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Activity of antifungal combinations against *Aspergillus* species evaluated by isothermal microcalorimetry☆,☆☆

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ABSTRACT

We evaluated the activity of antifungals alone or in combination against *Aspergillus fumigatus* and *Aspergillus terreus* by real-time measurement of fungal growth-related heat production. Amphotericin B, voriconazole, caspofungin, and anidulafungin were tested alone or in combination. Heat production was measured in Sabouraud dextrose broth containing 10^5 *Aspergillus* conidia/mL for 48 h at 37 °C. Antifungal activity was evaluated by measuring the heat detection time relative to the growth control. Against *A. fumigatus*, the voriconazole-echinocandin combination demonstrated longer heat detection time than each antifungal alone. Against *A. terreus*, the combination amphotericin B–echinocandin prolonged the heat detection time, compared to each antifungal alone. In contrast, the echinocandin-voriconazole combination did not increase the heat detection time, compared to voriconazole alone. None of the antifungal combinations decreased the heat detection time compared to the antifungals alone (e.g. antagonism was not observed). Microcalorimetry has the potential for real-time evaluation of antifungal combinations against *Aspergillus* spp.

1. Introduction

The prevalence of invasive mold infections is increasing, and the attributable mortality remains high (Lass-Florl, 2009; Pfaller and Diekema, 2010). Early and effective antifungal treatment can improve the outcome (Kontoyiannis, 2012). The introduction of new antifungal agents exhibiting different modes of action provided the opportunity to use combination treatment, which could potentially increase the efficacy, prevent emergence of resistance, and provide a broader spectrum of activity (Johnson et al., 2004). However, combination antifungal treatment may also exhibit antagonistic effect, increase drug toxicity, promote spread of resistance, and considerably increase the treatment expenses (Johnson et al., 2004). Therefore, the potential benefit or detriment of antifungal combinations needs to be evaluated carefully. In particular, it may guide the design of animal experiments and clinical trials to improve the outcome of invasive mold infections.

For testing the combination effect of 2 or more antifungal agents, the checkerboard microdilution broth assay is commonly used, and the interaction is expressed by the fractional inhibitory concentration (FIC) index. This index is calculated by dividing the MIC of each drug in combination by the MIC of each drug alone (Pillai et al., 2005). A FIC index of <0.5 indicates synergy, and an index >4 indicates antagonism. The majority of combination studies report results with FIC indices ranging between 0.5 and 4, suggesting indifferent or additive activity (Vazquez, 2008). However, the validity of the FIC index has been questioned by several experts, as a clear correlation with clinical outcome is lacking (Meletiadis et al., 2010). Other authors have suggested using time-kill studies to evaluate the activity of antifungal combinations. However, the investigation of conidia in these studies is questionable since conidia are generally absent in infected tissues, and the fungicidal activity against actively growing hyphae is more predictable for the treatment outcome than conidia (Krishnan et al., 2005; Pfaller et al., 2004).

In this study, we investigated isothermal microcalorimetry as a potential better tool for evaluation of antifungal drug combinations, based on measuring heat production, which is proportional to microbial metabolism and growth rate (Braissant et al., 2009; Wadsö, 2002). A bacterial or fungal culture constitutes a closed thermodynamic system that, under constant temperature and pressure, will exchange heat with its surrounding. By microcalorimetry, heat can be measured in the range of microwatt with high sensitivity and accuracy (Wadsö, 2001). In presence of an antimicrobial, the growth-related heat production is suppressed, which can be used to assess the antimicrobial susceptibility of the microorganism of interest. The use of isothermal microcalorimetry for antimicrobial susceptibility testing was recently reported for *Escherichia coli* and *Staphylococcus aureus* (von Ah et al., 2009) mycobacteria (Howell et al., 2012) and for evaluation of antiparasitic drugs against *Schistosoma mansoni* (Manneck et al., 2011),

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Table 1
Antifungal susceptibility of *A. fumigatus* and *A. terreus* determined by visual examination and microscopy in SDB and according to CLSI and EUCAST guidelines at 24 h and 48 h.

Strain	Time	MIC (μg/mL)						MEC (μg/mL)			
		Amphotericin B			Voriconazole			Caspofungin		Anidulafungin	
		SDB Visual	SDB Micro	CLSI/EUCAST	Visual	Micro	CLSI/EUCAST	SDB Micro	CLSI/EUCAST	Micro	CLSI/EUCAST
A. fumigatus	24 h	0.25	1	0.5/0.5	0.25	0.5	0.25/0.25	0.25	0.5/0.5	0.03	nd
	48 h	1	2	0.5/0.5	0.25	0.5	0.5/1	0.25	0.5/0.5	0.03	nd
A. terreus	24 h	2	4	0.5/0.5	0.25	0.5	0.5/0.5	0.5	0.5/0.5	0.03	nd
	48 h	8	16	1/2	0.5	0.5	0.5/0.5	0.5	1/0.5	0.03	nd

Trypanosoma brucei, and *Plasmodium falciparum* (Wenzler et al., 2012). In addition, we recently demonstrated the use of isothermal microcalorimetry for real-time antifungal susceptibility testing of *Aspergillus* (Furustrand Tafin et al., 2012a) and non-*Aspergillus* species (Furustrand Tafin et al., 2012b). In these studies, amphotericin B, triazoles, and echinocandins affected the heat flow profiles of *Aspergillus* spp. in different manners depending on their ability to inhibit or kill molds (i.e. express fungistatic or fungicidal activity, respectively). These effects were observed particularly with echinocandins when using Sabouraud dextrose broth (SDB), but not Roswell Park Memorial Institute medium, which is recommended for routine antifungal susceptibility by both CLSI (CLSI, 2008) and EUCAST guidelines (EUCAST, 2008).

