JAC

Comparative *in vivo* activity of BAL4815, the active component of the prodrug BAL8557, in a neutropenic murine model of disseminated *Aspergillus flavus*

Peter A. Warn[1]*, Andrew Sharp[1], Juan Mosquera[1], Jochen Spickermann[2], Anne Schmitt-Hoffmann[2], Markus Heep[2] and David W. Denning[1,3]

[1]*School of Medicine, University of Manchester, 1.800 Stopford Building, Oxford Road, Manchester M13 9PT, UK;* [2]*Basilea Pharmaceutica, Ltd, PO Box 3255, 4002 Basel, Switzerland;* [3]*Wythenshawe Hospital, Southmoor Road, Manchester M23 9PL, UK*

Background: BAL8557 (WSA) is the water-soluble prodrug of the triazole BAL4815 with *in vitro* anti-*Aspergillus* activity. We compared the activity of oral BAL8557 with oral itraconazole, oral voriconazole and intravenous caspofungin in a temporarily neutropenic murine model of disseminated *Aspergillus flavus*.

Methods: Mice were immunosuppressed using cyclophosphamide, then infected. Mice were treated either 2 h pre-infection (PRE), or 4 or 24 h post-infection (4POST and 24POST, respectively). Treatment was for 10 days followed by 4 days of observation. Surviving mice were killed and liver, kidneys, lungs and brain cultured. BAL8557 groups included doses corresponding to ∼30, 15, 6 and 3 mg/kg of the active BAL4815; comparators included itraconazole 25 and 10 mg/kg/dose, voriconazole (plus oral grapefruit) 25 and 10 mg/kg/day or caspofungin 1 mg/kg/day. In a simultaneous tissue burden study mice were treated for 3 days, kidneys removed and homogenized and burden measured by quantitative culture and quantitative PCR using fluorescence resonance energy transfer (FRET).

Results: Control mice had 83–100% mortality. Over 66% of BAL8557-treated mice survived after >6 mg/kg PRE or >15 mg/kg POST. In the PRE models BAL8557 (6 mg/kg) and caspofungin were 100% protective and itraconazole 67% protective, but voriconazole 10 mg/kg had 100% mortality ($P = 0.0016$). In the 4POST and 24POST models survival was >66% with BAL8557 30 and 15 mg/kg/dose and similar to voriconazole or itraconazole. In the 24POST groups, sterilization of all organs was achieved in 11/16 survivors treated with BAL8557. The quantitative PCR correlated with kidney fungal burden ($r^2 = 0.59$). Earlier treatment reduced burdens.

Conclusions: BAL8557 demonstrated impressive antifungal activity against *A. flavus* in this model, in both survival and tissue burden.

Keywords: antifungal, susceptibility, mouse, itraconazole, voriconazole, caspofungin, amphotericin B, water-soluble azole, WSA

Introduction

Aspergillus spp. are ubiquitous airborne saprophytic fungi with low pathogenicity for humans and rarely invade immunologically competent hosts.[1] Whilst advances have been made in the diagnosis and treatment of invasive aspergillosis, the incidence is still increasing and mortality rates remain stubbornly high at 30–50%.[2] *Aspergillus flavus* is recognized as the second most common cause of invasive aspergillosis both in neutropenic and non-neutropenic patients with incidences ranging from 6% to 65% of all cases.[3–6]

The disease spectrum caused by *A. flavus* mirrors that caused by *Aspergillus fumigatus* in many studies and is implicated in invasive pulmonary aspergillosis and disseminated aspergillosis in both bone marrow transplant and solid organ transplant patients.[4,5,7] In contrast the incidence of *A. flavus* in sinus and soft tissue infections appears disproportionately high being regularly the most commonly recovered *Aspergillus* species.[6,8–11]

---

*Corresponding author. Tel: +44-161-606-7251; Fax: +44-161-275-5656; E-mail: peter.warn@.manchester.ac.uk

