC), S164 is considered to be susceptible.

In contrast, time-kill curves in AM3 showed different AMB-killing patterns (Fig. 2). AMB concentrations of 1× MIC (Fig. 2A) resulted in a faster and shorter MDK99 of 12 h ($P < 0.0001$) for S164 compared to other isolates, which failed to reach the 99%-killing detection limit within 48 h at 1× MIC. In addition, S164 showed time- and concentration-dependence, with a MDK99 of 6h at 20× ($P < 0.0001$) and 40× MIC ($P < 0.0001$). T81 and T31 showed tolerant phenotypes at 20× ($P = 0.0101$ and $P < 0.0001$, respectively) and 40× MIC ($P = 0.0125$ and $P < 0.0001$, respectively), displaying MDK99s of 36 h and 18 h, respectively (Fig. 2B and C). R134 was shown to be AMB-resistant by not reaching the 99%-killing detection limit in any of the concentrations and time points tested (Fig. 2A to C).

***A. terreus* showed an increased germination rate in AM3 compared to that in RPMI 1640.** All isolates showed significantly higher rates of germination in AM3 compared to those in RPMI 1640. Following 12 h of incubation in AM3, the average germination rates for S164, R134, T81, and T31 were 95.3% ($P = 0.0059$), 86.6% ($P = 0.0016$), 100% ($P = 0.0023$), and 90.3% ($P = 0.0040$), respectively. In contrast, the germination
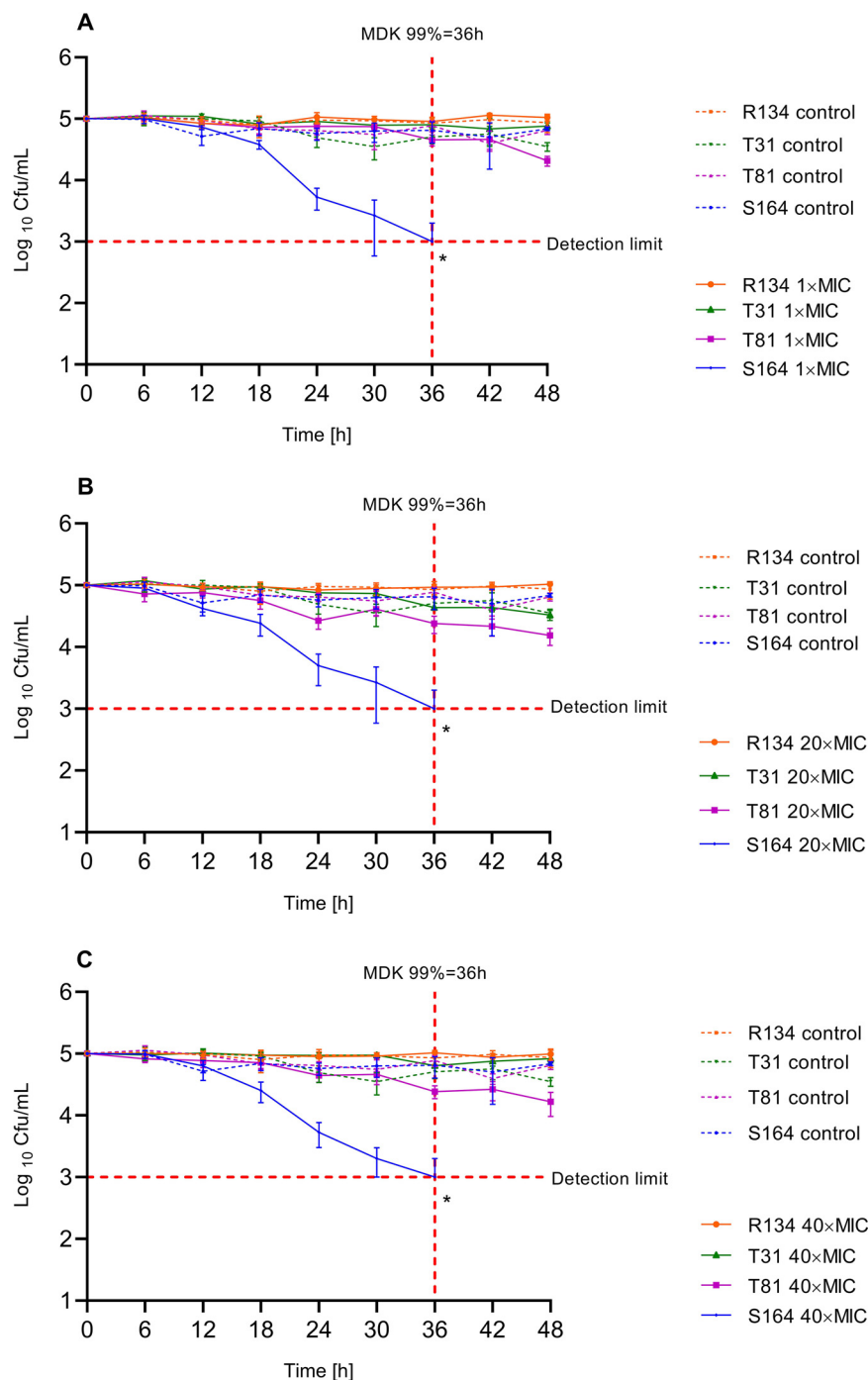
FIG 1 Time-kill kinetics of selected *A. terreus* isolates following exposure to different concentrations of amphotericin B at 1× (A), 20× (B), and 40× MIC (C) in RPMI 1640 medium supplemented with 2% glucose. Red horizontal dotted lines indicate limit of detection (99% of the initial population). Red vertical dotted lines show minimum duration of killing times needed to kill 99% of the initial inoculum (MDK99). All data represent mean values of three independent experiments (*, $P < 0.05$). For clarity, plots illustrate only the statistical significance of the first time points which reached the detection limit.

rates were lower in RPMI 1640: 12.6% for S164, 10.6% for R134, 21% for T81, and 20% for T31.

