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Review Article

Review Article

Aspergillus terreus: Novel lessons learned on amphotericin B resistance

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Abstract

The polyene antifungal amphotericin B (AmB) exerts a powerful and broad activity against a vast array of fungi and in general displays a remarkably low rate of antimicrobial resistance. *Aspergillus terreus* holds an exceptional position among the *Aspergilli* due to its intrinsic AmB resistance, *in vivo* and *in vitro*. Until now, the underlying mechanisms of polyene resistance were not well understood. This review will highlight the molecular basis of *A. terreus* and AmB resistance recently gained and will display novel data on the mode of action of AmB. A main focus is set on fundamental stress response pathways covering the heat shock proteins (Hsp) 90/Hsp70 axis, as well as reactive oxygen species detoxifying enzymes in response to AmB. The effect on main cellular functions such as fungal respiration will be addressed in detail and resistance mechanisms will be highlighted. Based on these novel findings we will discuss new molecular targets for alternative options in the treatment of invasive infections due to *A. terreus*.

Key words: *Aspergillus terreus*, intrinsic amphotericin B resistance, resistance mechanisms, stress response.

Introduction

The frequency of invasive fungal infections (IFI) is still rising and represents a considerable threat as IFIs are still accompanied by poor prognosis and high mortality rates. Approximately 1.5 million people die of fungal infections each year.[1] Effective antifungal therapy has become particularly important as the number of immunocompromised patients increased in parallel with life-threatening IFIs. Treatment of fungal infections is challenging due to an immunocompromised status of the majority of patients, and also due to the increasing emergence of drug resistant strains. *Aspergillus sp*. as opportunistic pathogens account for the most frequent mould infections especially in patients with oncohematological malignancies, individuals undergoing allogenic hematopoetic stem cell or solid organ transplantation.[2,3] Other populations at risk for invasive aspergillosis (IA) comprise patients with prolonged immnosuppressive therapy (e.g., corticosteroids) and critically ill patients in intensive care units with respective co-morbidities like severe liver disease, respiratory diseases like chronic obstructive pulmonary disease. The clinical manifestations of disease caused by aspergilli cover a broad spectrum from allergic

reactions to invasive pulmonary infections and systemic infections.[4] The genus *Aspergillus* is one of the best-studied genera of filamentous fungi, largely due to medical relevance as pathogens (*A. fumigatus* and *A. terreus*), food spoilage and toxin production (*A. flavus*, *A. parasiticus*), and industrial and biotechnologic applications (*A. niger, A. oryzae*). The most frequently occurring human pathogenic *Aspergillus* species are *Aspergillus fumigatus* (67–73%), followed by *A. flavus* (10–16%), *A. niger* (5–9%), *A. terreus* (3–4%), and other *Aspergillus sp*.[4,5] However, the epidemiology of invasive fungal disease is shifting as non–*A. fumigatus Aspergillus sp*. and other moulds have become more frequent in most geographical areas and clinical settings.[2–6] Additionally geographical accumulations of certain opportunistic pathogens are observed, like *A. terreus* in Innsbruck, Austria or Houston, Texas.[7,8] *A. terreus* constitutes an emerging opportunistic fungus and invasive infections with this pathogen are associated with disseminated infections and high mortality rates.[3,8,9] A prospective international multicenter surveillance study including 21 countries and 38 centers detected an overall prevalence of 5.2% of *A. terreus* infections among mold infections.[3] *A. terreus* holds an outstanding position among aspergilli due to its intrinsic resistance against the polyene AmB, thereby challenging treatment.[8,10–12] Morphologically *A. terreus* can be noticeably distinguished from other *Aspergillus sp*. due to the presence of aleuroconidia,[13] produced under *in vitro* and *in vivo* conditions.

There are only five classes of antifungal drugs used to treat systemic infections: (i) azoles, (ii) polyenes, (iii) echinocandins, (iv) pyrimidine analogues and mitotic inhibitors, and (v) allylamines.[14]

Polyenes comprise more than 200 compounds mainly produced by *Streptomyces* sp. AmB-deoxycholate, AmB-lipid formulations, nystatin and natamycin are known in the clinical setting. AmB was developed in the 1950s and was the only antifungal agent available for the treatment of invasive fungal diseases for decades. AmB is an exceptional resistance-evasive antifungal that has remained the last line of defence in treating fungal infections, including aspergillosis, cryptococcal meningitis, candidiasis and mucormycosis, for over half a century due to its broad activity spectrum.[15] Albeit resistance to AmB is a rare finding, a few fungal species possess AmB resistance such as *Candida* sp., *Cryptococcus neoformans*, *A. tanneri, Fusarium* sp., and *Scedosporium prolificans*[7,16–18]; see Table 2.

