

Effect of hypoxic conditions on *in vitro* susceptibility testing of amphotericin B, itraconazole and micafungin against *Aspergillus* and *Candida*

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Objectives: The aim of this study was to investigate the effect of hypoxic conditions on *in vitro* susceptibility testing of amphotericin B, itraconazole and micafungin against *Aspergillus* (four species) and *Candida* (six species).

Methods: *In vitro* susceptibility tests were set up according to NCCLS M27-A2 and M38-A recommendations, but incubation atmospheres were either air plus 5% CO₂, 1% oxygen/5% CO₂/94% nitrogen or 0.25% oxygen/5% CO₂/94.75% nitrogen.

Results: In all *Aspergillus* species, the MIC of amphotericin B was reduced but the MFC remained unaltered with reduced oxygen. The MICs and MFCs of itraconazole and micafungin were unaltered in hypoxic conditions but interpretation of the MIC was much simpler for micafungin with 1% and 0.25% oxygen. Against *Candida*, conditions modelling hypoxia had little effect on the MICs and MFCs of any of the agents.

Conclusions: This simple adaptation of susceptibility testing may have important consequences for understanding how antifungal drugs work and for endpoint reading.

Keywords: antifungals, hypoxia, susceptibility tests

Introduction

Invasive fungal disease represents a major threat to life in immunocompromised patients and is now one of the most common causes of infection in this group^{1–4} with incidences from 5% to more than 20% in high risk groups.^{5–7} In this patient group, it is essential that effective antifungal agents be administered rapidly. There is a limited range of antifungal agents available to treat disease caused by *Aspergillus* or *Candida*, including the polyenes, flucytosine, azoles and more recently the echinocandins. A variety of techniques have been recommended for the susceptibility testing of fungi to antifungal agents with NCCLS M38-A⁸ and NCCLS M27-A2⁹ widely accepted as the reference methods even though there are problems correlating the *in vitro* response with the *in vivo* outcome particularly for amphotericin B¹⁰ against *Aspergillus* and the azoles against *Candida*.¹¹ Many other methods have been suggested including Etest^{12,13} and disc diffusion but all these methods have the common feature that the *in vitro* activity is tested in air or air supplemented with 5% CO₂.

It has been recognized for many years that even though atmospheric air contains approximately 21% O₂ (pO₂ is 19.7 kPa at sea level), the level in the alveoli of healthy lungs is nearer to 14% (pO₂ is 13.2 kPa). Despite an arterial pO₂ of 13.2 kPa, in actuality the available oxygen is nearer to 7.9 kPa due to the high affinity of haemoglobin for oxygen. By the time oxygen has reached the capillaries and diffused into the tissues its availability is much lower with levels of 2–4% (2–4 kPa) being common (pO₂ may be as low as 0.13 kPa owing to the tension gradient and distance from the capillary).¹⁴ At the same time as the available O₂ decreases, a corresponding increase in CO₂ occurs with normal levels in venous blood near to 5.3 kPa.

Additionally it is clear that the available O₂ within abscesses and infected wounds is lower than the surrounding tissue^{15,16} resulting in extremely hypoxic conditions. The situation within aspergillomas or infarcted lung tissue is unknown but other infarcted tissues such as brain have extremely hypoxic tissue conditions. Further it has previously been noted that the activity of the aminoglycosides is suppressed under low oxygen conditions similar to those likely to occur in infected tissues¹⁷ with 10-fold increases in the MIC. During investigations of amphotericin B-induced damage and killing of *Candida*

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albicans, it was noted that there was a dramatic reduction in protoplast lysis under hypoxic conditions¹⁸ indicating that lysis as a result of amphotericin B required oxygen. This finding is supported by evidence of Gudewicz *et al.*¹⁹ who demonstrated increased *in vitro* efficacy of amphotericin B when used with hyperbaric oxygen.

In this study, we compared the *in vitro* activity of amphotericin B (AMB), itraconazole (ITC) and micafungin (MICA) in two different hypoxic conditions to their activity in air against 37 isolates of *Aspergillus* and 24 isolates of *Candida*.