AMB efficacy in *A. terreus*-infected *G. mellonella* larvae showed a good correlation with *in vitro* MDK99 compared to MIC. Treatment with AMB was only successful in larvae infected with S164 (Fig. 3A), as shown by their prolonged survival compared to
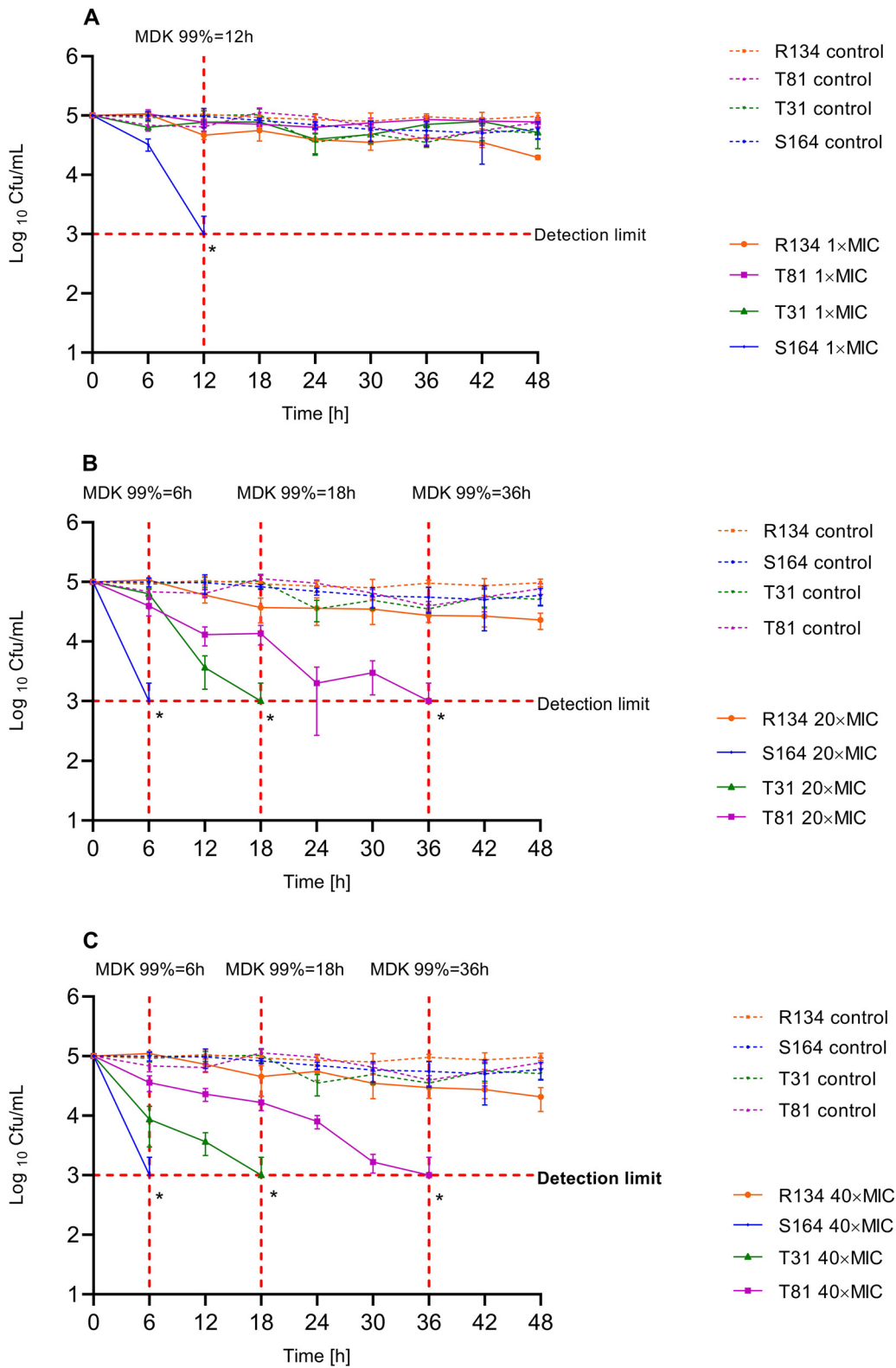
FIG 2 Time-kill kinetics of selected *A. terreus* isolates following exposure to different concentrations of amphotericin B at 1× (A), 20× (B), and 40× MIC (C) in antibiotic medium 3 (AM3). Red horizontal dotted lines indicate limit of detection (99% of the initial population). Red vertical dotted lines indicate minimum duration of killing time needed to kill 99% of the initial inoculum (MDK99). All data represent mean values of three independent experiments (*, $P < 0.05$). For clarity, plots illustrate only the statistical significance of the first time points which reached the detection limit.