The main target of AmB is ergosterol of the fungal plasmamembrane, and it is thought that AmB forms aggregates, which are incorporated in the lipid bilayers (channel formation), thereby permeabilizing and disrupting the plasmamembrane and accomplishing the fungicidal activity.[19,20] In contrast to the ion channel model, more recent studies highlight that the main mechanism of action is sterol binding and that channel formation represents a secondary mode of action.[21] These findings have been confirmed and extended to the 'sterol sponge' model,[22] in which extra-membranous AmB aggregates extract ergosterol from phospholipid bilayers and thereby killing fungal cells. This in turn explains the toxicity in human cells, where AmB extracts cholesterol and novel attempts are made to minimize cholesterol binding.[23]

Several studies emphasize that AmB induces oxidative stress and thereby causes fungal cell death.[19,24–26] This approach is completely different to the sterol model, but it could contribute to the potent antifungal activity of AmB. Furthermore this hypothesis also indicates that AmB´s mechanism of action might be more complex and multifaceted. Moreover auto-oxidation of AmB and subsequent formation of free radicals was reported.[27]

Our laboratory became interested in the intrinsic AmB resistance of *A. terreus* because *A. terreus* infections occur especially frequently at our institution. This review will focus on the basis of the intrinsic resistance to AmB of *A. terreus* (see Table 1), its particularities and discuss the perspective of modulating this intrinsic resistance.

Assessing AmB resistance

Mechanisms of AmB resistance - ergosterol content alterations

Acquired resistance to AmB has been extensively studied in yeasts. In *Candida albicans* AmB resistance stemmed from deletions of ergosterol biosynthetic genes. Validated resistance mechanisms involve a double loss of ERG3 and ERG11 genes (C-5 sterol desaturase and lanosterol 14α-demethylase, respectively) in *Candida albicans*.[28,29] In other *Candida* sp. AmB resistance resulted from deletions in ERG2, a C-8 sterol isomerase, and ERG6, C-24 sterol methyltransferase. A comprehensive and systematic investigation of AmB resistance-evolution was accomplished by Vincent et al. in *C. albicans*.[30] Here, the authors on the one hand selected for *in vitro* AmB resistant strains and performed whole genome sequencing to pin down the respective mutations conferring AmB resistance. On the other hand, this study validated the respective genes in deletion mutants in laboratory strains. Already known ergosterol biosynthetic gene mutations, which confer AmB resistance, were confirmed with this dual approach and additional ergosterol biosynthetic genes were identified, like the aforementioned ERG6 (C-24 sterol methyltransferase). All the AmB resistant mutants displayed a lack of the C5-C6 and / or C7-C8 conjugation peaks in spectrophotometric analysis of sterol extracts. Indicating that

Table 1. Overview of targets that interfere with the intrinsic AmB resistance in *A. terreus*.

Mechanisms	Objective	*In vitro* effects of inhibition	Effects of supplementation
Mitochondrial functions			
Oxygen consumption	lower in ATR		
Electron transport chain	Complex I	decreased susceptibility in susceptible isolates	
	Complex II	increased susceptibility	
	Complex III	full susceptibility	
	Complex IV	full susceptibility	
	alternative oxidases	increased susceptibility	
Cellular redox status			
	anti-oxidants (N-acetyl-cysteine)		decreased susceptibility
	pro-oxidants (L-ascorbic acid)		full susceptibility
Stress response			
	HSP90	full susceptibility	
	HSP70	increased susceptibility	
	Calcineurin	increased susceptibility	
ROS detoxification			
	Superoxide dismutases	increased susceptibility	
	Catalases	full susceptibility	

Table 2. Amphotericin B resistance in other fungi (References compiled from[7,16–18]).

	Mechanisms
Candida albicans	altered sterol, phospholipids in fungal membrane, increased catalase activity
Candida glabrata	altered sterol in fungal cell membrane
Cryptococcus neoformans	altered ergosterol in membrane, defective Δ-8,7, isomerase
Fusarium species	unknown
Scedosporium species	unknown

ergosterol is missing in the membranes of resistant mutants and confirming that AmB resistant fungal species accumulate ergosterol intermediates, which are not targeted by AmB.