In this proof-of-concept study, we evaluated the additive effect of caspofungin or anidulafungin, when added to voriconazole or amphotericin B, on 2 *Aspergillus* spp. with distinctive susceptibility pattern, *A. fumigatus* and *A. terreus*. The combination activity was evaluated in parallel by isothermal microcalorimetry and by microscopical evaluation in broth. Antifungal combinations were chosen on the basis of clinical guidelines; recommending voriconazole as first choice for the treatment of invasive aspergillosis and lipid formulation of amphotericin B is a valuable alternative (Segal and Walsh, 2006; Walsh et al., 2008). In addition, adding an echinocandin, such as caspofungin, micafungin, or anidulafungin, is considered in patients not responding to single-drug therapy (Walsh et al., 2008). The availability of an accurate and simple assay may provide important information about antifungal combinations and help to predict their clinical outcome.

2. Methods

2.1. Test organisms

A. fumigatus ATCC 204305 and *A. terreus* ATCC 10690 were used. Molds were subcultured for 3–5 days prior to testing on Sabouraud dextrose agar at 37 °C. Stocks of each strain were maintained in water at 4 °C for short-term storage and in SDB 20% glycerol at −80 °C for long-term storage. An inoculum of ~5×10^7 conidia/mL was prepared in sterile 0.9% saline. The exact inoculum size was determined by microscopic enumeration of conidia using a hemocytometer (Neubauer chamber; Assistent, Sondheim, Germany).

2.2. Antifungals

Solubilized amphotericin B (Sigma, St. Louis, MO, USA) and pure powder of caspofungin (Merck & Co., Whitehouse Station, NJ, USA) were dissolved in sterile water according to the manufacturer's instruction. Pure powder of voriconazole and anidulafungin (Pfizer Pharma AG, Zürich, Switzerland) was dissolved in dimethyl sulfoxide.

2.3. Determination of antifungal susceptibility by microbroth dilution and microscopy

Microdilution broth was performed in SDB (Oxoid CM0147; Basingstoke, Hampshire, UK). After inoculation (2.5×10^5 spores/mL), microdilution plates were incubated at 37 °C and read after 24 h and 48 h. MIC values for amphotericin B and triazoles were determined visually as the lowest concentration of drug that caused complete inhibition of fungal growth and by inverted contrast light microscopy (Nikon Eclipse TS100, 40×/0.65) as the lowest concentration of drug leading to absence of germinated conidia, compared to the growth control. The minimum effective concentration (MEC) for caspofungin and anidulafungin was determined by inverted contrast light microscopy and was defined as the lowest drug concentration at which short, stubby, and highly branched hyphae were observed (Kurtz et al., 1994). In parallel MICs and MICs at 24 h and 48 h were

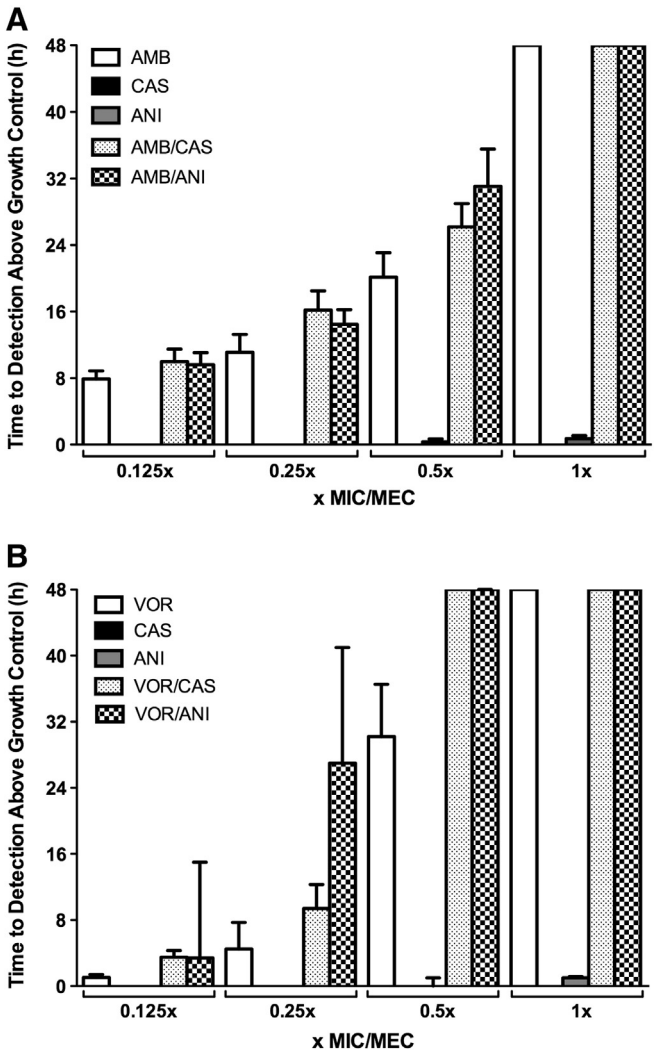

Fig. 1. Time to heat detection of *A. fumigatus* in the presence of single or 2 antifungals (above the growth control): amphotericin B and caspofungin or anidulafungin (A), voriconazole and caspofungin or anidulafungin (B). Detection limit was 20 μW. Endpoint of the assay was 48 h. Data are shown as median and interquartile ranges. The median detection time of the growth control was 8.0 h.