Materials and methods

Organisms and preparation of inoculum

Susceptibility tests were carried out on 37 isolates of *Aspergillus* belonging to four different species and 24 isolates of *Candida* belonging to six species; all were recent clinical isolates from a variety of patient types. The group comprised 20 *A. fumigatus*, seven *A. terreus*, four *A. flavus*, six *A. niger*, five *C. albicans*, five *C. tropicalis*, four *C. parapsilosis*, four *C. guilliermondii*, three *C. glabrata* and three *C. krusei*. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were tested with each set of experiments to ensure quality control.⁹ The isolates were subcultured from frozen stock and were grown on Sabouraud dextrose agar (Difco, Surrey, UK) at 37°C in air for up to 10 days. *Aspergillus* conidia were harvested by washing the surface of the culture with 25 mL of phosphate buffered saline (Life Technologies, Paisley, UK) plus 0.05% Tween 80 (Sigma, Poole, UK) (PBST). *Candida* blastoconidia from three or four colonies were collected and suspended in PBST. The conidia and blastoconidia were counted using a haemocytometer and diluted as required in PBST.

Antifungal agents

ITC was obtained from Janssen-Cilag Ltd (High Wycombe, UK), AMB was obtained from Sigma (Poole, Dorset, UK) and MICA was provided by Fujisawa (Osaka, Japan).

ITC was first dissolved in acetone (Sigma) and then 0.2 M hydrochloric acid (Sigma) was added to provide a stock solution of 3200 mg/L.

AMB was dissolved in dimethyl sulphoxide (Sigma), to provide a stock solution of 3200 mg/L (after adjusting for potency).

MICA powder was dissolved in 5% glucose (in water), to produce a stock solution of 1280 mg/L.

All drug stocks after preparation were dispensed into aliquots and stored in glass vials, protected from the light, at -70°C until required.

Susceptibility testing

Microdilution susceptibility tests for AMB, ITC and MICA were carried out as recommended by the NCCLS M38-A or M27-A2 using RPMI 1640 (Sigma) buffered with MOPS (Melford Laboratories Ltd, Ipswich, Suffolk, UK) to pH 7.0, the medium was modified by the addition of 2% glucose (Sigma).²⁰ Final drug concentrations used for ITC and AMB were 0.008–8 mg/L and for MICA 0.004–4 mg/L (a subgroup of *Aspergillus* isolates were tested at MICA concentrations of 0.000125–4 mg/L); all drug dilutions were made in RPMI 1640. Final inocula were $0.5\text{--}5 \times 10^4$ conidia/mL (*Aspergillus*) and $0.5\text{--}2.5 \times 10^3$ blastoconidia/mL (*Candida*). All microdilution plates were prepared in triplicate (on at least two occasions for *Aspergillus* and three occasions for *Candida*) and incubated in different atmospheres. Plates were incubated at 37°C for 48 h.

Minimum inhibitory concentrations (MICs) for *Aspergillus* were read visually with a no growth endpoint for AMB and ITC. For MICA, plates were examined using oblique lighting; the first well in which there was a prominent reduction in growth was taken as the MIC. MICs for *Candida*

were read spectrophotometrically (at 490 nm) with an 80% reduction in growth endpoint for ITC and a no growth endpoint for AMB and MICA.

Minimum fungicidal concentrations (MFCs) were determined by culturing 100 µL from each well in the microdilution plate that had no visible growth; the MFC was taken as the first well with less than 5 cfu (99.99% kill for *Aspergillus* and a 99% kill for *Candida*).

Gas mixtures in incubation atmosphere

During incubation, the microdilution plates were sealed in gas-tight jars and gas flow was maintained over the plates at a steady rate of 0.25 bar above atmospheric pressure.

Three different gas mixtures were used to generate the incubation atmosphere in the study (BOC Special Gases, Manchester, UK):

- (i) 20% oxygen/5% carbon dioxide/75% nitrogen;
- (ii) 1% oxygen/5% carbon dioxide/94% nitrogen;
- (iii) 0.25% oxygen/5% carbon dioxide/94.75% nitrogen.

Statistical analysis

Data were analysed by the Kruskal–Wallis test (pairwise comparison Conover–Inman) using the computer package StatsDirect (Ashwell, UK). Box plots were generated using the computer package Analyse-It (Leeds, UK).