---

For many years the drug of choice for therapy of invasive fungal disease was amphotericin B but dose-limiting renal toxicity is a major concern and many patients require transfer to alternative antifungal treatment.[12] The new lipid-based formulations of amphotericin whilst less toxic still have significant side effects that often lead to withdrawal of therapy; additionally whilst these compounds can be administered at higher doses than conventional amphotericin B the improvement in outcome has not been remarkable.[12]

The introduction of the echinocandins with their novel drug target and low toxicity offers an alternative to cell-wall-targeted antifungals. The activity of the echinocandins against moulds is mostly limited to *Aspergillus*. They are predominantly fungistatic and cause fragmentation of the fungal mass; this has led to concerns about the use of the echinocandins as first-line therapy in neutropenic patients with aspergillosis.[13] The introduction of new triazoles with improved anti-*Aspergillus* activity has increased the choice of antifungals available for first-line therapy, but their efficacy is far from ideal and could be improved in terms of superior cidal activity, pharmacokinetic/pharmacodynamic characteristics, drug interactions and/or protein binding.

BAL8557 (WSA), the precursor of BAL4815, is the first extended-spectrum triazole that is highly water soluble. In contrast to itraconazole, voriconazole and posaconazole, oral and intravenous (iv) preparations of WSA do not require the addition of cyclodextrins or other excipients to enhance solubility. After administration either as an oral or iv preparation WSA is cleaved into an active component BAL4815 and an inactive prodrug cleavage product BAL8728. The cleavage is rapid and complete with little of the parent compound detectable in serum after administration. After oral administration as the prodrug, BAL4815 has a relative bioavailability >90% in humans.

In this study we tested WSA in a temporarily immunocompromised mouse model of disseminated aspergillosis caused by *A. flavus*. The activity of WSA was compared with that of voriconazole, itraconazole and caspofungin. The efficacy was compared both in survival and tissue burden studies (measured both by culture and quantitative PCR), and as prophylaxis and in the treatment of established disease.

Materials and methods

Test strain

A. flavus AFL8 was isolated from a patient with AIDS who had received 6 weeks itraconazole treatment and has been shown to be susceptible to amphotericin B and itraconazole using *in vivo* models.[14] The strain was maintained on slopes of Sabouraud dextrose agar (SAB) (Oxoid Limited, Basingstoke, UK) supplemented with 0.5% (w/v) chloramphenicol and for long-term storage at −70°C in 15% glycerol.

In vitro susceptibility testing

WSA (Basilea Pharmaceutica, Basel, Switzerland) was provided as a pure powder from the manufacturer. Itraconazole (Janssen Pharmaceuticals, Beerse, Belgium) and amphotericin B (Sigma, Poole, UK) were obtained as pure compounds. Voriconazole (Vfend; Pfizer Ltd, Sandwich, UK) and caspofungin (Cancidas; Merck Sharp & Dohme Ltd, Hoddesdon, UK) were obtained in vials for iv administration (Salford Royal Hospital, Manchester, UK). Stock solutions (3200 mg/L)

of all drugs were prepared using appropriate solvents—WSA, voriconazole and caspofungin (sterile distilled water), itraconazole and amphotericin B (dimethyl sulphoxide)—and adjusted for potency when necessary. Susceptibility tests were performed according to the broth microdilution modified method of the CLSI M38-A accepted standard using RPMI 1640 medium (Sigma) buffered to pH 7.0 with MOPS (Sigma).[15] In brief, final drug ranges (in mg/L) were WSA, voriconazole and itraconazole 0.0078–8, caspofungin and amphotericin B 0.0156–4.

Inoculum suspensions were prepared from 8 day cultures grown on SAB at 37°C and adjusted using a counting chamber. The final inoculum was between 0.5×10^4 and 5×10^4 cfu/mL as demonstrated by quantitative colony counts. Drug-free and cell-free controls were included. WSA, itraconazole, voriconazole and amphotericin B microdilution plates were incubated in air; and caspofungin microdilution plates were incubated in 1% O_2/5% CO_2/94% N_2 to aid reading.[16] Readings were made after 48 h of incubation at 37°C. The MIC endpoints for WSA, itraconazole, voriconazole and amphotericin B were read visually as the lowest drug concentration that prevents any discernible growth. The MIC endpoints for caspofungin were read visually and taken as that which reduced growth by 80% compared with the drug-free control.