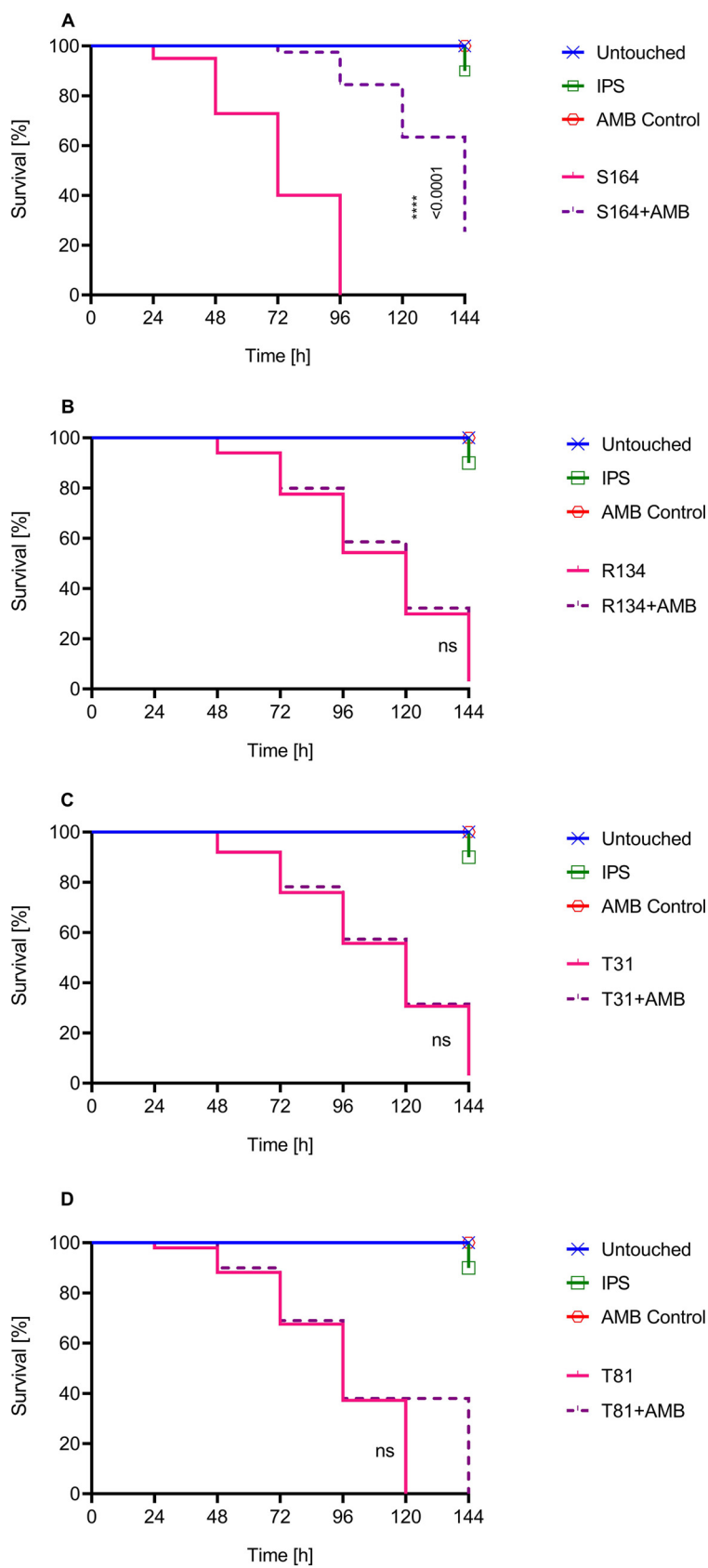**FIG 3** Kaplan-Meier survival curve for *G. mellonella* larvae following inoculation with different *A. terreus* isolates: S164 (A), R134 (B), T31 (C), and T81 (D). Larvae were infected with 1×10^7 spores; 2 h later, either

groups that did not receive AMB ($P < 0.0001$). AMB administration did not influence the survival of larval infected with R134 (Fig. 3B), T31 (Fig. 3C), or T81 (Fig. 3D).

DISCUSSION

The discordance between MICs in a susceptible range and poor therapy outcomes (31, 37) may be attributable to drug tolerance, which is not detectable by AFST (33, 38, 39). AMB MIC distributions in RPMI 1640 showed a broad range, which is in line with the results of previous studies (17, 36, 40–43); in comparison, MIC_{50} and MIC_{90} shifted to higher concentrations when applying AM3 (Table 1). In alignment with previous studies, AMB did not show any fungicidal activity in RPMI 1640 at any concentration tested (41, 44). In contrast, fungicidal activities of the polyene were observed to utilize AM3 (Table 1). This indicates that for selected *A. terreus* strains, AMB may act fungistatic in RPMI 1640 and fungicidal in AM3. These findings underline the importance of considering any effects of nutrient media when assessing biomedical tests (34, 45–47).

We hypothesized that a lack of discrimination between AMB-susceptible and AMB-tolerant *A. terreus* phenotypes in the application of standard AFST could be a reason for treatment failure in patients infected with susceptible isolates (20, 37, 48–50). MIC alone is not an adequate metric for tolerance detection, and relying solely on MICs might lead to suboptimal or inappropriate therapy (33, 38, 39). Tolerance in bacteria or/and yeasts is a multifactorial phenomenon, either associated with a genetic basis (inherited or non-inherited) or conferred by environmental and nutritional factors (33, 38, 51–53). To our knowledge, no study has addressed the detection of tolerance in molds; however, attempts have been made to develop different methods for tolerance detection based on the type of microorganism and the cidal or static activity of the agent (33, 51, 54, 55). Based on recent studies, we aimed to assess AMB tolerance among *A. terreus* s.s. by applying MDK99 detection (33).

In our study, two strains of *A. terreus* s.s., with MICs in the susceptible range, demonstrated tolerant phenotypes in AM3, but not in RPMI 1640, according to MDK99 (Fig. 1 and 2). Polyene antifungal activity may be influenced by medium composition (44, 56, 57). Besides this, nutrient-limited media affects fungal growth and, in consequence, tolerance (33, 38). An optimal nutrient environment provides sufficient nutrients to allow molds to grow and germinate without restriction (58). In agreement with the results of the previous study, our results confirmed that utilizing a nutrient-limited media such as RPMI 1640, although supplemented with 2% glucose, causes fungi to germinate at a lower rate than in AM3, which contains complex nutrients (58). This, in turn, might result in deviating results for the fungicidal activity of AMB in nutrient-limited media (34, 35, 54, 56, 58). The use of AM3 resulted in higher germination rates as well as improved detection of the fungicidal activity of AMB. Our data suggest AM3 be superior to RPMI 1640 when evaluating AMB activity; however, further studies are necessary until new recommendations can be made for AFST.

These data support the existence of AMB-tolerant phenotypes in *A. terreus* s.s. T81 and T31 required longer times (36 and 18 h, respectively) to reach the 99%-killing detection limit in high AMB concentrations (20× and 40× MIC), although they displayed MICs in a susceptible range. Irrespective of the medium, the AMB-susceptible isolate was killed in 1× MIC of AMB, while tolerant isolates survived at this concentration, similar to the resistant isolate, by not reaching the 99%-killing detection limit (Fig. 1A and Fig. 2A). Only tolerant isolates could withstand high concentrations of AMB for a long time (Fig. 2B and C). These findings verify that longer exposure to an agent, rather than a higher concentration, is required to produce the same level of killing in a tolerant strain as in a susceptible one (33, 38). Besides this, the MIC-based resistant iso-

FIG 3 Legend (Continued)

amphotericin B (AMB; 0.5 μg per larva) or insect physiological saline (IPS) was administered, and larvae were incubated at 37°C. Survival was monitored every 24 h over 144 h (6 days). Curves represent the average of three independent experiments (60 larvae in total). *P* values for significantly different results are shown ($P < 0.05$; Mantel-Cox test); otherwise, results are marked as not significant (ns).

late could not be killed by AMB, irrespective of medium, concentration, or duration of treatment (Fig. 1 and Fig. 2A–C).

