

## Phase-dependent antifungal activity against *Aspergillus fumigatus* developing multicellular filamentous biofilms

Eilidh Mowat<sup>1</sup>, Sue Lang<sup>1</sup>, Craig Williams<sup>2</sup>, Elaine McCulloch<sup>2</sup>, Brian Jones<sup>3</sup>  
and Gordon Ramage<sup>4\*</sup>

<sup>1</sup>Department of Biological and Biomedical Sciences, Glasgow Caledonian University, Glasgow, UK; <sup>2</sup>Microbiology Department, Royal Hospital for Sick Children, Glasgow, UK; <sup>3</sup>Glasgow Royal Infirmary, Glasgow, UK; <sup>4</sup>Section of Infection and Immunity, Glasgow Dental School, Faculty of Medicine, University of Glasgow, 378 Sauchiehall Street, Glasgow G2 3JZ, UK

Received 8 June 2008; returned 7 August 2008; revised 26 August 2008; accepted 2 September 2008

**Objectives:** *Aspergillus fumigatus* undergoes morphological transition throughout its growth and development. These changes have direct implications for the effectiveness of antifungal treatment. Here we report the *in vitro* antifungal activity of voriconazole, amphotericin B and caspofungin against three specific phases of multicellular development of *A. fumigatus*.

**Methods:** *A. fumigatus* conidia were propagated for 8, 12 and 24 h prior to antifungal challenge. The resultant activity of the three agents tested was determined using an XTT reduction assay to assess both endpoint and time–kill susceptibility profiles.

**Results:** Endpoint susceptibility testing demonstrated a time-dependent decrease in efficacy for all three antifungal agents as the complexity of the *A. fumigatus* hyphal structure developed. Overall, amphotericin B exhibited the best spectrum of activity at each phase of growth, but was comparable to voriconazole against germinated conidial growth (8 h). Later, both voriconazole and caspofungin were ineffective against complex mycelial structures (12 and 24 h). Time–kill studies demonstrated that amphotericin B was significantly more efficacious at reducing *A. fumigatus* metabolism than both voriconazole and caspofungin for all three growth phases examined, most notably after 1 h of drug exposure ( $P < 0.001$ ).

**Conclusions:** Overall, the data presented demonstrate that treatment of actively growing *A. fumigatus* cells with antifungal agents is more efficacious than treating mature structures *in vitro*. Amphotericin B was consistently more effective against each phase and displayed rapid effects, and therefore may be a suitable option for managing patient groups at risk from aspergillosis infections.

**Keywords:** *A. fumigatus*, pharmacokinetics, invasive aspergillosis

### Introduction

The incidence of systemic mycoses caused by filamentous fungi has risen dramatically in the last decade mainly due to the increased use of immunosuppressive therapy. Pulmonary disorders, corticosteroid use and diabetes mellitus are three of the most common conditions that cause patients to be at increased risk of *Aspergillus* infection. The principal cause of invasive aspergillosis is *Aspergillus fumigatus*, which is associated with at least a 40% mortality rate in some patient populations.<sup>1</sup>

The initial establishment of an *A. fumigatus* infection involves the germination of inhaled conidia with subsequent

hyphal invasion of the lung tissues. Histology and microscopic examination of bronchopulmonary lavage samples indicate the presence of numerous *A. fumigatus* hyphae which often intertwine and form complex multicellular structures. We have recently developed an *in vitro* model to investigate the filamentous growth and development of *A. fumigatus* as a multicellular entity and shown that the antifungal susceptibility profiles of conidia (as assessed by the CLSI) and multicellular filamentous structures are quite disparate, suggesting that morphological status may influence the susceptibility profile.<sup>2</sup> However, conflicting reports indicate that the morphological state may, or may not, alter susceptibility profiles of filamentous fungi.<sup>3,4</sup>

\*Corresponding author. Tel: +44-141-211-9752; Fax: +44-141-211-1593; E-mail: g.ramage@dental.gla.ac.uk

The purpose of this study was to determine the antifungal susceptibility profiles of *A. fumigatus* during different phases of growth and development of complex filamentous biofilm communities and to investigate how rapidly the three classes of antifungal agent act upon these.