Minimum fungicidal concentrations (MFCs) were also determined for all drugs (other than caspofungin). For each isolate, 100 µL was removed from all wells without visible growth. Each aliquot was spot-inoculated onto SAB, and the liquid was allowed to soak into the agar. When dry, the plate was streaked to separate any conidia and to remove them from the drug source. The plates were incubated at 37°C for 48 h. The MFC was defined as the lowest drug concentration that allowed the growth of 50 or fewer colonies (99% kill).

In vivo studies

Animals

All mice included in this study were part of ongoing studies performed under UK Home Office project licence PIL40/2356 entitled Invasive Fungal Infections. Male CD1 mice, 4–5 weeks old and weighing between 22 and 24 g were purchased from Charles River UK Ltd (Margate, Kent, UK). The mice were virus-free and were allowed free access to food and water.

Immunosuppression

Cyclophosphamide (Pharmacia, Milton Keynes, UK) was administered iv via the lateral tail vein to all animals at a dose of 200 mg/kg 3 days prior to infection. This generates a state of profound neutropenia 3 days after administration. White cell counts begin to recover 4 days after this nadir.[17]

Preparation of inoculum

For the experiment the isolate was cultured on SAB for 8 days. The conidia were harvested in 25 mL of sterile phosphate-buffered saline (PBS) (Life Technologies, Paisley, UK) plus 0.05% Tween® 80 (Sigma). The count was determined by serial dilution and culture onto SAB. The conidia were stored at 4°C for 4 days until used.

Infection of mice

Prior to the experiment, inoculum-finding studies (LD_{90} 14 days post-infection) for the isolate were performed using iv injections via the lateral tail vein of 0.2 mL of a range of inocula. The inoculum was administered iv on day 0 (3 days post-immunosuppression). Post-infection viability counts were performed to ensure the correct

inoculum had been given. These studies determined an inoculum of 2.4×10^4 conidia per mouse was required (for all treatment groups).

Antifungal therapy

Mice were treated 2 h pre-infection (PRE), 4 h post-infection (4POST) or 24 h post-infection (24POST). Treatment for the survival models was continued for 10 days followed by 4 days observation. Treatment in the tissue burden models was for three complete days.

Caspofungin acetate (Cancidas) was reconstituted in the vial as per the manufacturer's instructions in sterile distilled water. It was further diluted in 5% glucose to prepare a solution of 0.1 mg/mL.

Mice treated with voriconazole were also treated orally twice daily with 0.25 mL of grapefruit juice started 3 days before infection and continued for 10 days post-infection. Grapefruit juice was administered 30 min before voriconazole treatment.[18] Voriconazole (Vfend) was reconstituted as per the manufacturer's instructions in sterile distilled water. It was further diluted in 5% glucose to provide solutions of 1.0 mg/mL and 2.5 mg/kg.

Itraconazole (2.5 g) was dissolved in 10 mL of 1,2 propanediol (Sigma) + 0.95 mL of concentrated hydrochloric acid (Sigma). (2-Hydroxypropyl)-β-cyclodextran (HPBC; Fluka, Poole, Dorset, UK) (60 g) was dissolved in 40 mL of water. The itraconazole and HPBC solutions were then combined and the pH adjusted to 1.9–2.1 with 10 M NaOH.[19] Water was then added to adjust the volume to 100 mL; this was further diluted in HPBC to provide solutions of 1.0 and 2.5 mg/mL. Mice were treated three times daily orally on days 1 and 2 then twice daily on days 3–10.