For data analysis, MICs and MFCs of >8 mg/L were recorded as 16 mg/L.

Results

Summaries of the *in vitro* susceptibility tests of *Aspergillus* are presented in Table 1 and Figure 1.

Antifungal susceptibilities for the control organisms varied by no more than three two-fold dilutions. For *C. krusei* ATCC 6528, the MICs of itraconazole ranged between 0.06 and 0.25 mg/L, and those of amphotericin B ranged between 0.5 and 2 mg/L. For *C. parapsilosis* ATCC 22019, the MICs of itraconazole ranged between 0.03 and 0.12 mg/L, and those of amphotericin B ranged between 0.5 and 2 mg/L. The final optical densities of the control strains were a mean of 0.610 in 20% oxygen, 0.464 in 1% oxygen and 0.411 in 0.25% oxygen indicating reduced growth in hypoxic conditions.

Combined data from all the *Aspergillus* species for AMB demonstrated a stepwise reduction in the MIC indicating that strains were more susceptible (as measured by MIC) in hypoxic conditions ($P = 0.03$ for 20% versus 1% and $P < 0.0001$ for 20% versus 0.25%). This trend could be seen in *Aspergillus* species but some comparisons did not reach statistical significance (this trend is clear in Figure 1 of a box-whisker plot of the effect of oxygen on the MIC of amphotericin). Little change occurred in the MFC over the hypoxic range tested. Changes in MFC could not be determined for *A. flavus* as some MFCs were in excess of 8 mg/L.

Combined data from all the *Aspergillus* species for ITC demonstrated little change in the MIC. The MFCs for *A. terreus* of ITC demonstrated a stepwise increase in MFC with hypoxia. It was not possible to assign MFCs for *A. flavus* or *A. niger* as some MFCs were in excess of 8 mg/L.

Data for MICA MICs did not demonstrate a trend with increasing hypoxia with all MICs being ≤ 0.004 mg/L in all conditions. The subgroup of the *Aspergillus* isolates retested at MICA concentrations lower than 0.004 mg/L demonstrated a trend towards increased susceptibility when oxygen was reduced to 1% (this was not further increased on reduction of oxygen to 0.25%). Assigning an MIC to the susceptibility in air was difficult because of the high level of residual

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Table 1. *In vitro* susceptibility to AMB, ITC and MICA of 37 *Aspergillus* isolates in air and hypoxic conditions

Species (number of isolates)	Oxygen concentration (%)	MIC AMB median (mg/L)	MFC AMB median (mg/L)	MIC ITC median (mg/L)	MFC ITC median (mg/L)	MIC MICA median (mg/L)	MFC MICA median (mg/L)
<i>A. fumigatus</i> (20)	20	0.5	2	0.25	2	<0.004 ^a	>4.0
	1	0.5	4	0.125	1.5	0.001	>4.0
	0.25	0.25	2	0.125	2	0.0009	>4.0
<i>A. terreus</i> (7)	20	1	4	0.0625	2	<0.004 ^a	>4.0
	1	1	4	0.0625	2	0.0009	>4.0
	0.25	1	4	0.125	8	0.0009	>4.0
<i>A. niger</i> (6)	20	1	4	1	>8	<0.004 ^a	>4.0
	1	0.1875	2.5	1	>8	0.0009	>4.0
	0.25	0.25	3	1	>8	0.0007	>4.0
<i>A. flavus</i> (4)	20	2	8	1.5	>8	<0.004 ^a	>4.0
	1	0.75	8	1.5	>8	0.0006	>4.0
	0.25	0.375	4	1.25	>8	0.0009	>4.0
All isolates (37)	20	1	4	0.25	2	<0.004 ^a	>4.0
	1	0.5*	4	0.125	4	0.001	>4.0
	0.25	0.25**	4	0.25	4	0.0009	>4.0

^aThis value is the MEC; an exact value is not assigned because of high levels of residual growth.

* $P = 0.03$, 20% versus 1% oxygen.