An initial study on the intrinsic AmB resistance of *A. terreus* by Blum et al. used clinical isolates of *A. terreus* and compared those AmB resistant isolates with AmB susceptible *A. fumigatus* clinical isolates with respect to their ergosterol content, using a spectrophotometric approach, which allowed to detect ergosterol intermediates.[31] Interestingly the fungal ergosterol content was similar in both species, with even slightly increased ergosterol levels in *A. terreus* isolates (4.4 ± 1.4 and 5.1 ± 2.4 μg/ml in *A. fumigatus* and *A. terreus*, respectively). These findings suggest that ergosterol as target of AmB is present in the membranes and that other ergosterol intermediates in the membrane are not the cause of the intrinsic resistance. A continuing study, including a new rare AmB susceptible *A. terreus* (ATS) clinical isolate, confirmed that ergosterol levels of resistant and susceptible isolates were almost identical, and ATS responds to AmB treatment in a murine infection model.[16]

Mechanisms of AmB resistance –cell wall composition

The connection between AmB resistance and components of the fungal cell wall is poorly described, but is observed in different fungi. A study in *A. flavus* isolated and characterized a highly AmB resistant derivative in an AmB resistance screen. This resistant offspring displayed a similar ergosterol content to its progenitor but an altered cell wall composition. [32] Phenotypically this mutant is slow growing but exhibits growth up to 100 μg/ml AmB; protoplasts, however, lose the ability to grow in the presence of AmB and behaved like the susceptible progenitor strain, indicating that cell wall components might confer AmB resistance in *A. flavus*. The major difference in the cell wall composition was a significantly increased ß-1-3 glucan content in the mutant. A more recent work describes an increased ß-1-3 glucan content in AmB resistant *C. tropicalis* isolates along with thicker cell walls.[33] Here other alterations were found, too, in AmB resistant strains, like respiration defects (discussed below), which might impact on AmB resistance. Another study using *C. albicans* reports that removing of ß-1-3 glucans with enzymes increases AmB susceptibility.[33]

Blum et al. illustrated that protoplasts are equally resistant as conidia and that the cell wall of *A. terreus* has no impact on AmB efficacy.[31]

Modulators of AmB resistance: molecular chaperones

Fungal pathogens have developed robust stress responses to counteract and overcome antimicrobial defence mechanisms encountered within hosts. One of the most powerful and conserved adaptation mechanism to various stressors is the heat shock response. Heat shock protein 90 (Hsp90) is an essential and highly conserved molecular chaperone that acts as sensor of cell integrity by regulating the folding, transport, maturation, and degradation of a diverse set of client proteins, many of them are involved in signalling pathways like transcription factors, kinases and other signal transducers.[34] Hsp90 directly interacts with calcineurin and keeps this client protein balanced, as calcineurin is required for numerous responses to environmental stimuli.

In fungi, Hsp90 is a major player in stress responses and Hsp90 is essential for survival as well as for pathogenicity, assessed in *C. albicans* and *A. fumigatus*. Virulence of *C. albicans* and *A. fumigatus* is completely lost when Hsp90 is genetically repressed.[35,36] The seminal work by Cowen and Lindquist illustrates that Hsp90 is involved in the emergence and maintenance of drug resistance in diverse fungal species in different ways[37]: (i) when Hsp90 function is compromised new genetic variations of drug resistance may persist, (ii) misfolded or mutated proteins may result in a loss of function of resistance, and (iii) Hsp90 may chaperone regulators of cell signalling (e.g., calcineurin) and thereby open the way for new adaptive phenotypes.[37] Hsp90 has distinct effects on drug resistance in evolutionary distant fungi. In *A. terreus* the susceptibility to the echnocandin drug caspofungin is increased with Hsp90 inhibitors with an equally strong effect as with calcineurin inhibitors.[38] In *C. albicans*, in contrast, where Hsp90 inhibition did not alter caspofungin sensitivity, inhibition of Hsp90 rather caused the susceptibility to the azoles fluconazole and voriconazole, which is unaffected in *A. terreus*.[37]