To analyze whether AMB tolerance correlated with the *in vivo* efficacy of AMB, we performed treatment studies utilizing *G. mellonella* larvae (Fig. 3). Our results showed that AMB administration increased the survival of larvae infected with the susceptible strain (Fig. 3A); this finding correlates with those of previous studies (24, 30). In contrast, AMB treatment did not improve the survival of larvae infected with resistant (Fig. 3B) or tolerant *A. terreus* representatives (Fig. 3C and D). In line with our results, a recent study (51) showed that fluconazole treatment was not effective in *G. mellonella* infected with fluconazole-tolerant *Candida albicans*. Further clinical studies regarding the rate of tolerance and its outcome are lacking.

The results of this study indicate that MDK99s are better predictors of treatment outcome than MICs alone. Tolerant isolates required longer MDK99s to reach the 99%-killing detection limit (Fig. 2A–C) than susceptible ones (Fig. 2A–C); in addition, tolerant phenotypes behaved as resistant isolates in larvae upon AMB treatment. The appearance of an AMB-tolerant phenotype might explain the discordance of MIC values obtained by standardized assays and the lack of AMB efficacy *in vivo*.

In conclusion, we suggest that AMB efficacy is affected by the use of fungal growth medium (36, 45, 46, 58), which results in an increased MIC_{50} and MIC_{90} and in broader MFC ranges in AM3 than in RPMI 1640 when testing 100 clinical isolates of section *Terrei*. We underline the presence of tolerant phenotypes within the *A. terreus* population, showing MICs of ≤1 mg/L. *In vivo*, tolerant phenotypes responded to AMB similarly to resistant strains, despite showing AMB MICs in a susceptible range. Hence, distinguishing tolerance from susceptibility may be adequate before starting AMB treatment for *A. terreus*-related infections in selected cases.

MATERIALS AND METHODS

Fungal strains, growth conditions, and inoculum preparation. A total of 100 clinical and sequenced isolates of *Aspergillus* section *Terrei*, including *A. terreus* s.s. (*n* = 78), *A. hortai* (*n* = 10), *A. citrinoterreus* (*n* = 11), and *A. alabamensis* (*n* = 1) were analyzed. Strains were collected and previously molecularly classified by the ISHAM-ECMM-EFISG TerrNet Study group (www.isham.org/working-groups/aspergillus-terreus) (10, 17). Isolates were cultured from 10% glycerol frozen stocks (–80°C) on Sabouraud dextrose agar (SDA) (BD, Difco) at 37°C for 3 to 5 days; conidia were harvested by applying spore suspension buffer (0.9% NaCl, 0.01% Tween 20 [Sigma-P1379]). Isolate S164 acts as external susceptible control and has not been included in the 100 clinical isolates panel (30).

Antifungal agent. Deoxycholate amphotericin B (Sigma-Aldrich, A2411) was utilized in this study. AMB was dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich).

Antifungal susceptibility testing. Antifungal susceptibility testing for AMB was carried out according to EUCAST guidelines (www.EUCAST.org) (59), using two different nutrition media, including RPMI 1640 medium (R6504, Sigma) supplemented with 2% glucose buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS; Sigma), as a standard medium recommended by EUCAST, and AM3 as a complex medium (pH 7.0) (Oxoid, Hampshire, United Kingdom), providing adequate growth and a broader distribution of MICs and MFCs (47, 58, 60).

Fungicidal-activity testing. After MIC determination, MFCs were determined by removing 10 μL from all wells displaying no visible growth and from the growth control (drug-free medium), followed by preparing 1:100 dilutions in spore suspension buffer (*n* = 100) (41, 61). Afterward, 100 μL was spread on SDA plates using a Whitley Automated Spiral Plater (model WASP 2, Don Whitley, Shipley, United Kingdom). SDA plates were incubated at 37°C for 48 h, and CFU/mL was counted. The MFC of AMB was defined as the lowest drug concentration which approximately killed 99% of the inoculum.

Determination of tolerant phenotypes. (i) Definitions. Due to the lack of clinical breakpoint (CBP) for AMB and *A. terreus*, categorization in the present study was adopted according to the CBP defined for AMB and *A. fumigatus*, which is ≤1 mg/L (62, 63). Isolates with AMB MICs of ≤1 mg/L were categorized as susceptible, and isolates with AMB MICs of >1 mg/L as resistant. Tolerance defines the ability of a phenotypical susceptible isolate (AMB MICs of ≤1 mg/L) to survive high AMB concentrations for a longer time than the susceptible control. Hence, the MDK99 of a tolerant strain is longer than the MDK99 of a susceptible one (33, 38, 39).

Determination of the minimum duration of killing by time-kill assay. Four isolates of *A. terreus* *sensu stricto* (s.s.) with different susceptibility profiles in a susceptible (≤1 mg/L) and resistant (>1 mg/L) range, which showed identical MICs in both RPMI 1640 and AM3 based on EUCAST method, were chosen for the time-kill assay: *A. terreus* 164 (S164, susceptible control, MIC = 0.5 mg/L; *A. terreus* s.s. 31 (T31), MIC = 1 mg/L; *A. terreus* s.s 81 (T81), MIC = 1 mg/L; and *A. terreus* s.s 134 (R134, resistant control), MIC = 4 mg/L. The time-kill analyses were performed as previously described with minor modifications using RPMI 1640 and AM3 (23). The time-kill assays were conducted in RPMI 1640 and AM3 using three

concentrations of AMB (1×, 20×, and 40× MIC) and an untreated growth control. Freshly harvested spores of each isolate of *A. terreus* s.s. were prepared, and the inoculum of each isolate (1×10^5 spores/mL) was added to 20 mL of each medium, RPMI 1640 and AM3. At different time points (0, 6, 12, 18, 24, 30, 36, 42, and 48 h), 100-μL aliquots were taken, diluted 100-fold in spore buffer, and 100 μL were cultured on SDA plates at 37°C (48h) for counting CFU/mL. The detection limit was defined to be 1×10^3 CFU/mL. MDK99 was defined as the minimum time needed to reach a 99% reduction of the total number of CFU/mL from the initial inoculum at a specific concentration. Killing curves were constructed by plotting the $\log_{10}$ CFU/mL versus time over 48 h of each test condition against the control in Graph Pad Prism (version 8.0) software. Experiments were done three times and the mean values of three independent counts were used.