WSA was reconstituted in distilled water to produce a stock solution of 5 mg/mL of the prodrug which is equivalent to 3 mg/mL of the active compound. This was further diluted in 5% glucose to provide additional solutions of 2.5, 1.0 and 0.5 mg/mL of WSA. Mice were treated three times daily orally on days 1 and 2 then twice daily on days 3–10. All subsequent doses and serum concentrations of WSA refer to the amount of active compound BAL4815.

Control mice were infected but received no active treatment. Groups received 5% glucose iv, 5% glucose orally, 5% glucose and grapefruit juice orally or HPBC solution orally.

Each treatment or control group consisted of six mice for survival studies or three mice for tissue burden studies.

Treatment groups

PRE: caspofungin 1 mg/kg iv once daily, voriconazole 10 mg/kg oral once daily, itraconazole 10 mg/kg oral three times a day on days 1 and 2 then twice a day, WSA 25, 10 or 5 mg/kg oral three times a day on days 1 and 2 then twice a day (15, 6.0 and 3 mg/kg/dose of the active compound).

4POST and 24POST: caspofungin 1 mg/kg iv once daily, voriconazole 25 mg/kg oral once daily, itraconazole 25 mg/kg oral three times a day on days 1 and 2 then twice a day, WSA 50, 25 or 10 mg/kg oral three times a day on days 1 and 2 then twice a day (30, 15 or 6.0 mg/kg/dose of the active compound).

Experimental endpoints (all mouse groups)

Mice were examined at least four times daily. Any infected animals with severely reduced mobility, unable to reach the drinker or otherwise in substantial distress were humanely terminated. Attention was paid to postural changes, torticollis and staggering or staining of the anal region, as these were indicators of imminent deterioration. All mice demonstrating signs of disease also had their temperature measured by infra-red telemetry;[20] mice with temperatures below 33°C were humanely terminated.

Tissue burden model. Mice were treated for three complete days followed by 12 h (WSA and itraconazole) or 24 h of observation (voriconazole and caspofungin). All mice were then humanely terminated on the fourth day post-infection using an overdose of halothane.

Survival model. On day 14 post-infection (10 days of therapy and 4 days of observation) all remaining mice were humanely terminated using an overdose of halothane.

Organ culture

The kidneys, liver, lungs, brain and spleen (only the kidneys were examined in the 3 day treatment tissue burden study) were removed and transferred into 2 mL of sterile PBS. The organs were homogenized in a tissue grinder (Polytron, Kinematica AG, Luzern, Switzerland) for 2–3 s. Colony counts were determined using serial 10-fold dilutions plated on the surface of SAB with 0.5% (w/v) chloramphenicol. Plates were incubated at 37°C in a moist atmosphere and examined daily for up to 5 days. Plates were examined and the *A. flavus* colonies counted. Single colonies were accorded a negative result, because of the possibility of airborne contamination. This method detected *A. flavus* at >30 cfu/organ.

DNA extraction from kidney homogenates

After the kidneys were homogenized a sample was removed and immediately frozen at –20°C. The frozen homogenates were thawed, and 100 µL was added to 400 µL of lysing matrix D (FastDNA® kit; Qbiogene-Alexis, Nottingham, UK) and processed by mechanical disruption in a Fastprep FP220A (Qbiogene-Alexis) for 180 s (3×60 s pulses, placed on ice between pulses) at speed setting 5, then placed on ice. The homogenates were processed as detailed in the FastDNA® application manual. DNA was further purified using a Geneclean Turbo kit (Qbiogene-Alexis) as per the application manual. The extracted DNA (a mixture of mouse and *Aspergillus* DNA) was quantified using a NanoDrop 3300 (Wilmington, USA).