Our laboratory investigated the effect of Hsp90 inhibitors on AmB susceptibility in *A. terreus* AmB resistant clinical isolates (ATR) as well as one rare AmB susceptible *A. terreus* (ATS) clinical isolate and in *A. fumigatus in vitro*.[39] Synergism of AmB was observed for the Hsp90 inhibitors geldanamycin and the synthetic but less toxic derivative, 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-AAG), and the calcineurin inhibitors cyclosporine A and FK506 using 5 and 10 μM of each inhibitor. Cotreatment with the Hsp90 and calcineurin inhibitors rendered to be ATR susceptible to AmB. Fungal growth was completely abolished when Hsp90 inhibitors were used at concentrations of 20 μM and in *A. fumigatus* inhibitors at higher concentrations (20 μM). However, in *A. fumigatus* the synergism of AmB and geldanamycin was not confirmed by Lamoth et al., probably because of differences in susceptibility testing methods.[40] Hsp90 is induced by various stressors to react on cellular disturbances. At a transcriptional level ATS displayed slightly increased basal *hsp90* levels and short term AmB treatment caused a moderate response in the two groups. Hsp90 transcripts were slightly and not significantly decreased in ATR but increased in ATS. A marked increase of *hsp90* transcripts was observed in *A. fumigatus* after 15 min AmB treatment, but basal hsp90 transcript levels were higher in *A. terreus*.[39]

The *in vitro* data could not be confirmed in an *in vivo* murine *A. terreus* infection model using combinatorial treatment of AmB and 17-AAG. Murine models for invasive candidiasis also failed to demonstrate any benefit of cotreatment because of the toxicity of Hsp90 inhibitors.[41] *In vivo* the Hsp90 inhibitor 17-AAG at the higher concentration (60 mg/kg daily) alone decreased survival and synergistic effects were not found. Combinatorial treatment with 17-AAG (20 mg/kg daily) and AmB decreased fungal burden in the lung but did not impact on survival rates. A similar phenotype was observed for the calcineurin inhibitor cyclosporine A (50 mg/kg daily), using cyclosporine alone promote fungal burden in the lungs by two log levels, probably due to the immunomodulatory capacity of the inhibitor.[39]

Another major player in the chaperone machinery in eukaryotic cells is the of heat shock protein 70 family, Hsp70. Together with Hsp90 Hsp70 members hold a broad and fundamental role in the fungal cellular surveillance network.[42] Similar to Hsp90, Hsp70 members are ATP-dependent chaperones, but unlike Hsp90, Hsp70 proteins promiscuously interact with all proteins in their unfolded, aggregated or misfolded forms. Hsp70 members are involved in the folding and activation of client proteins via a chaperone cycle depending on Hsp90. Our laboratory got interested in Hsp70 family, since the Hsp70 member SSB was identified to be highly up-regulated by AmB treatment in resistant strains in a 2D SDS-PAGE proteome analysis coupled to MS/MS analysis of AmB treated versus untreated ATS and ATR. Western blot experiments revealed that ATR exhibit higher basal levels of SSA and SSB Hsp70 proteins and that AmB induces a robust induction of these Hsp70 members in all resistant isolates tested, while the three tested ATS isolates display an obviously attenuated response.[43]

A. terreus possesses nine Hsp70 members belonging to the distinct seven subfamilies of Hsp70 proteins described in *S. cerevisiae*.[44] Expression analysis confirmed the high

induction of *ssa* and *ssb* transcripts in ATR confronted with AmB. Consistent with the higher expression levels of *ssa* and *ssb* in ATR, the respective putative nucleotide exchange factors of the Hsp70 family, *sse* and *ssz*, were also elevated. The majority of Hsp70 genes were immediately induced in ATR strains by AmB treatment, pointing to a role of Hsp70 stress response in AmB resistance mechanisms and suggesting that ATR is better able to cope with cellular stress. This assumption is confirmed by increased levels of ubiquitinylated proteins in ATS isolates when confronted with AmB.[43]