Determination of spore germination rate. Freshly harvested conidia from each of the four strains (S164, T81, T31, and R134) were washed three times with spore buffer and adjusted to 1×10^5 spores/mL in RPMI 1640 and AM3. A 200-μL volume of each solution was placed in a 96-well plate and incubated at 37°C for 12 h. The germination rate was determined microscopically at each time by determining conidia that already had formed a germ-tube out of 100 randomly chosen conidia. Assays were carried out three times, and these three independent counts were used to calculate the percentage of germination.

Assessing AMB efficacy against *A. terreus* isolates in *G. mellonella*. To examine the relevance of the *in vitro* MDK99 results and the *in vivo* efficacy of AMB, four isolates (S164, T81, T31, and R134) exhibiting susceptible, tolerant, and resistant profiles were evaluated in the *G. mellonella* insect model as described previously (30). Briefly, groups of 20 larvae (0.3 to 0.4 g; SAGIP, Italy) were stored in wood shavings in the dark at 18°C for 24 h prior to the experiment. Three control groups were included: larvae injected with 20 μL sterile insect physiological saline (IPS; 150 mM NaCl, 5 mM KCl, 10 mM EDTA, and 30 mM sodium citrate in 0.1 M Tris-HCl [pH 6.9]), larvae that received 0.5 μg AMB, and untouched larvae. Larvae were infected with 1×10^7 conidia/larva of each of the four isolates of *A. terreus* s.s. (S164, T31, T81, and R134), and injected with 0.5 μg AMB in a volume of 20 μL of IPS per larva at 2 h postinfection. The survival rate was monitored for up to 144 h at 37°C. Experiments were conducted in triplicate, and the data from all experiments (60 larvae in total) were combined to calculate the average survival rates determined every 24 h for a 144-h duration.

Statistical analysis. Survival rates of *G. mellonella* were determined using Kaplan-Meier survival curves and analyzed with the log-rank (Mantel-Cox) method. Furthermore, one-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test (for time-kill assay) and a paired *t* test (for germination rate determination) were used to determine statistical differences, using GraphPad Prism version 9.0.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). MIC$_{50}$, MIC$_{90}$, MFC$_{50}$, and MFC$_{90}$ values were calculated using Microsoft Office Excel 2016. *P* values of <0.05 were considered significant.

REFERENCES

1. Brown GD, Denning DW, Gow NAR, Levitz SM, Netea MG, White TC. 2012. Hidden killers: human fungal infections. Sci Transl Med 4:165rv13. https://doi.org/10.1126/scitranslmed.3004404.
2. Vazquez J, Tovar-Torres M, Hingwe A, Cheema F, Welch V, Ford K. 2016. The changing epidemiology of invasive aspergillosis in the non-traditional host: risk factors and outcomes. Pulm Crit Care Med 1:67–71. https://doi.org/10.15761/PCCM.1000114.
3. Kosmidis C, Denning DW. 2015. The clinical spectrum of pulmonary aspergillosis. Thorax 70:270–277. https://doi.org/10.1136/thoraxjnl-2014-206291.
4. Binder U, Lass-Flörl C. 2013. New insights into invasive aspergillosis from the pathogen to the disease. Curr Pharm Des 19:3679–3688. https://doi.org/10.2174/13816128113199990366.
5. Patterson TF, Thompson GR, Denning DW, Fishman JA, Hadley S, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Nguyen MH, Segal BH, Steinbach WJ, Stevens DA, Walsh TJ, Wingard JR, Young J-AH, Bennett JE. 2016. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis 63:e1–e60. https://doi.org/10.1093/cid/ciw326.
6. Arvanitis M, Mylonakis E. 2015. Diagnosis of invasive aspergillosis: recent developments and ongoing challenges. Eur J Clin Invest 45:646–652. https://doi.org/10.1111/eci.12448.
7. Perfect JR. 2017. The antifungal pipeline: a reality check. Nat Rev Drug Discov 16:603–616. https://doi.org/10.1038/nrd.2017.46.
8. McCarthy MW, Kontoyiannis DP, Cornely OA, Perfect JR, Walsh TJ. 2017. Novel agents and drug targets to meet the challenges of resistant fungi. J Infect Dis 216:S474–S483. https://doi.org/10.1093/infdis/jix130.
9. Walsh TJ, Anaissie EJ, Denning DW, Herbrecht R, Kontoyiannis DP, Marr KA, Morrison VA, Segal BH, Steinbach WJ, Stevens DA, van Burik J-A, Wingard JR, Patterson TF, Infectious Diseases Society of America (IDSA). 2008. Treatment of aspergillosis: clinical practice guidelines of the Infectious Diseases Society of America. Clin Infect Dis 46:327–360. https://doi.org/10.1086/525258.
10. Zoran T, Sartori B, Sappl L, Aigner M, Sánchez-Reus F, Rezusta A, Chowdhary A, Taj-Aldeen SJ, Arendrup MC, Oliveri S, Kontoyiannis DP, Alastruey-Izquierdo A, Lagrou K, Cascio GL, Meis JF, Buzina W, Farina C, Drogari-Apiranthitou M, Grancini A, Tortorano AM, Willinger B, Hamprecht A, Johnson E, Klingspor L, Arsic-Arsenijevic V, Cornely OA, Meletiadis J, Prammer W, Tullio V, Vehreschild J-J, Trovato L, Lewis RE, Segal E, Rath P-M, Hamal P, Rodriguez-Iglesias M, Roilides E, Arikan-Akdagli S, Chakrabarti A, Colombo AL, Fernández MS, Martin-Gomez MT, Badali H, Petrikkos G, Klimko N, Heimann SM, Uzun O, Roudbary M, de la Fuente S, Houbraken J, Risslegger B, Lass-Flörl C, Lackner M. 2018. Azole-resistance in *Aspergillus terreus* and related species: an emerging problem or a rare phenomenon? Front Microbiol 9:516. https://doi.org/10.3389/fmicb.2018.00516.
11. Arendrup MC, Jensen RH, Grif K, Skov M, Pressler T, Johansen HK, Lass-Flörl C. 2012. *In vivo* emergence of *Aspergillus terreus* with reduced azole susceptibility and a *Cyp51a M217I* alteration. J Infect Dis 206:981–985. https://doi.org/10.1093/infdis/jis442.
12. Yoshii N, Yamada K, Niki M, Imoto W, Yamairi K, Shibata W, Namikawa H, Sakatoku K, Sato K, Nakai T, Yamada K, Watanabe T, Asai K, Kakeya H, Kawaguchi T. 2021. Invasive pulmonary aspergillosis caused by *Aspergillus terreus* diagnosed using virtual bronchoscopic navigation and endobronchial ultrasonography with guide sheath and successfully treated with liposomal amphotericin B. Infection 49:1049–1054. https://doi.org/10.1007/s15010-020-01545-x.

