Prior *in vitro* exposure to voriconazole confers resistance to amphotericin B in *Aspergillus fumigatus* biofilms

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RUNNING TITLE: Azole induced *Aspergillus fumigatus* biofilm resistance

Abstract

Triazoles are the mainstay treatment for aspergillosis, though resistance to these antifungal agents may be associated with treatment failure. Refractory infections often necessitates the switch to other antifungal agents, including amphotericin B (AMB), though these infections may not resolve. The aim of this present study was to investigate the effect of prior azole exposure to AMB sensitivity in *Aspergillus fumigatus* biofilms. We hypothesised that sequential antifungal therapy has the potential to impact adaptive resistance mechanisms. Antifungal sensitivity was determined for each isolate against amphotericin B ± voriconazole (VRZ) exposure by a broth microdilution method and a XTT metabolic assay. To analyse the role of extracellular DNA (eDNA) and Hsp90 activation, sensitivity to AMB ± DNA digesting enzyme (DNase) and Hsp90 inhibitor (geldanamycin [GDA]) was also tested. Finally, scanning electron microscope imaging was performed to assess phenotypic changes. Our *in vitro* data revealed that *A. fumigatus* sensitivity to AMB was decreased when it was tested in combination with VRZ. In addition, a 2- to 4-fold decreased sensitivity to AMB was recorded against VRZ exposed germlings compared to controls. It was also shown that depletion of eDNA by DNase treatment enhanced AMB activity against VRZ exposed cells by 8-fold, which visually could be explained by the destabilisation of the biofilm when examined microscopically. Pharmacological inhibition of Hsp90 by GDA significantly improved biofilm susceptibility to AMB 4- to 8-fold. In conclusion, *A. fumigatus* pre-exposure to VRZ drug concomitantly induces eDNA release and activates the stress response, which collectively confers AMB resistance *in vitro*.

KEYWORDS: *Aspergillus fumigatus*, biofilm, azole, polyene, eDNA, Hsp90

Introduction

Aspergillus fumigatus is the most common species associated with invasive aspergillosis (IA), an opportunistic infection associated with high morbidity and mortality. Triazoles such as voriconazole (VRZ) and posaconazole are the mainstay of oral therapy and prophylaxis of aspergillosis. These compounds are fungistatic, inhibiting the enzyme complexes involved in the production of ergosterol, a key molecule involved in cell membrane integrity. However, the emergence of acquired azole resistance mechanisms among *A. fumigatus* isolates, such as alterations in the *Cyp51a* locus, is increasingly common and associated with treatment failure, leading to mortality rates of >88% in Europe [1]. Amphotericin B (AMB) is an alternative therapeutic option for IA, such as in patients who cannot tolerate VRZ or those who fail to respond to azoles.

AMB is a fungicidal compound that actively binds to ergosterol and directly disrupts membrane integrity. Polyene resistance has not been a major clinical problem to date. However, there have been an increasing number of reports of clinically significant AMB resistance in fungal pathogens. A meta-analysis by Sterling and Merz (1998) reported that 59% of AMB resistance was acquired during treatment, and 86% of these cases did not clear infection [2]. The mechanism of AMB resistance in *A. fumigatus* is not yet fully understood. Recent studies have reported that *A. fumigatus* has the capacity to form biofilms, and these display adaptive antifungal resistance mechanisms [3-5]. It has been shown that these mechanisms confer altered resistance phenotypes depending on the phase of biofilm growth. A mutation in the ergosterol biosynthesis pathway has been reported to cause azole resistance and consequently triggers cross-resistance to AMB in

Candida spp. [6]. Treating *Candida* isolates with sub-inhibitory levels of azoles was shown to increase tolerance to AMB *in vitro* [7]. However, in *A. fumigatus* the number of azole resistance isolates with non-*Cyp51a* mutation has increased over past few years and the impact of azole resistance on susceptibility to AMB remains unclear [8].

The potential mechanisms of AMB resistance include alteration of cell wall components and reduced binding of AMB to membrane sterols. Based on our previous studies on adaptive resistance mechanisms in *A. fumigatus* biofilms, we hypothesised that azole selection pressure activates heat shock proteins (HSP90) and induces extracellular DNA (eDNA) release in *A. fumigatus* biofilms, therefore affecting subsequent AMB sensitivity.

Materials and methods

Strains and media

A. fumigatus Af293, and three clinical strains isolated from the Royal Hospital for Sick Children (Yorkhill Division, Glasgow) were used throughout this study. For conidial preparation isolates were propagated on Sabouraud dextrose agar for 72h and conidia harvested, quantified and standardised to 1×10^5 conidia/mL in MOPS buffered RPMI for all subsequent studies, as described elsewhere [3].

Susceptibility testing

Time kill study was performed on Af293 to investigate the kinetics of VRZ (Pfizer Pharmaceuticals, NY, USA) and AMB (Sigma Aldrich, UK) either alone or in combination (VRZ-AMB), against 8h grown biofilm (germlings) at $2 \times MIC_{90}$ (1 mg/L) of this strain. At selected time points (1, 4, 12 and 24h) the cells were washed and the proportion of metabolic reduction compared to unchallenged cells assessed by XTT (2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-caboxanilide) assay [9]. Eight replicates for each time point were performed on two separate occasions.