Real-time quantitative PCR

The oligonucleotide primers and fluorogenic hybridization probes complementary to the *A. flavus* ITS1 region of the rDNA gene (Figure 1) were designed using Roche Applied Science Light Cycler Probe Design Software and were supplied by Tib Molbiol (Berlin Germany): (i) forward primer,
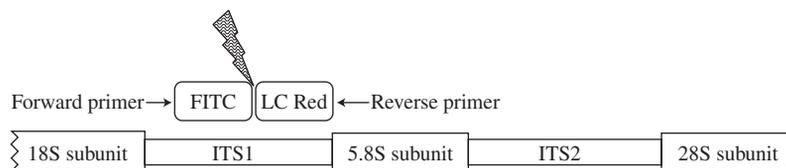
Figure 1. rDNA schematic showing the conserved 18S, 5.8S and 28S regions and less-conserved ITS1 and ITS2 regions of *A. flavus* (not to scale). The locations of the quantitative PCR primers and probes are also indicated.

5′-ACCACGAACTCTGTCTGATCTA-3′; (ii) reverse primer, 5′-ACGGAATTCTGCAATTCACAC-3′; (iii) probe 1, 5′-AACAAT-GGATCTCTTGGTTCCGGC-fluorescein-3′; and (iv) probe 2, 5′-LC red 640-CGATGAAGAACGCAGCGAAATGCG-phosphate-3′.

PCR conditions

DNA samples were analysed on a Roche Lightcycler 2 (20 µL capillary) (Roche, Lewes, UK). The mixture consisted of 2 µL of primers (1 µM), 0.5 µL of probes (0.1 µM), 4 µL of mastermix (LC FastStart DNA Master Plus HP; Roche), 10 µL of water and 1 µL of DNA.

The quantitative PCR cycle conditions were: (i) pre-incubation at 95°C for 10 min; (ii) amplification for 50 cycles [denaturation at 95°C for 0 s; annealing at 58°C for 5 s; and extension at 72°C for 15 s (with data acquisition)]; and (iii) cooling at 40°C for 30 s. Internal controls with known template concentration and blank samples were included in all amplification runs.

Validation of quantitative PCR and quantification of DNA concentration

Genomic DNA was extracted from *A. flavus* AFL8 as described above and a standard curve of template number versus crossing point (CP) was generated. The template numbers generated from the quantitative PCRs of the test samples were corrected for by normalizing against the mouse DNA concentration in the extracted sample.

Pharmacokinetics

Blood samples were collected from a separate group of immunosuppressed and infected mice by cardiac puncture to determine pharmacokinetics of voriconazole, itraconazole and WSA. In all cases three mice were sampled for every drug at every time point. For the determination of the serum concentrations of BAL4815 (the active component of WSA) samples were collected 1.5, 4 and 8 h post-dose; for voriconazole blood samples were collected 5 min and 3, 6 and 9 h post-dose after three complete days of treatment; and for itraconazole samples were collected 5 min and 1, 3 and 6 h post-dose after three complete days of treatment (after third dose of voriconazole and the eighth dose of itraconazole and WSA). WSA plasma samples were stabilized by the addition of 10 µL of 2 M citric acid per mL of plasma then stored at –20°C until analysed.

For voriconazole and itraconazole measurement, samples were thawed and analysed as a batch in bioassays using RPMI MOPS agar (Sigma) and the *Candida kefyr* San Antonio strain.[21] BAL4815 concentration was determined using a validated LC-MS/MS method by Basilea Pharmaceutica Ltd.

Statistical analysis

Mortality data were analysed using the Mann–Whitney *U*-test or the Kruskal–Wallis test if the Mann–Whitney was not possible (i.e. if all values are identical in one group). Two-sided *P* values are given.

All data analysis was performed using the computer package StatsDirect (Ashwell, Herts, UK).

Results

In vitro susceptibility

The MIC and MFC values for AFL8 are 1 and 2 mg/L (BAL4815), 0.5 and 0.5 mg/L (itraconazole), 1 and 1 mg/L (voriconazole), and 0.25 and >4 mg/L (caspofungin). This indicates that *in vitro* AFL8 is susceptible to all four antifungal agents.