13. Latgé J-P, Chamilos G. 2019. *Aspergillus fumigatus* and aspergillosis in 2019. Clin Microbiol Rev 33:e00140-18. https://doi.org/10.1128/CMR.00140-18.

14. Lass-Flörl C, Dietl A-M, Kontoyiannis DP, Brock M. 2021. *Aspergillus terreus* species complex. Clin Microbiol Rev 34:e0031120. 10.3343/alm.2015.35.6 .602. 10.1128/CMR.00311-20.

15. Dietl A-M, Vahedi-Shahandashti R, Kandelbauer C, Kraak B, Lackner M, Houbraken J, Lass-Flörl C. 2021. The environmental spread of *Aspergillus terreus* in Tyrol, Austria. Microorganisms 9:539. https://doi.org/10.3390/microorganisms9030539.

16. Lass-Flörl C, Cuenca-Estrella M. 2017. Changes in the epidemiological landscape of invasive mould infections and disease. J Antimicrob Chemother 72:i5–i11. https://doi.org/10.1093/jac/dkx028.

17. Risslegger B, Zoran T, Lackner M, Aigner M, Sánchez-Reus F, Rezusta A, Chowdhary A, Taj-Aldeen SJ, Arendrup MC, Oliveri S, Kontoyiannis DP, Alastruey-Izquierdo A, Lagrou K, Lo Cascio G, Meis JF, Buzina W, Farina C, Drogari-Apiranthitou M, Grancini A, Tortorano AM, Willinger B, Hamprecht A, Johnson E, Klingspor L, Arsic-Arsenijevic V, Cornely OA, Meletiadis J, Prammer W, Tullio V, Vehreschild J-J, Trovato L, Lewis RE, Segal E, Rath P-M, Hamal P, Rodriguez-Iglesias M, Roilides E, Arikan-Akdagli S, Chakrabarti A, Colombo AL, Fernández MS, Martin-Gomez MT, Badali H, Petrikkos G, Klimko N, Heimann SM, Houbraken J, Uzun O, Edlinger M, de la Fuente S, Lass-Flörl C. 2017. A prospective international *Aspergillus terreus* survey: an EFISG, ISHAM and ECMM joint study. Clin Microbiol Infect 23:776.e1–776.e5. https://doi.org/10.1016/j.cmi.2017.04.012.

18. Tritz DM, Woods GL. 1993. Fatal disseminated infection with *Aspergillus terreus* in immunocompromised hosts. Clin Infect Dis 16:118–122. https://doi.org/10.1093/clinids/16.1.118.

19. Hachem R, Gomes MZR, El Helou G, El Zakhem A, Kassis C, Ramos E, Jiang Y, Chaftari A-M, Raad II. 2014. Invasive aspergillosis caused by *Aspergillus terreus*: an emerging opportunistic infection with poor outcome independent of azole therapy. J Antimicrob Chemother 69:3148–3155. https://doi.org/10.1093/jac/dku241.

20. Steinbach WJ, Perfect JR, Schell WA, Walsh TJ, Benjamin DK. 2004. *In vitro* analyses, animal models, and 60 clinical cases of invasive *Aspergillus terreus* infection. Antimicrob Agents Chemother 48:3217–3225. https://doi.org/10.1128/AAC.48.9.3217-3225.2004.

21. Singh S, Kaur H, Singh M, Rudramurthy SM, Chakrabarti A. 2019. *Aspergillus terreus* causing probable invasive aspergillosis in a patient with cystic fibrosis. Mycopathologia 184:151–154. https://doi.org/10.1007/s11046-018-0294-1.

22. Escribano P, Peláez T, Recio S, Bouza E, Guinea J. 2012. Characterization of clinical strains of *Aspergillus terreus* complex: molecular identification and antifungal susceptibility to azoles and amphotericin B. Clin Microbiol Infect 18:E24–E26. https://doi.org/10.1111/j.1469-0691.2011.03714.x.

23. Walsh TJ, Petraitis V, Petraitiene R, Field-Ridley A, Sutton D, Ghannoum M, Sein T, Schaufele R, Peter J, Bacher J, Casler H, Armstrong D, Espinel-Ingroff A, Rinaldi MG, Lyman CA. 2003. Experimental pulmonary aspergillosis due to *Aspergillus terreus*: pathogenesis and treatment of an emerging fungal pathogen resistant to amphotericin B. J Infect Dis 188:305–319. https://doi.org/10.1086/377210.

24. Blum G, Hörtnagl C, Jukic E, Erbeznik T, Pümpel T, Dietrich H, Nagl M, Speth C, Rambach G, Lass-Flörl C. 2013. New insight into amphotericin B resistance in *Aspergillus terreus*. Antimicrob Agents Chemother 57:1583–1588. https://doi.org/10.1128/AAC.01283-12.

25. Vahedi Shahandashti R, Lass-Flörl C. 2019. Antifungal resistance in *Aspergillus terreus*: a current scenario. Fungal Genet Biol 131:103247. https://doi.org/10.1016/j.fgb.2019.103247.

26. Tortorano AM, Prigitano A, Dho G, Biraghi E, Stevens DA, Ghannoum M, Nolard N, Viviani MA. 2008. *In vitro* activity of amphotericin B against *Aspergillus terreus* isolates from different countries and regions. J Chemother 20:756–757. https://doi.org/10.1179/joc.2008.20.6.756.

27. Won EJ, Choi MJ, Shin JH, Park Y-J, Byun SA, Jung JS, Kim SH, Shin MG, Suh S-P. 2017. Diversity of clinical isolates of *Aspergillus terreus* in antifungal susceptibilities, genotypes and virulence in *Galleria mellonella* model: comparison between respiratory and ear isolates. PLoS One 12:e0186086. https://doi.org/10.1371/journal.pone.0186086.