Due to the differences in Hsp70 expression between ATS and ATR the impact of Hsp70 inhibition was investigated. The Hsp70 inhibitors had only limited effects on ATS isolates but decreased MIC values in ATR. The adenosine derived Hsp70 inhibitor Ver155008, caused a significant MIC reduction and decreased also the MIC of ATS and *A. fumigatus*. However, this effect could result from the broad specificity of Ver155008 for other Hsp members. KNK232, a benzidine lactam compound, which specifically blocks the induction of Hsp70, Hsp72, and Hsp105, decreased MICs in ATR at all tested concentration, but this decline in the MIC was not sufficient to abrogate resistance in ATR. Pifithrin-μ, which inhibits chaperone activity by interactions with the substrate-binding site by allosteric inhibition, displayed the most effective outcome and decreased MICs in ATR and rendered ATR susceptible to AmB treatment.[43] Pifithrin-μ is also used in anti-cancer drug research.[45] Hence, this inhibitor was tested *in vivo* in the invertebrate *Galleria melonella* infection model and the results correlated well with the *in vitro* data. ATR-infected *G. mellonella* larvae displayed increased survival after AmB and pifithrin-μ treatment.

In *A. fumigatus* pifithrin-μ potentiated the effect of caspofungin and displayed additive effects with other interventions compromising the Hsp90-Hsp70 network.[42] Overexpression of *ssa* or *ssb* in ATS by implication had no effect on AmB susceptibility, but overexpression of each Hsp70 gene in ATS promoted sporulation rates, to levels comparable with ATR.[43]

Modulators of AmB resistance – mitochondrial functions and the cellular redox homeostasis

Several studies support the hypothesis that AmB induces reactive oxygen species within the fungal cell.[24,46–49] Furthermore, alterations in mitochondrial functions and content affect cellular networks targeted by antifungal drugs.[50] In yeasts, a connection between mitochondria and azole susceptibility is established; however, mitochondrial function affects azole susceptibility in *C. albicans* and *C. glabrata* in an antithetic manner. *C. glabrata* is capable to survive a complete loss of the mitochondrial genome, which results in azole resistance mainly due to up-regulation of the transcriptional regulator *CgPDR1*.[51] *C. albicans* in contrast cannot undergo mitochondrial genome loss and inactivation of mitochondrial complexes causes increased azole drug susceptibility[52] pointing toward a complex link between mitochondria and azole activity in fungal pathogens [reviewed in [53]].

Mitochondria harbour the oxidative phosphorylation (OXPHOS) system at the inner mitochondrial membrane, which is on the one hand the major source of ATP generation and on the other hand mitochondria are the major origin of intracellular reactive oxygen species (ROS) formation. Inappropriate electron transfer reactions in the mitochondrial electron transport chain (ETC, in complexes I–IV of the OXPHOS system) produce ROS, when electrons are released prematurely from the ETC and react with O_2 to superoxide anions O_2.[54] These highly reactive and toxic oxygen-containing species can cause cellular damage and impair vital cellular functions. The three available AmB susceptible ATS isolates available at our institution displayed a more than threefold higher oxygen consumption rate respirometric analyses over time along with elevated mitochondrial genome copy numbers. Interestingly, AmB treatment caused significantly diverging changes in the mitochondrial genome copy number with increased in ATS and decreased copy numbers in ATR, accentuating differences in mitochondrial activity.[55] This AmB-mediated up-regulation of the mitochondrial DNA content in ATS was also reselected by a significant increase in ROS. Since AmB might induce oxidative stress within fungal cells, AmB induced ROS generation was studied and H_2O_2 treatment served as positive control. Similar to H_2O_2 exposure, AmB mediated significantly higher ROS levels in ATS concurrent with decreased respiration rates after 15 min AmB exposure. ATR responded to AmB treatment with a moderate ROS increase and the initial decline in oxygen consumption recovered over time, whereas ATS oxygen consumption rates decreased constantly with AmB exposure through AmB´s fungicidal activity. The use of a mitochondrial ROS indicator validated a severe AmB-induced mitochondrial ROS generation in ATS but not in ATR isolates.[55]

On the basis of the differences in oxygen consumption rates and AmB-mediated ROS induction in mitochondria, we investigated the inhibition of the mitochondrial ETC components to see if mitochondrial alterations elicit divergent susceptibility patterns in distinct *A. tereus* isolates. Individual inhibitors were used in equimolar relations where radial growth was affected less than 20% using the individual inhibitors alone. Consistent with results in *A. fumigatus* and *Neurospora crassa*, inhibition of complex I, the NADH:ubiquinone oxidoreductase, had no effect on