In vivo results

Convention for expressing drug and prodrug dosing

Doses of BAL8557 (WSA) are expressed as mg/kg equivalent of the active drug BAL4815. For example, doses of 30 mg/kg BAL4815 were actually administered to mice as 50 mg/kg of the prodrug WSA.

Tissue burdens after 3 days of treatment

The organ burdens determined by both culture and quantitative PCR are detailed in Table 1. The sensitivity of the quantitative PCR was ≤10 copies of the target DNA and was measurable in the range 10^1–10^6 template copies (Figure 2).

Untreated mice had burdens of 1.3×10^4 cfu/g of tissue and 3.4×10^4 genome equivalents. Treatment reduced burdens to 424, 260, 1340 and 301 cfu/g and genome equivalents to 201, 194, 151 and 108 for WSA, voriconazole, itraconazole and caspofungin, respectively. No statistical difference was found between different treatment regimens (at the same dose of prodrug WSA and of active drug for voriconazole, itraconazole and caspofungin) but in most cases earlier treatment reduced burdens. The quantitative PCR correlated well with kidney fungal burden overall ($r^2 = 0.59$), the correlation within the treated mice groups was similarly good ($r^2 = 0.54$) (Figure 3a–f) but there were differences within the individual therapeutic arms. The r^2 for each treatment group was 0.41, 0.54, 0.58, 0.85 and 0.12 for solvent controls, WSA, voriconazole, itraconazole and caspofungin, respectively. This indicates that whilst after solvent therapy or triazole treatment quantitative PCR and culture burden were reasonably well correlated they were poorly correlated after caspofungin treatment.

Survival studies

The mortality curves shown in Figure 4 demonstrate that AFL8 caused lethal infections in mice. Mice receiving no active treatment had 83–100% mortality.

In the PRE model WSA (6 mg/kg) and itraconazole (10 mg/kg) were superior in terms of mortality to 10 mg/kg voriconazole ($P = 0.002$). WSA at 6 and 15 mg/kg/dose were superior to WSA 3 mg/kg/dose ($P < 0.001$). Voriconazole 10 mg/kg/dose was not significantly superior to its solvent control at improving survival. WSA 6 mg/kg and caspofungin were the only regimens with 100% survival in the PRE treated mice.

In the 4POST model all treatments (other than WSA 6 mg/kg/dose) were superior to control regimens ($P < 0.2$). WSA 15 and 30 mg/kg, itraconazole 25 and voriconazole 25 mg/kg/dose were numerically superior to caspofungin in terms of survival but this did not reach statistical significance. WSA 15 and 30 mg/kg/dose were superior to the 6 mg/kg/dose at improving survival ($P < 0.02$). No treatment had 100% survival but WSA 15 mg/kg/dose, itraconazole 25 mg/kg/dose and voriconazole 25 mg/kg/dose were all able to achieve 83% survival.

In the 24POST model all active treatments were superior to control regimens ($P < 0.02$). There were no significant statistical differences between the active treatments. WSA 15 and 30 mg/kg/dose and caspofungin were the only regimens to give 100% survival.

Table 1. Summary of *A. flavus* tissue burden 3–4 days post-infection measured by culture and quantitative PCR for all treatment groups

	PRE			4POST			24POST		
	mean cfu burden	standard error cfu burden	mean genome equivalents	mean cfu burden	standard error cfu burden	mean genome equivalents	mean cfu burden	standard error cfu burden	mean genome equivalents
WSA solvent	15 470	15 015	13 497	8490	5026	6259	9298	8826	5255
WSA 30 mg/kg[a]	NA	NA	NA	45	45	71	490	248	626
WSA 15 mg/kg[a]	292	204	359	1140	217	226	152	91	40
WSA 6 mg/kg[a]	35	35	337	2070	144	8273	1995	924	536
WSA 3 mg/kg[a]	175	72	277	NA	NA	NA	NA	NA	NA
ITC solvent	233	42	647	26 250	7967	56 819	7840	6056	1483
ITC 25 mg/kg	NA	NA	NA	105	15	110	490	245	6
ITC 10 mg/kg	187	11	162	NA	NA	NA	NA	NA	NA
VRC solvent	1272	256	4451	1080	652	8113	8552	2839	1071
VRC 25 mg/kg	NA	NA	NA	90	69	11	255	75	98
VRC 10 mg/kg	3663	3420	1173	NA	NA	NA	NA	NA	NA
CAS solvent	583	192	18 775	14 670	11 118	3274	47 850	14 756	58 036
CAS 1 mg/kg	93	51	150	300	156	103	510	18	72