28. Heo MS, Shin JH, Choi MJ, Park Y-J, Lee HS, Koo SH, Lee WG, Kim SH, Shin M-G, Suh S-P, Ryang D-W. 2015. Molecular identification and amphotericin B susceptibility testing of clinical isolates of *Aspergillus* from 11 hospitals in Korea. Ann Lab Med 35:602–610. https://doi.org/10.3343/alm.2015.35.6.602.

29. Speth C, Blum G, Hagleitner M, Hörtnagl C, Pfaller K, Posch B, Ott HW, Würzner R, Lass-Flörl C, Rambach G. 2013. Virulence and thrombocyte affectation of two *Aspergillus terreus* isolates differing in amphotericin B susceptibility. Med Microbiol Immunol 202:379–389. https://doi.org/10.1007/s00430-013-0300-7.

30. Maurer E, Browne N, Surlis C, Jukic E, Moser P, Kavanagh K, Lass-Flörl C, Binder U. 2015. *Galleria mellonella* as a host model to study *Aspergillus terreus* virulence and amphotericin B resistance. Virulence 6:591–598. https://doi.org/10.1080/21505594.2015.1045183.

31. Pastor FJ, Guarro J. 2014. Treatment of *Aspergillus terreus* infections: a clinical problem not yet resolved. Int J Antimicrob Agents 44:281–289. https://doi.org/10.1016/j.ijantimicag.2014.07.002.

32. Mortensen KL, Johansen HK, Fuursted K, Knudsen JD, Gahrn-Hansen B, Jensen RH, Howard SJ, Arendrup MC. 2011. A prospective survey of *Aspergillus* spp. in respiratory tract samples: prevalence, clinical impact and antifungal susceptibility. Eur J Clin Microbiol Infect Dis 30:1355–1363. https://doi.org/10.1007/s10096-011-1229-7.

33. Brauner A, Fridman O, Gefen O, Balaban NQ. 2016. Distinguishing between resistance, tolerance and persistence to antibiotic treatment. Nat Rev Microbiol 14:320–330. https://doi.org/10.1038/nrmicro.2016.34.

34. Rex JH, Cooper CR, Merz WG, Galgiani JN, Anaissie EJ. 1995. Detection of amphotericin B-resistant *Candida* isolates in a broth-based system. Antimicrob Agents Chemother 39:906–909. https://doi.org/10.1128/AAC.39.4.906.

35. Oliver BG, Silver PM, White TC. 2008. Polyene susceptibility is dependent on nitrogen source in the opportunistic pathogen *Candida albicans*. J Antimicrob Chemother 61:1302–1308. https://doi.org/10.1093/jac/dkn101.

36. Chandrasekar P, Abraham O, Manavathu E, Nune U, Kanuri K. 2000. Effect of test medium on *in vitro* susceptibility testing results for *Aspergillus fumigatus*. Rev Iberoam Micol 17:107–110.

37. Baddley JW, Marr KA, Andes DR, Walsh TJ, Kauffman CA, Kontoyiannis DP, Ito JI, Balajee SA, Pappas PG, Moser SA. 2009. Patterns of susceptibility of *Aspergillus* isolates recovered from patients enrolled in the transplant-associated infection surveillance network. J Clin Microbiol 47:3271–3275. https://doi.org/10.1128/JCM.00854-09.

38. Berman J, Krysan DJ. 2020. Drug resistance and tolerance in fungi. Nat Rev Microbiol 18:319–331. https://doi.org/10.1038/s41579-019-0322-2.

39. Delarze E, Sanglard D. 2015. Defining the frontiers between antifungal resistance, tolerance and the concept of persistence. Drug Resist Updat 23:12–19. https://doi.org/10.1016/j.drup.2015.10.001.

40. Sutton DA, Sanche SE, Revankar SG, Fothergill AW, Rinaldi MG. 1999. *In vitro* amphotericin B resistance in clinical isolates of *Aspergillus terreus*, with a head-to-head comparison to voriconazole. J Clin Microbiol 37:2343–2345. https://doi.org/10.1128/JCM.37.7.2343-2345.1999.

41. Borman AM, Fraser M, Palmer MD, Szekely A, Houldsworth M, Patterson Z, Johnson EM. 2017. MIC distributions and evaluation of fungicidal activity for amphotericin B, itraconazole, voriconazole, posaconazole and caspofungin and 20 species of pathogenic filamentous fungi determined using the CLSI broth microdilution method. J Fungi (Basel) 3:27. https://doi.org/10.3390/jof3020027.

42. Lass-Flörl C, Kofler G, Kropshofer G, Hermans J, Kreczy A, Dierich MP, Niederwieser D. 1998. *In-vitro* testing of susceptibility to amphotericin B is a reliable predictor of clinical outcome in invasive aspergillosis. J Antimicrob Chemother 42:497–502. https://doi.org/10.1093/jac/42.4.497.

43. Vaezi A, Fakhim H, Arastehfar A, Shokohi T, Hedayati MT, Khodavaisy S, Rezaei-Matehkolaei A, Badiee P, Hagen F, Lass-Flörl C, Dannaoui E, Meis JF, Badali H. 2018. *In vitro* antifungal activity of amphotericin B and 11 comparators against *Aspergillus terreus* species complex. Mycoses 61:134–142. https://doi.org/10.1111/myc.12716.

44. Meletiadis J, Antachopoulos C, Stergiopoulou T, Pournaras S, Roilides E, Walsh TJ. 2007. Differential fungicidal activities of amphotericin B and voriconazole against *Aspergillus* species determined by microbroth methodology. Antimicrob Agents Chemother 51:3329–3337. https://doi.org/10.1128/AAC.00345-07.

45. Tortorano AM, Dannaoui E, Meletiadis J, Mallie M, Viviani MA, Piens M, Rigoni AL, Bastide JM, Grillot R, Network E, EBGA Network. European Group for Research on Biotypes and Genotypes of Aspergillus fumigatus. 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. https://doi.org/10.1179/joc.2002.14.3.246.

46. Dannaoui E, Lortholary O, Raoux D, Hoinard D, Dromer F. 2007. Comparison of RPMI and AM3 medium for *in vitro* susceptibility testing of caspofungin against *Candida* spp. by EUCAST broth microdilution method. 17th European Congress of Clinical Microbiology and Infectious Diseases (ECCMID) and 25th International Congress of Chemotherapy (ICC). ECCMID, Basel, Switzerland.