AmB susceptibility in ATR, however increased the MIC in ATS.[56] Complex I can be circumvented, since electrons may enter the ETC from either side of the membrane via internal and external alternative NADH dehydrogenases, and thus reduce ROS generated by complex I. Complex II of the OXPHOS system, the succinate dehydrogenase complex, interconnects the tricarboxylic acid cycle with the respiratory chain and inhibition resulted in a marked MIC decline in ATR isolates, however, still in the resistant range and ATS isolated were unaffected. Inhibition of complex III and IV decreased MIC to a susceptible range in all tested ATR isolates and ATS isolated did not respond to inhibition again. Fungi possess accessory complexes in their ETC, like internal and external NADH dehydrogenases or alternative oxidases (AOX). AOXs constitute nuclear encoded terminal oxidases present in plants, algae, protists and fungi (*S. cerevisiae* and *S. pombe* are notable exceptions). They are located in the inner mitochondrial membrane and catalyse the reduction of oxygen to water receiving electrons from reduced ubiquinone, thereby circumventing complexes III and IV. Inhibition of the AOX rendered ATR susceptible to AmB treatment and again no effect on ATS isolates. Owing to their high AmB susceptibility and abnormal mitochondrial function, no apparent response to inhibition of the ETC complexes could be observed in ATS. In contrast, ATR isolates, which are able to cope better with oxidative stress compared with ATS, were heavily affected in AmB susceptibility by the inhibition of the ETC components, indicating the OXPHOS system as targets for antifungal drugs. At a transcriptional level, AmB induced the expression of mitochondrial ETC genes in all tested strains. However, this induction was significantly more pronounced in the ATS isolates tested, except for the AOX, in which the upregulation of mitochondrial activity resulted in overproduction of ROS to toxic levels.[55]

With regard to the potential influence of antioxidants and redox scavenging agents, the ability of N-acetyl-cysteine (NAC) as an unspecific thiol donor of to counteract ROS was investigated. In support of the hypothesis that AmB increases ROS levels, NAC rescued AmB-induced ROS increase as well as AmB tolerance in ATS in a dose-dependent manner. NAC rescued AOX activities in ATS at transcriptional and translational levels, thus suggesting a participation in AmB-induced ROS detoxification responses. Furthermore, an ortholog of the yeast activator protein, *yap1* in *S. cerevisiae,* was transcriptionally induced in ATS more than 26-fold with NAC/AmB cotreatment, and in hand with this, downstream targets of Yap1 such as glutathione metabolic genes and thioredoxin-related genes were increased too. Similar results were obtained in yeast, where NAC increased the reduced glutathione pool.[57,58] NAC was used in previous studies to counteract AmB-induced nephrotoxicity or to alleviate invasive pulmonary aspergillosis-associated lung injury in a neutropenic mouse model.[59,60]

In contrast to NAC/AmB co-treatment, supplementation with L-ascorbic acid (AA) resulted in a contrary outcome concerning AmB susceptibility *in vitro* and *in vivo*. AA, an important bioactive substance, was recently revisited in human cancer studies to exert pro-oxidant activity.[61,62] A pro-oxidant mechanism of AA was furthermore suggested in a study on *C. albicans* and *Cryptococcus neoformans*.[47] Recently, beneficial effects of AA/AmB co-treatment increasing survival in a murine model for the reappearance of candidemia during sepsis were reported.[63] AA was used in the range of pharmaceutical concentrations that are obtainable with intravenous injection of AA in the human body.[64] At this concentration, AA together with AmB exhibited pro-oxidative capacities and the ROS levels in ATR doubled, which was accompanied by a significant decrease of AmB MIC, thereby rendering resistant strains susceptible.[55] Catalase (CAT) and Mn Superoxide (SOD) dismutase activities increased in all tested strains due to AA treatment pointing to higher levels of oxidative stress and a pro-oxidant capacity of AA. Co-treatment of AmB resistant *C. albicans* and *A. flavus* clinical isolates with AA/AMB displayed a sharp decline in MIC values, however not always to a susceptible range for the tested *A. flavus* isolates. Interestingly, AA had no impact to the susceptibility of azole drugs. Importantly, these results strongly suggest an involvement of ROS in AmB´s fungicidal activity. Additionally, *in vivo* experiments in *G. mellonella* larvae revealed that so far resistant ATR isolates rendered susceptible upon co-treatment with AA and AmB.[55]