## Materials and methods

### Isolates

*A. fumigatus* Af293 was used throughout this study. This was stored on Sabouraud dextrose (SAB) agar slopes (Oxoid) at 4°C. For conidial preparation, Af293 was propagated on SAB agar for 72 h and conidia were harvested and quantified as previously described.<sup>2</sup>

### Susceptibility testing

Commercially available amphotericin B (Sigma-Aldrich, Gillingham, Dorset, UK), voriconazole (Pfizer Pharmaceuticals, NY, USA) and caspofungin (Merck Sharp Dohme Ltd, NJ, USA) were used throughout this study. Each antifungal drug was prepared at stock concentrations of 10 mg/mL in sterile water and used within 24 h of reconstitution. The endpoint activity of these antifungal drugs was determined. Af293 conidial inoculum ( $1 \times 10^5$  conidia/mL) was dispensed into flat-bottomed 96-well microtitre plates as described previously and incubated over selected time points (8, 12 and 24 h) at 37°C.<sup>2</sup> Cells were washed and each antifungal drug was diluted to working concentrations in MOPS-buffered RPMI, and then serially double-diluted across each adjacent well of the microtitre plate to give a concentration range of 0.008–256 mg/L. A series of antifungal-free wells and cell-free wells were also included to serve as positive and negative controls, respectively. Each challenged multicellular population was incubated for 24 h at 37°C. Following challenge, the antifungal drugs were removed and the antifungal activity was determined. Time–kill studies were also performed on Af293 for each phase of filamentous growth (8, 12 and 24 h) with defined concentrations of each antifungal (voriconazole, 1 mg/L; amphotericin B, 1 mg/L; caspofungin, 8 mg/L). At each time point (0, 1, 2, 4, 8, 12 and 24 h), the cells were washed and their metabolic activity was assessed using an XTT [2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide] assay.<sup>2</sup>

### Quantification of antifungal activity

Antifungal activity of each growth phase was quantified using an XTT reduction assay, as described previously.<sup>2</sup> Cellular viability is calculated as a function of metabolism, which is indicated by a change from a colourless solution to orange depending on the relative viability of the filamentous population.<sup>2</sup> The metabolic activity of

each phase of filamentous growth was quantified in a microtitre plate reader (FLUOstar OPTIMA, BMG Labtech, Buckinghamshire, UK) at 490 nm. The XTT absorbance value, after normalizing for background absorbance levels, was used to assess the effectiveness of each antifungal agent relative to the unchallenged positive control. Sessile MICs (SMICs) were determined at 50% and 90% reductions in XTT absorbance compared with the untreated control. Testing of each isolate was performed in triplicate.

### Statistical analysis

For time–kill analysis, a one-way ANOVA with Bonferroni's post-test for multiple comparisons was performed on normalized proportional data using GraphPad Prism version 4.0 for Windows (GraphPad Software, CA, USA).

## Results

Table 1 summarizes the *in vitro* endpoint testing of amphotericin B, voriconazole and caspofungin against Af293. All three antifungal agents exhibited good overall activity against germinated conidia (8 h), with voriconazole and amphotericin B exhibiting the most efficacious characteristics (SMIC<sub>90</sub> = 0.5 mg/L). Against the monolayer of intertwined hyphae (12 h), the efficacy of all three antifungal agents decreased. Amphotericin B was the most efficacious agent (SMIC<sub>90</sub> = 4 mg/L), whereas voriconazole and caspofungin were less active (SMIC<sub>90</sub> = 8 mg/L). Against 24 h growth (dense intertwined hyphae), the efficacy of the three antifungal agents tested was further reduced. Amphotericin B was again the most effective (SMIC<sub>90</sub> = 32 mg/L), whereas voriconazole and caspofungin activity was markedly reduced (SMIC<sub>90</sub> = >256 mg/L). However, voriconazole did retain residual activity (SMIC<sub>50</sub> = 16 mg/L).