** $P < 0.0001$, 20% versus 0.25% oxygen.

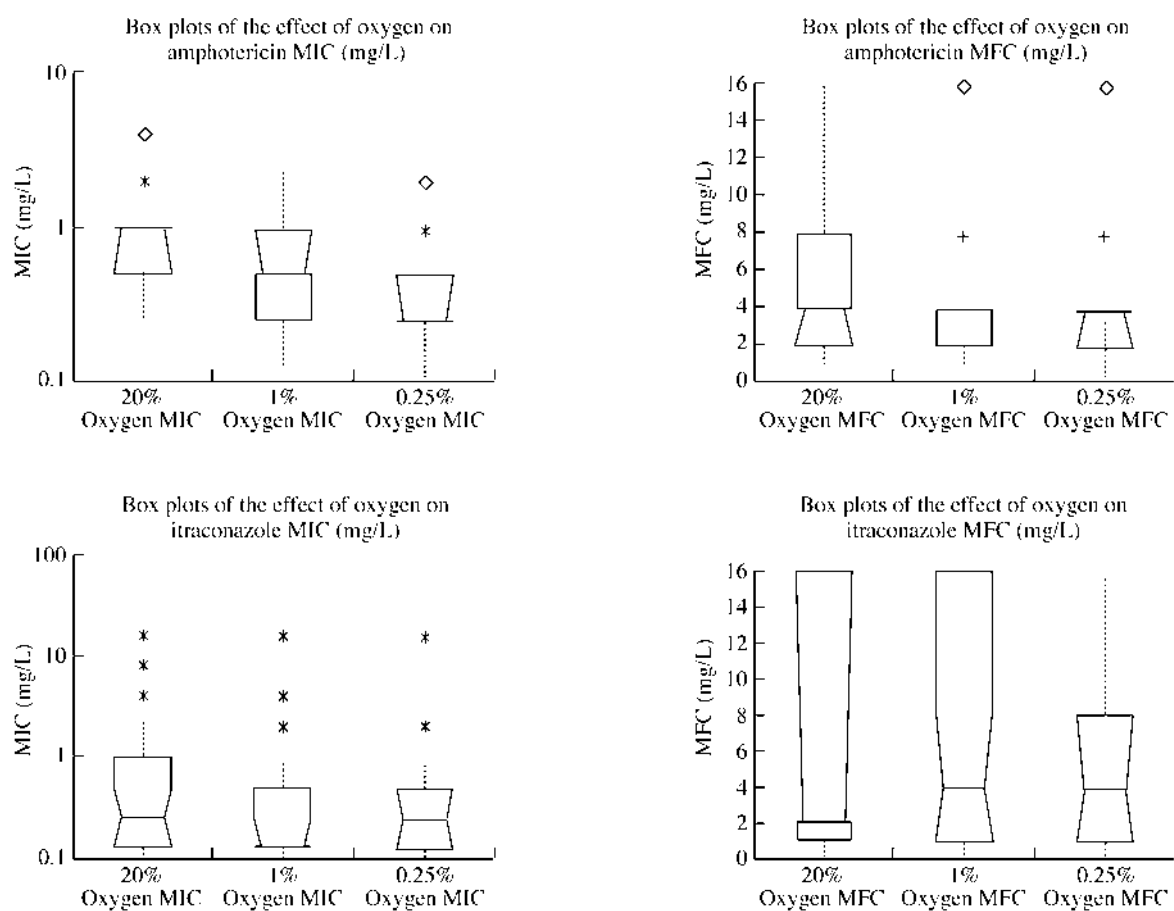


Figure 1. Box-whisker plots of the effect of oxygen on either the MIC or MFC of amphotericin and itraconazole. The box indicates the interquartile range (IQR) (upper/lower quartile), the centre-line is the median, and the crosses and diamonds indicate near outliers (1.5–3.0 IQR) and far outliers (greater than 3.0 IQR), respectively.