Blum et al. reported higher catalase activity in ATR compared to *A. fumigatus* or the rare AmB susceptible *A. terreus* isolates.[16,31] At a transcriptional level ATS and ATR displayed distinct expression and different patterns upon AmB treatment. In ATR the mitochondrial Me/Fe SOD was significantly up-regulated by AmB treatment, while ATS isolated increased transcription of the cytosolic Cu/Zn SOD levels and superoxide anions increased in ATS mitochondria.[65] Tackling SOD or CAT functions with inhibitors rendered resistant *A. terreus* isolates susceptible to AmB treatment, too.

terreus in vivo models and host determinants impacting on AmB efficacy

A. terreus infections are associated with high dissemination rates of the pathogen and increased lethality rates. Speth et al. investigated and compared the virulence potential of a rare susceptible *A. terreus* isolate with ATR in a systemic murine infection model over a period of 24 days.[66] The investigated strains showed distinct virulence patterns and

interestingly ATS infected mice showed higher mortality rates and died earlier. The fungal burden was significantly higher in ATS infected mice, especially in the liver and in the lungs, indicating that the rare AmB susceptibility has no effects on the ability to induce infection or virulence. Moreover, ATS infected animals exhibited decreased thrombocyte numbers in the course of infection.

In vitro tests with freshly isolated human thrombocytes incubated with *A. terreus* ATS and ATR conidia, respectively, were performed as *A. terreus* frequently enters the blood stream of infected individuals and might induce thrombosis.[8] In this comparative analysis a marked release of CD62P from alpha granules especially with ATS conidia was reported. Additionally, ATS conidia caused a significant release of CD63P from dense granules in contrast to ATR conidia. A similar platelet activation and attachment of platelets was also found for aleuroconidia and hyphal cell wall components of ATS but not ATR. AmB treatment of ATS infected mice, however, improved survival rates by 75% but proofed ineffective for ATR.[16]

Consistent with the murine model, the elevated virulence potential of ATS isolates was confirmed in the *G. mellonella* infection model.[67] In the *G. mellonella* infection model virulence was inoculation dose and temperature-dependent, but ATS isolates again showed increased virulence potential compared to ATR.[67] AmB treatment increased survival in the ATS infected groups in a dose dependent manner, but no effect was observed on ATR isolates. AmB administration alone increased the number of haemocytes, immune cells in *G. mellonella* resembling phagocytes. The hemocyte density was discerned in the ATS and ATR infected groups, respectively. ATR infected larvae displayed hemocyte numbers comparable to the uninfected control groups, while hemocyte numbers decreased in ATS infected larvae, supporting the findings obtained in the murine infection model. In hand with decreased hemocyte numbers, melanization, as one of the first immune responses of larvae, occurred earlier in the ATS infected group.[67]

Fungal growth may induce hypoxic microenvironments in infected tissue with consequences on the fungal metabolism, cell wall and membrane composition and in turn innate immune responses but also drug efficacy.[68,69] The consequences of reduced oxygen levels on antifungal susceptibility of clinical relevant *Aspergillus* species were examined *in vitro*.[69] Growth rates under hypoxia were reduced by 20% for *A. terreus* and *A. flavus*, while growth of *A. fumigatus* was unaffected. The growth reduction could result from delayed germination rates of conidia. AmB´s MICs and epidemiological cut off values (ECOFF) were reduced in *A. terreus*; however, the reduction was dependent on the test methods used (MIC distribution Etest 12 $\log_2$ dilutions, MIC distribution microdilution 2 $\log_2$ dilutions, comparing hypoxia vs. normoxia). Interestingly, ergosterol or cholesterol supplementation abolished the effects under hypoxia. Minimal fungicidal concentrations (MFCs) were not altered under hypoxia for *A. fumigatus* and *A. flavus*. MFCs of *A. terreus* for azoles were either increased or could not be determined, and for AmB more colonies could be recovered under hypoxia, although no MFCs could be determined under either oxygen condition suggesting a shift to fungistatic activity under low oxygen levels, in part explaining the high failure rate of antifungal therapy *in vivo*.[69]

Antifungal drug resistance is becoming an increasing problem, as acquired resistance to azole drugs is rising and possibly becoming more common in *Aspergillus* species.[2] AmB has proven to be extremely resistance evasive, and the mechanism of AmB action was further unravelled in the recent past. Fundamental insights in the mode of AmB fungicidal mode of action regarding ergosterol binding have been gained and additional fungicidal effects by oxidative damage induction were discovered.[22,23,46] Elegant studies demonstrated that acquired AmB resistance mechanisms come at great costs—that is, the loss of virulence, highlighting the singularity of AmB as antifungal.[30]

The rare intrinsic AmB resistance is noted for a few pathogenic species; however, the underlying mechanisms are still poorly understood as conventional targets as for instance altered sterol composition or content can be ruled out for *A. terreus*.