Time–kill methodology was used to investigate the antifungal rate of kill on the three phases of Af293 filamentous growth at  $2 \times$  SMIC<sub>90</sub> (Figure 1). These data indicated that throughout the duration of antifungal drug exposure (0–24 h), amphotericin B and voriconazole were significantly less effective the more complex the filamentous populations became ( $P < 0.001$ ). However, for caspofungin, this was only the case for over 12 h of exposure (12–24 h) ( $P < 0.001$ ). Amphotericin B was significantly more effective against germinated conidia after immediate drug exposure (0–4 h) than against intermediate and complex filamentous populations ( $P < 0.001$ ). However, as amphotericin B exposure time increased (8–24 h), there was no significant difference in activity between early and intermediate cellular populations. These data also indicated that immediate

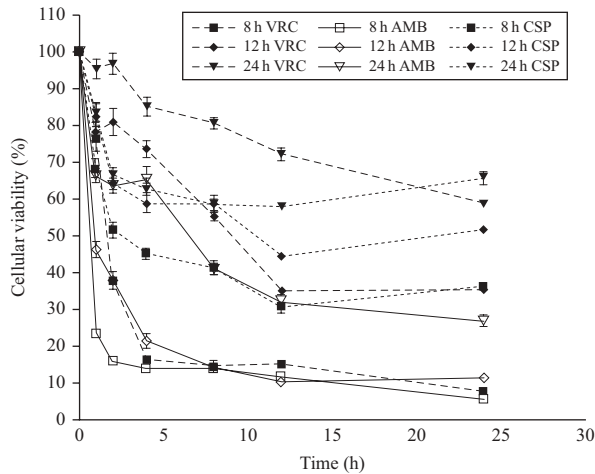
**Table 1.** Antifungal activities of voriconazole, amphotericin B and caspofungin against 8, 12 and 24 h *A. fumigatus* cultures

	Voriconazole (mg/L)			Amphotericin B (mg/L)			Caspofungin (mg/L)		
	8 h	12 h	24 h	8 h	12 h	24 h	8 h	12 h	24 h
SMIC <sub>50</sub> <sup>a</sup>	0.125	0.25	16	0.0625	0.0625	0.125	<0.007	2	128
SMIC <sub>90</sub> <sup>b</sup>	0.5	8	>256	0.5	4	32	4	8	>256

<sup>a</sup>50% reduction in metabolism of the entire cellular population.

<sup>b</sup>90% reduction in viability of the entire cellular population.

## In vitro killing of *Aspergillus fumigatus*



**Figure 1.** Pharmacodynamics of voriconazole (VRC), amphotericin B (AMB) and caspofungin (CSP) against multicellular structures of *A. fumigatus*. The figure shows the killing kinetics of voriconazole (1 mg/L), amphotericin B (1 mg/L) and caspofungin (8 mg/L) against pre-formed *A. fumigatus* cells developed for 8, 12 and 24 h. The results are the averages 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 error of means.

exposure to amphotericin B (0–4 h) was significantly more effective at killing germinated conidia than both voriconazole and caspofungin ( $P < 0.001$ ). However, there were no significant differences in activity between voriconazole and amphotericin B after prolonged exposure (8–24 h). Still, both were significantly superior to caspofungin ( $P < 0.001$ ). Overall, amphotericin B was the most rapid and effective against all cell populations, whereas voriconazole, although highly effective against germinating conidia, was poorer as filamentation increased. Caspofungin was consistently ineffective against all populations investigated.

## Discussion

Although invasive forms of aspergillosis are often diagnosed histologically for the presence of hyphae, current clinical practices rely on conidia for susceptibility testing to inform physicians on the most appropriate antifungal therapy. This study has shown that morphological status plays an essential role in the response to antifungal treatment. To our knowledge, this is the first study to examine three distinct growth phases in relation to antifungal susceptibility, which utilized a timely and reproducible XTT high-inoculum-based assay that provided susceptibility profiles for *Aspergillus* spp., as previously described by Antachopoulos *et al.*<sup>5</sup> The data presented herein clearly demonstrates that *A. fumigatus* becomes increasingly resistant to polyene, azole and echinocandin antifungal agents throughout morphological differentiation, a key characteristic of aspergillosis. Recent studies of multicellular *A. fumigatus* growth have revealed properties including matrix production and reduced susceptibility to antifungal challenge.<sup>2,6–8</sup> In addition, three key developmental phases were identified, which are analogous to the key phases of *Candida albicans* biofilm growth.<sup>7,9</sup> Analysis of these phases indicated that amphotericin B was overall the most efficacious, reducing the metabolic activity of all three