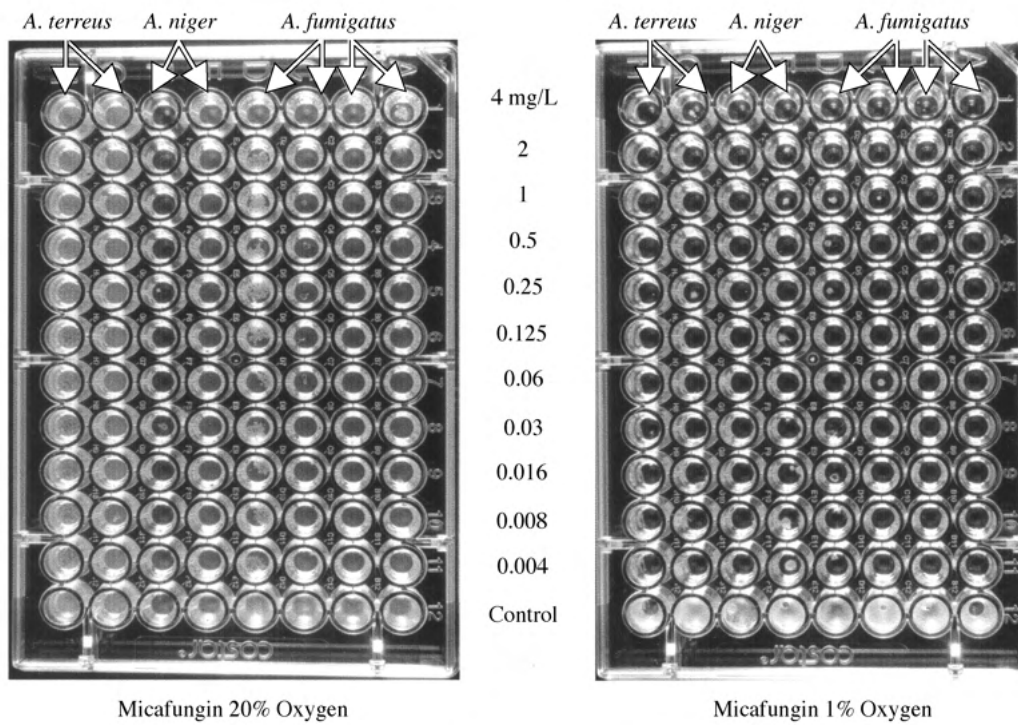


Figure 2. *Aspergillus* susceptibility test for micafungin in 20% and 1% oxygen.

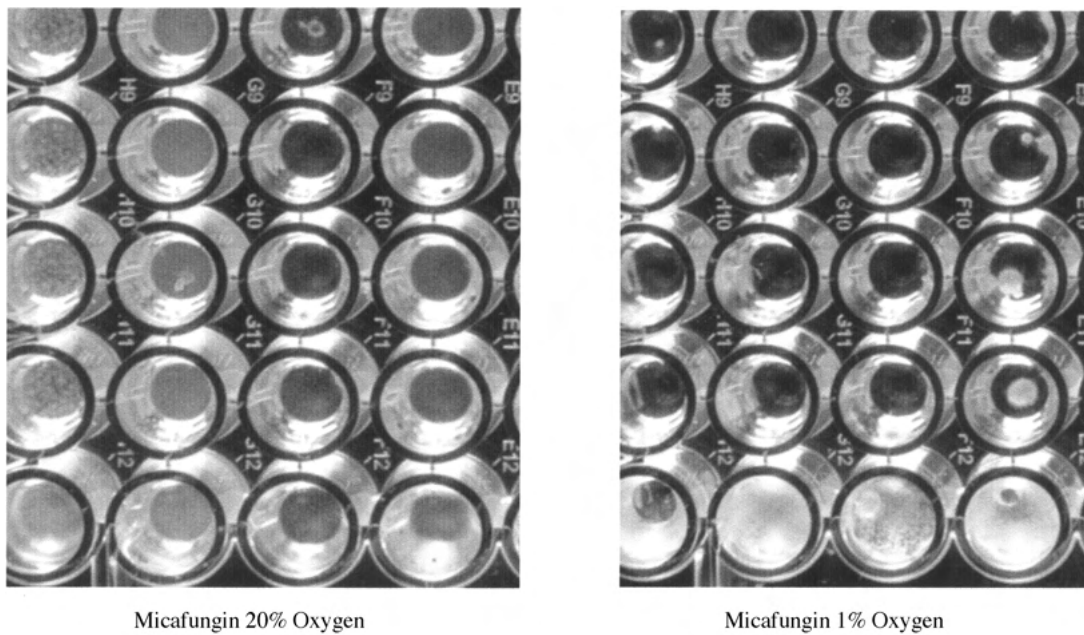


Figure 3. *Aspergillus* susceptibility test for micafungin in 20% and 1% oxygen with enlarged view of the zones of MIC/MEC.

growth. Furthermore, MICA was not fungicidal against any *Aspergillus* strain under any atmospheric conditions.