Aspergillus terreus is the prototype of an AmB resistant mould and offers the possibility to explore the features that confer the outstanding intrinsic AmB resistance. Scarce AmB susceptible clinical *A. terreus* facilitate to dissect AmB resistance traits. These susceptible strains are able to infect their host and display higher virulence in *in vivo* models but respond to AmB treatment. A common motif for the intrinsic AmB resistance is that various stress responses hold key roles required to overcome AmB fungicidal effects and alterations are observed in ATS (see Fig. 1). Impairing the mediators of stress responses through pharmacologic inhibition caused sharp declines in resistance. Nevertheless inhibiting may cause collateral damage, exemplified for Hsp90 inhibition in a murine infection model.[39] Mitochondrial functions displayed striking differences in ATS and ATR isolates and impact on the cellular redox status as pro- and anti-oxidants modulate AmB activity in an antithetic manner and a mitochondria dependent mode.[55] These findings show that the pro-oxidative character of ascorbic acid overpowers AmB resistance and that combinatorial treatment strategies can overcome resistance. Fungal mitochondria hold some unique features that are currently under investigation and hold great potential for the development of novel antifungals [reviewed in[70]].

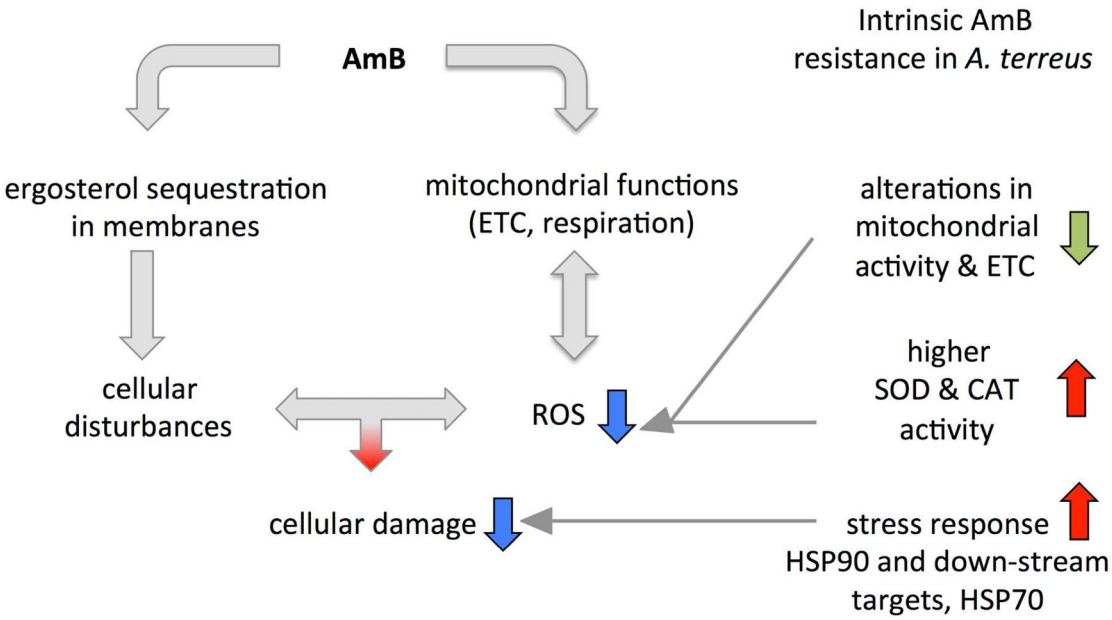

Figure 1. Mechanisms contributing to the intrinsic AmB resistance in *A. terreus*. CAT, catalase. ETC, electron transport chain. HSP, heat shock proteins. ROS, reactive oxygen species. SOD, superoxide dismutase. This Figure is reproduced in color in the online version of *Medical Mycology*.

Apart from the attempt to identify novel drug targets exciting research is performed to develop less toxic, more effective and resistance evasive AmB-urea derivatives, which have to be tested on intrinsically resistant species.[71]

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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