different cell populations, and was also the most rapid by causing a significant reduction of metabolic activity within 1 h of exposure compared with the other antifungal agents for each respective phase of growth. Amphotericin B binds directly to membrane sterols causing the formation of channels or pores, resulting in cytoplasmic and ion leakage leading to cell death. Therefore, its ability to kill cells rapidly and reduce metabolic activity is not directly influenced by the growth phase *per se*. However, as structural complexity increased, a reduction in activity was observed, which suggests that the matrix material, which includes galactomannan and  $\alpha$ -1,3-glucans, is associated with this growth phase and plays a role in impeding drug action, a phenomenon reported by our group and others.<sup>6,7</sup> It has been reported that persister cells can exist in fungal biofilms, which may be an alternative explanation to the effect observed in *A. fumigatus* cells following amphotericin B challenge.<sup>10</sup>

Voriconazole was also effective, but primarily against actively growing hyphae associated with early cell populations, although overall significantly less rapid at reducing metabolism than amphotericin B. This data is consistent with its mechanism of action, i.e. the requirement for actively growing cells in order for it to interfere with ergosterol biosynthesis. Voriconazole was ineffective against complex filamentous growth, which is in part due to a reduced growth rate, but also potentially due to the presence of matrix material. Although caspofungin exhibited initial activity, targeting the cell wall of growing hyphal tips of the germinated conidia, both our SMIC data and time–kill data showed caspofungin to be generally ineffective against highly filamentous forms of *A. fumigatus*, which therefore limits its potential to prophylaxis rather than a defined treatment *per se*.

In conclusion, this study has demonstrated that amphotericin B has a rapid and wide spectrum of activity against various morphological forms of *A. fumigatus* *in vitro*. Furthermore, both cell wall and cell membrane active agents are more effective against *A. fumigatus* during its early development than against complex filamentous forms, and this has implications for early antifungal therapy against suspected aspergillosis.

## Acknowledgements

We would like to thank Dr Colin Murray for his critical evaluation of the manuscript and Dr Andrea Sherrieff for statistical advice.

## Funding

This work was supported by an unrestricted educational grant from Pfizer Pharmaceuticals.

## Transparency declarations

None to declare.

## References

1. Denning DW, Marinus A, Cohen J *et al.* An EORTC multicentre prospective survey of invasive aspergillosis in haematological

patients: diagnosis and therapeutic outcome. EORTC Invasive Fungal Infections Cooperative Group. *J Infect* 1998; **37**: 173–80.

2. Mowat E, Butcher J, Lang S *et al.* Development of a simple model for studying the effects of antifungal agents on multicellular communities of *Aspergillus fumigatus*. *J Med Microbiol* 2007; **56**: 1205–12.

3. Guarro J, Llop C, Aguilar C *et al.* Comparison of *in vitro* antifungal susceptibilities of conidia and hyphae of filamentous fungi. *Antimicrob Agents Chemother* 1997; **41**: 2760–2.

4. Pujol I, Fernandez-Ballart J, Guarro J. Effect of inoculum form on *in vitro* antifungal susceptibilities of *Aspergillus* spp. *J Antimicrob Chemother* 2001; **47**: 715–8.

5. Antachopoulos C, Meletiadiis J, Sein T *et al.* Use of high inoculum for early metabolic signalling and rapid susceptibility testing of *Aspergillus* species. *J Antimicrob Chemother* 2007; **59**: 230–7.

6. Beauvais A, Schmidt C, Guadagnini S *et al.* An extracellular matrix glues together the aerial-grown hyphae of *Aspergillus fumigatus*. *Cell Microbiol* 2007; **9**: 1588–600.

7. Mowat E, Williams C, Jones B *et al.* The characteristics of *Aspergillus fumigatus* mycetoma development: is this a biofilm? *Med Mycol* 2008; doi:10.1080/13693780802238834.

8. Seidler MJ, Salvenmoser S, Muller FM. *Aspergillus fumigatus* forms biofilms with reduced antifungal drug susceptibility on bronchial epithelia cells. *Antimicrob Agents Chemother* 2008; doi:10.1128/AAC.00234-08.

9. Chandra J, Kuhn DM, Mukherjee PK *et al.* Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* 2001; **183**: 5385–94.

10. LaFleur MD, Kumamoto CA, Lewis K. *Candida albicans* biofilms produce antifungal-tolerant persister cells. *Antimicrob Agents Chemother* 2006; **50**: 3839–46.