Figures 2 and 3 show the microdilution plates used in MICA susceptibility tests after incubation. The plate incubated in air demonstrates the characteristic slight reduction in growth at the MIC of the isolate. As normally is seen, the exact well which is assigned the MIC is open to debate with the possibility of inter-operator errors in the interpretation. The microdilution plate incubated in 1% O₂ demon-

strates a very clear endpoint with only occasional colonies growing on the surface of the liquid in the wells at concentrations higher than the MIC.

Summaries of the *in vitro* susceptibility tests of *Candida* are presented in Table 2.

MICs and MFCs for *Candida* (both individual species and data combined from all species) of AMB, ITC and MICA demonstrated no statistical differences between incubation conditions. In most

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Table 2. *In vitro* susceptibility to AMB, ITC and MICA of 24 *Candida* isolates in air and hypoxic conditions

Species (number of isolates)	Oxygen concentration (%)	MIC AMB median (mg/L)	MFC AMB median (mg/L)	MIC ITC median (mg/L)	MFC ITC median (mg/L)	MIC MICA median (mg/L)	MFC MICA geometric mean (mg/L)
<i>C. albicans</i> (5)	20	0.25	0.25	0.25	>8	0.004	0.012
	1	0.125	0.1875	0.25	>8	0.004	0.016
	0.25	0.125	0.1875	0.125	>8	0.004	0.012
<i>C. tropicalis</i> (5)	20	0.5	1	0.25	>8	0.004	0.016
	1	0.25	0.75	0.25	>8	0.004	0.016
	0.25	0.25	0.5	0.125	>8	0.004	0.03
<i>C. parapsilosis</i> (4)	20	0.5	1	0.125	0.75	0.375	4
	1	0.5	1	0.5	1	0.5	4
	0.25	0.5	1	0.5	1	0.125	3
<i>C. guilliermondii</i> (4)	20	0.3125	1	0.125	2	0.125	¼
	1	0.25	0.5	0.5	2	0.06	¼
	0.25	0.125	1	0.1875	2	0.125	¼
<i>C. glabrata</i> (3)	20	0.25	1	1	>8	0.004	0.008
	1	0.25	0.5	1	>8	0.004	0.008
	0.25	0.25	0.5	0.5	4	0.004	0.008
<i>C. krusei</i> (3)	20	0.5	2	0.5	2	0.06	0.125
	1	0.5	2	0.5	2	0.06	0.125
	0.25	0.5	2	0.25	2	0.06	0.125
All isolates (24)	20	0.5	1	0.25	>8	0.004	0.23
	1	0.25	0.5	0.5	4	0.004	0.23
	0.25	0.5	0.5	0.25	4	0.004	0.03

species treated with AMB, there was a trend towards reduced MICs in hypoxic conditions. For both AMB and ITC, there was a trend towards reduction in MFC with increasingly hypoxic conditions.

MICA demonstrated no trends in either MIC or MFC between incubation conditions. Both the MIC and MFC of MICA were much higher in all conditions for *C. parapsilosis* and *C. guilliermondii*.

Atmospheres generated with either anaerobic or microaerophilic gas packs (data not shown) do not replicate the growth patterns seen in 1% or 0.25% oxygen.

Discussion

One aim of antifungal susceptibility testing is to provide data which will guide the clinician treating the patient with information on isolates that is predictive of therapeutic success or failure. It is clear that currently there is a large gap between *in vitro* and *in vivo* response to some antifungal agents. Reliable susceptibility data are an essential aid in the selection of therapy for fungal disease. The effectiveness of the treatment of invasive or disseminated disease is at best moderate. This is reflected in the high mortality rates associated with invasive fungal disease even when appropriate treatment (as predicted by *in vitro* susceptibility) is aggressively administered.⁵ For many years, amphotericin B deoxycholate has been the standard treatment for invasive aspergillosis even though responses are sub-optimal (less than 40%) in severely immunocompromised patients.^{21,22} The decision to treat with amphotericin B is not always simple as treatment is associated with multiple side effects (even though these may be reduced with the lipid formulations),²³ but if the *in vitro* susceptibility data are unreliable, this makes the decision even more difficult. It has recently been reported that voriconazole may be an effective alternative to amphotericin B in the primary treatment of

invasive aspergillosis²⁴ but even in this study, survival at 12 weeks was only 71% (with just 21% demonstrating a complete response). It is therefore clear that there is scope for large improvements in the efficacy of treatment in this patient group.