To investigate the effect of sequential treatment, biofilms were first treated with VRZ, followed by AMB. Briefly, the standardised conidial inoculum for each strain was dispensed into flat bottomed 96 well microtitre plates and incubated for 8h at 37^{o}C. The biofilms were then washed and treated with VRZ at a sub-MIC_{50} concentration of 0.06 mg/L for 16h. Biofilms were then challenged with AMB serially double diluted across each adjacent well of the microtitre plate to produce a concentration range of 0 to 64

mg/L. Next, to investigate the effect of VRZ pre-treatment and the role of eDNA or HSP90 in AMB sensitivity, a DNA digesting enzyme DNAse (128 mg/L, [Sigma]) or HSP90 inhibitor geldanamycin (GDA [50 mg/L], Invivogen) was tested in combination with AMB against VRZ exposed germlings, as described elsewhere [4, 5]. Each challenged biofilm was incubated for 24h at 37^{o}C. Following challenge the antifungal drugs were washed 3X in PBS and the MIC_{50} determined by XTT assay, as described above.

Scanning electron microscopy

For scanning electron microscopy (SEM), representative biofilms grown and treated on Thermanox coverslips were processed as previously described [4]. Briefly, VRZ pre-treated and control biofilms grown on coverslips were left untreated or treated with AMB (1 mg/L) in the presence and absence of DNase (128 mg/L) or GDA (50 mg/L) for 24h. Following treatment biofilms were fixed in 2% paraformaldehyde, 2% glutaraldehyde, and 0.15% [w/v] alcian blue in 0.15 M sodium cacodylate (pH 7.4). The biofilms were sputter coated with gold and viewed under a JEOL JSM-6400 scanning electron microscope.

Statistics

Analysis of variance (ANOVA) and *t* tests were used to investigate independent sample data. Bonferroni's correction for multiple comparisons was applied to the data where appropriate. GraphPad Prism (version 4; GraphPad, La Jolla, CA) was used for production of the figures. *P* values of <0.05 were considered significant.

Results

Time-kill analysis of early phase biofilm (Af293 - 8 h germlings) challenged with VRZ and AMB, either alone or in combination, demonstrated that at after 1 h AMB was significantly more effective than VRZ ($p<0.001$), but thereafter there were no significant differences observed (Figure 1). When the efficacy of VRZ and AMB used alone was compared to the combination, diminished activity was observed for each antifungal at 1, 4, 12 and 24 h of treatment, with combination therapy being less effective in all cases.

To examine the underlying mechanism involved in resistance to antifungal combinations, we investigated eDNA release and activation of HSP90 using appropriate inhibitors. Assessment of AMB sensitivity against germlings (Af293 and three clinical isolates) displayed a significantly higher MIC_{50} (the concentration that inhibited 50% XTT metabolism) by 2- to 8-fold, with VRZ pre-treatment (PT) compared to untreated germlings ($p<0.05$) (Figure 2). When treating the VRZ exposed germlings with AMB in the presence of DNase the MIC_{50} was significantly reduced by 4- to 8-fold compared to absence of DNase. In addition, pharmacological compromise of HSP90 by GDA also showed a synergistic effect of enhancing AMB activity by 4- to 16-fold (Figure 2A).

To further investigate the impact of VRZ pretreatment, the morphology of *A. fumigatus* biofilm was investigated using SEM imaging. Striking architectural changes of *A. fumigatus* biofilms were observed upon VRZ pretreatment at the sub-MIC concentration. Biofilm treated with VRZ displayed increased level of ECM (indicated by white arrow in Figure 2B), and hyphal production was observed. Treating VRZ exposed biofilms with

AMB alone resulted in minimal damage compared to unexposed controls, however, the addition of AMB with DNase or GDA caused more hyphal cell damage throughout the biofilm (indicated by arrow head in Figure 2B). Treating biofilm with DNase on its own display less ECM and disrupted biofilm architecture. The HSP90 inhibitor GDA on its own had less or no effect on biofilm morphology. Taken together, these results indicate that exposure of germlings to VRZ induces changes in morphology of *A. fumigatus* biofilms, which impacts AMB activity, depletion of eDNA and HSP90 inhibition enhancing the efficacy of AMB.

Discussion

In vitro the results of this study establish the adverse effect of VRZ exposure on subsequent AMB treatment in *A. fumigatus*. Resistance of *A. fumigatus* biofilms to antifungal drugs including the azoles is increasingly common, often requires surgical removal of the infected substrate. The mechanisms of antifungal resistance in fungal species are complex and some are shown to be adaptive responses. The mechanisms including mutation in *CYP51A* gene, efflux pump activity, HSP90 activated calcineurin signalling pathway and presence of eDNA have been reported to be associated with antifungal resistance in *A. fumigatus* [3-5, 8]. Although, some of the reported mechanisms were induced and contributed to azole resistance, their role in cross-resistance to other antifungal drugs remains unclear. In *C. albicans*, it was demonstrated that biofilms pretreated with fluconazole significantly decreases the efficacy of sequential caspofungin treatment, which was shown to be mediated by HSP90 and calcineurin signalling pathway [10]. Kelly and colleagues (1997) established that defective sterol $\Delta^{5,6}$ - desaturation causes fluconazole resistance in clinical isolates which results in reduced levels of ergosterol in the cell membrane, which in turn causes cross-resistance to AMB [6]. In this study, we have shown that azole selection pressure activates other resistance mechanisms such as HSP90 and eDNA in *A. fumigatus* which influences subsequent AMB treatment.