47. Park BJ, Arthington-Skaggs BA, Hajjeh RA, Iqbal N, Ciblak MA, Lee-Yang W, Hairston MD, Phelan M, Plikaytis BD, Sofair AN, Harrison LH, Fridkin SK, Warnock DW. 2006. Evaluation of amphotericin B interpretive breakpoints for *Candida* bloodstream isolates by correlation with therapeutic outcome. Antimicrob Agents Chemother 50:1287–1292. https://doi.org/10.1128/AAC.50.4.1287-1292.2006.

48. Lewis RE, Albert NP, Liao G, Wang W, Prince RA, Kontoyiannis DP. 2013. High-dose induction liposomal amphotericin B followed by de-escalation is effective in experimental *Aspergillus terreus* pneumonia. J Antimicrob Chemother 68:1148–1151. https://doi.org/10.1093/jac/dks521.

49. Herbrecht R, Denning DW, Patterson TF, Bennett JE, Greene RE, Oestmann J-W, Kern WV, Marr KA, Ribaud P, Lortholary O, Sylvester R, Rubin RH, Wingard JR, Stark P, Durand C, Caillot D, Thiel E, Chandrasekar PH, Hodges MR, Schlamm HT, Troke PF, de Pauw B, Invasive Fungal Infections Group of the European Organisation for Research and Treatment of Cancer and the Global *Aspergillus* Study Group. 2002. Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. N Engl J Med 347:408–415. https://doi.org/10.1056/NEJMoa020191.

50. Walsh TJ, Pappas P, Winston DJ, Lazarus HM, Petersen F, Raffalli J, Yanovich S, Stiff P, Greenberg R, Donowitz G, Schuster M, Reboli A, Wingard J, Arndt C, Reinhardt J, Hadley S, Finberg R, Laverdière M, Perfect J, Garber G, Fioritoni G, Anaissie E, Lee J. 2002. Voriconazole compared with liposomal amphotericin B for empirical antifungal therapy in patients with neutropenia and persistent fever. N Engl J Med 346:225–234. https://doi.org/10.1056/NEJM200201243460403.

51. Rosenberg A, Ene IV, Bibi M, Zakin S, Segal ES, Ziv N, Dahan AM, Colombo AL, Bennett RJ, Berman J. 2018. Antifungal tolerance is a subpopulation effect distinct from resistance and is associated with persistent candidemia. Nat Commun 9:2470. https://doi.org/10.1038/s41467-018-04926-x.

52. Xu Y, Lu H, Zhu S, Li W-Q, Jiang Y, Berman J, Yang F. 2021. Multifactorial mechanisms of tolerance to ketoconazole in *Candida albicans*. Microbiol Spectr 9:e00321-21. https://doi.org/10.1128/Spectrum.00321-21.

53. Delarze E, Brandt L, Trachsel E, Patxot M, Pralong C, Maranzano F, Chauvel M, Legrand M, Znaidi S, Bougnoux M-E, d'Enfert C, Sanglard D. 2020. Identification and characterization of mediators of fluconazole tolerance in *Candida albicans*. Front Microbiol 11:591140. https://doi.org/10.3389/fmicb.2020.591140.

54. Barchiesi F, Maracci M, Baldassarri I, Spreghini E, Giannini D, Scalise G. 2004. Tolerance to amphotericin B in clinical isolates of *Candida tropicalis*. Diagn

Microbiol Infect Dis 50:179–185. https://doi.org/10.1016/j.diagmicrobio.2004.06.002.

55. Seidenfeld SM, Cooper BH, Smith JW, Luby JP, Mackowiak PA. 1983. Amphotericin B tolerance: a characteristic of *Candida parapsilosis* not shared by other *Candida* species. J Infect Dis 147:116–119. https://doi.org/10.1093/infdis/147.1.116.

56. Gale EF, Johnson AM, Kerridge D, Koh TY. 1975. Factors affecting the changes in amphotericin sensitivity of *Candida albicans* during growth. J Gen Microbiol 87:20–36. https://doi.org/10.1099/00221287-87-1-20.

57. Bosch C, Toplis B, Vreulink J-M, Volschenk H, Botha A. 2020. Nitrogen concentration affects amphotericin B and fluconazole tolerance of pathogenic cryptococci. FEMS Yeast Res 20:foaa010. https://doi.org/10.1093/femsyr/foaa010.

58. Meletiadis J, Meis JFGM, Mouton JW, Verweij PE. 2001. Analysis of growth characteristics of filamentous fungi in different nutrient media. J Clin Microbiol 39:478–484. https://doi.org/10.1128/JCM.39.2.478-484.2001.

59. Arendrup MC, Meletiadis J, Mouton JW, Lagrou K, Hamal P, Guinea J, Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST). 2020. Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. EUCAST definitive document E.Def. 9.3.2, p. 1–23. EUCAST, Växjö, Sweden. Available from https://www.eucast.org/astoffungi/methodsinantifungalsusceptibilitytesting/ast_of_moulds/.

60. Peyron F, Favel A, Michel-Nguyen A, Gilly M, Regli P, Bolmström A. 2001. Improved detection of amphotericin B-resistant isolates of *Candida lusitaniae* by Etest. J Clin Microbiol 39:339–342. https://doi.org/10.1128/JCM.39.1.339-342.2001.

61. Espinel-Ingroff A, Fothergill A, Peter J, Rinaldi MG, Walsh TJ. 2002. Testing conditions for determination of minimum fungicidal concentrations of new and established antifungal agents for *Aspergillus* spp.: NCCLS collaborative study. J Clin Microbiol 40:3204–3208. https://doi.org/10.1128/JCM.40.9.3204-3208.2002.

62. European Committee on Antimicrobial Susceptibility Testing (EUCAST). 2020. Breakpoint tables for interpretation of MICs for antifungal agents version 10.0, valid from 4 February 2020. EUCAST, Växjö, Sweden. Available from https://www.eucast.org/astoffungi/clinicalbreakpointsforantifungals/.

63. Guinea J. 2020. Updated EUCAST clinical breakpoints against *Aspergillus*, implications for the clinical microbiology laboratory. J Fungi (Basel) 6:343. https://doi.org/10.3390/jof6040343.