MIC methodologies are designed to predict the *in vivo* susceptibility of fungi infecting patients. It has previously been demonstrated that MICs can vary depending on the test format and the fungal species tested.^{11,25} Multiple factors have been shown to influence the outcome of fungal MIC measurement including the format (macro- versus micro-dilution),^{26,27} culture medium,^{28,29} inoculum concentration,^{20,30} and temperature of incubation.^{31,32}

Methods by comparison with *in vivo* models have been published for a variety of fungi/antifungal agent combinations including the *Aspergillus*/itraconazole combination^{30,33,34} and the *Candida*/fluconazole combination.^{11,35} It has so far not been possible to validate a susceptibility test for the *Aspergillus*/amphotericin B combination using animal models^{10,30} but some degree of agreement was possible using correlation with clinical outcome.³⁶ Correlation of susceptibility results with clinical outcome has been possible with mucosal *Candida* infection (oropharyngeal thrush and oesophagitis), but is more difficult with disseminated candidiasis or aspergillosis as a result of the complex nature of the disease and poor outcome.

Susceptibility testing of *Candida* to the echinocandins using an adapted NCCLS M27-A2 protocol appears reliable^{37,38} with clear endpoints in susceptible strains and reduced *in vitro* susceptibility in resistant strains. Unfortunately, determining the MIC of the echinocandins for *Aspergillus* is not possible due to the incomplete nature of the inhibition.^{39,40} Therefore a revised interpretation of the MIC to the minimum effective concentration (MEC) has been suggested.⁴¹ The MEC is the point at which there is a transition of growth from a homogeneous hyphal mat to small spherical colonies.

This interpretation of the susceptibility test is currently not validated because of the lack of wild-type resistant *Aspergillus* isolates.

In this study, we have demonstrated that in hypoxic conditions similar to those that might occur in the tissues infected with *Aspergillus*,^{16,42} a reduction in the *in vitro* MIC (2–4 fold) of amphotericin B occurs. This would obviously have many advantages as drug delivery to some infected tissues is less than ideal and an increased ratio of tissue drug level to MIC would point to a therapeutic advantage for these drugs. Unfortunately, with amphotericin B we demonstrated no change in the MFC for *Aspergillus* in hypoxic conditions. It therefore appears that amphotericin is acting more as a fungistatic agent than a fungicidal agent. It would therefore seem good practice in patients with both aspergillosis and neutropenia to continue therapy until the neutropenia is resolved (particularly if the antifungal activity is predominantly fungistatic) but with amphotericin B this is often not possible because of adverse effects. In a recent study comparing treatment of invasive aspergillosis with voriconazole or amphotericin B,²⁴ amphotericin B therapy was used for a mean duration of only 10 days before therapy was changed; this in many cases would be before the resolution of neutropenia.

Hypoxic conditions did not alter either the MIC or the MFC of micafungin. However, growth of *Aspergillus* in the presence of micafungin in 1% oxygen was completely inhibited, aside from occasional surface or meniscus growth, which could represent inadequate exposure of individual conidia to the drug. This effect was also seen with caspofungin and anidulafungin (data not shown). So it is possible to assign an MIC with confidence rather than an MEC, using these conditions.

The echinocandins have been shown to be highly effective in treating both primary and refractory aspergillosis even though *in vitro* their inhibition of *Aspergillus* is incomplete. We have demonstrated that in conditions modelling hypoxia, the inhibition of hyphal growth by the echinocandins is almost total and is vastly superior to that occurring in air. It is possible that this high-level inhibition also occurs in tissues infected with *Aspergillus* as a result of the local hypoxic conditions caused by poor oxygen delivery.

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