Previous studies show that both VRZ and AMB exhibit their greatest activity against newly germinated conidia compared to multicellular hyphae [9]. However, AMB is generally more effective than VRZ against the fully formed hyphal populations, which

may be due to the fact that AMB does not require actively growing cells to elicit its action. This is clear from time kill studies showing that AMB displayed rapid and highly significant antifungal activity compared to VRZ after only 1 h (Figure 1). Nevertheless, increased resistance to all agents tested was observed in older hyphal populations which may be due in part to the presence of extrapolymeric material [11].

Due to the resistance of older hyphal populations, we focussed on the early germinated hyphal phase of growth to determine how rapidly each drug elicits its action. The data indicated that, while AMB was significantly more active at 1 h, both drugs were highly effective with no significant differences between them at 4, 12 and 24 h. When both drugs were tested in combination, a significant reduction in activity compared to either drug alone was seen. This is in contrast to studies performed with planktonic cells, where Perkhofer *et al* (2007) investigated *in vitro* antifungal combinations, demonstrating both indifference and synergism with combinations of VRZ-AMB [12]. In an *in vivo* model of IA Kirkpatrick and colleagues (2006) reported that VRZ alone or in combination with AMB was most effective in reducing the mortality and fungal tissue burden, noting no antagonism between the drugs [13]. A second report concluded there was no significant difference between groups of guinea pigs receiving VRZ alone or VRZ-AMB, and activity was neither enhanced nor reduced with the combination of two antifungal agents [14].

Secondly we have shown that pre-exposure of *A. fumigatus* germlings to sub-inhibitory concentration of VRZ increases tolerance to AMB by 2 to 4 fold. This is similar to the findings by Vazquez and colleagues (2007) who demonstrated a pronounced increase in

resistance to AMB with overnight exposure to azoles in *Candida* spp. The kill rate of *Candida* by AMB has shown to be significantly reduced after pre-exposure to either fluconazole or itraconazole for 16h [7]. When it comes to the mechanism of resistance SEM images suggest that VRZ treatment increases ECM production (Figure 2B), which directly impacts the AMB binding with sterols. The molecular chaperone HSP90 regulates azole resistance through different mechanisms including marked control over biofilm ECM production [4]. Pharmacological depletion of HSP90 significantly reduced AMB MIC in VRZ exposed cells, providing a compelling mechanism by which VRZ might confer AMB resistance. Recent studies established the presence of eDNA in fungal biofilms plays a role in biofilm stability and antifungal resistance [5, 15]. Stimulation of eDNA release by antifungals is not known to occur in fungal biofilms. However in bacterial biofilms low levels of β-lactam antibiotics induce eDNA release and biofilm formation. In this study, DNase was shown to enhance the efficacy of AMB following VRZ exposure suggesting that VRZ might also stimulate resistance by inducing eDNA release.

In conclusion, our data signify that *A. fumigatus* grown in the presence of VRZ induces tolerance to AMB. Our *in vitro* data suggest that inappropriate use of azoles may cause sequential AMB treatment be counterproductive. Finally, the use of DNase or Hsp90 inhibitor in combination with AMB appears promising to overcome the problem.

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Figure Legends

Figure 1. Pharmocodynamics of voriconazole, amphotericin B alone and in combination against *A. fumigatus* germinated conidia. Figure shows the time-kill kinetics of AMB (1 mg/L), VRZ (1 mg/L) either alone or in combination against Af293 germinated conidia (8h). Results are the average of eight replicates carried out on two separate occasions and are expressed as the proportion of cellular viability determined by the XTT assay. Error bars represent the standard deviation of the means. Significant differences are represented by an asterix (* $p<0.001$).

Figure 2. Pre exposure to voriconazole stimulates AMB tolerance. *A. fumigatus* germlings was grown in RPMI at 37^{o}C and pre-treated with VRZ (+VRZ PT) or vehicle (-VRZ PT) for up to 24h. (A) MIC_{50} of AMB ± DNase (128 mg/L) and AMB ± GDA (50 mg/L) was determined by broth micro dilution method for each isolates. Bar graph shows the mean MIC_{50} value of all tested isolates (Af293 and three clinical isolates). Error bars represent the standard deviation of the means. Significant differences are represented by an asterix (*$p<0.05$). (B) VRZ pre-treated and control biofilms grown on coverslips were left untreated or treated with AMB ± DNase and AMB ± GDA for 24h. Following drug exposure, biofilms were fixed and imaged by SEM. Representative biofilm from each group is shown in figure. The white arrow indicates biofilm matrix and arrowhead indicates burst and broken hyphae in the biofilms.