CAS, caspofungin; ITC, itraconazole; VRC, voriconazole; NA, not available (this treatment regimen not used in this model).
[a]All WSA concentrations refer to the active compound.
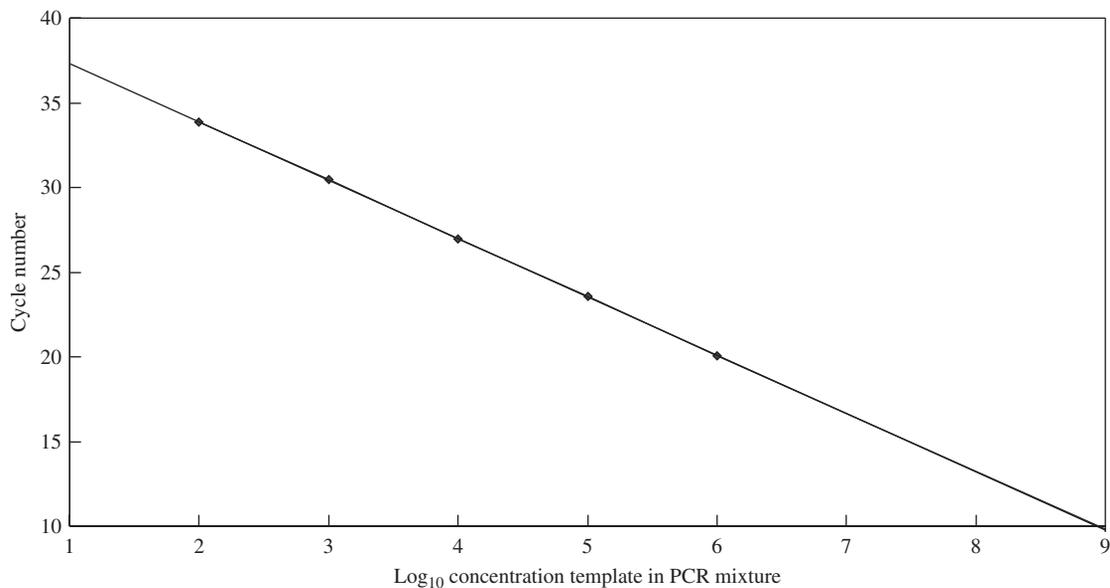
Figure 2. Standard curve of the crossing point against template number in the PCR.

Fourteen day organ burdens

Organ burdens recovered from mice killed 14 days post-infection were relatively low with the kidneys being the most heavily infected (as has been previously noted[14]).

In the PRE model, clearance of all organs was achieved in 2/5, 0/6 and 0/1 survivors at 15, 6 and 3 mg/kg/dose WSA, and in 3/4 of the itraconazole and 2/6 of the caspofungin groups. In most cases *A. flavus* was only recovered from the kidneys.

In the 4POST model, clearance of all organs was achieved in 3/4, 3/5 and 0/1 survivors at 30, 15 and 6 mg/kg/dose WSA, and in 3/5 of the itraconazole, 2/5 of the voriconazole and 0/3 of the caspofungin groups. No treatment was able to clear the burden completely from all mice in the treatment group.

In the 24POST model, clearance of all organs was achieved in 5/6, 3/6 and 3/4 survivors at 30, 15 and 6 mg/kg/dose WSA, and in 3/5 of the itraconazole, 2/5 of the voriconazole and 6/6 of the caspofungin groups.
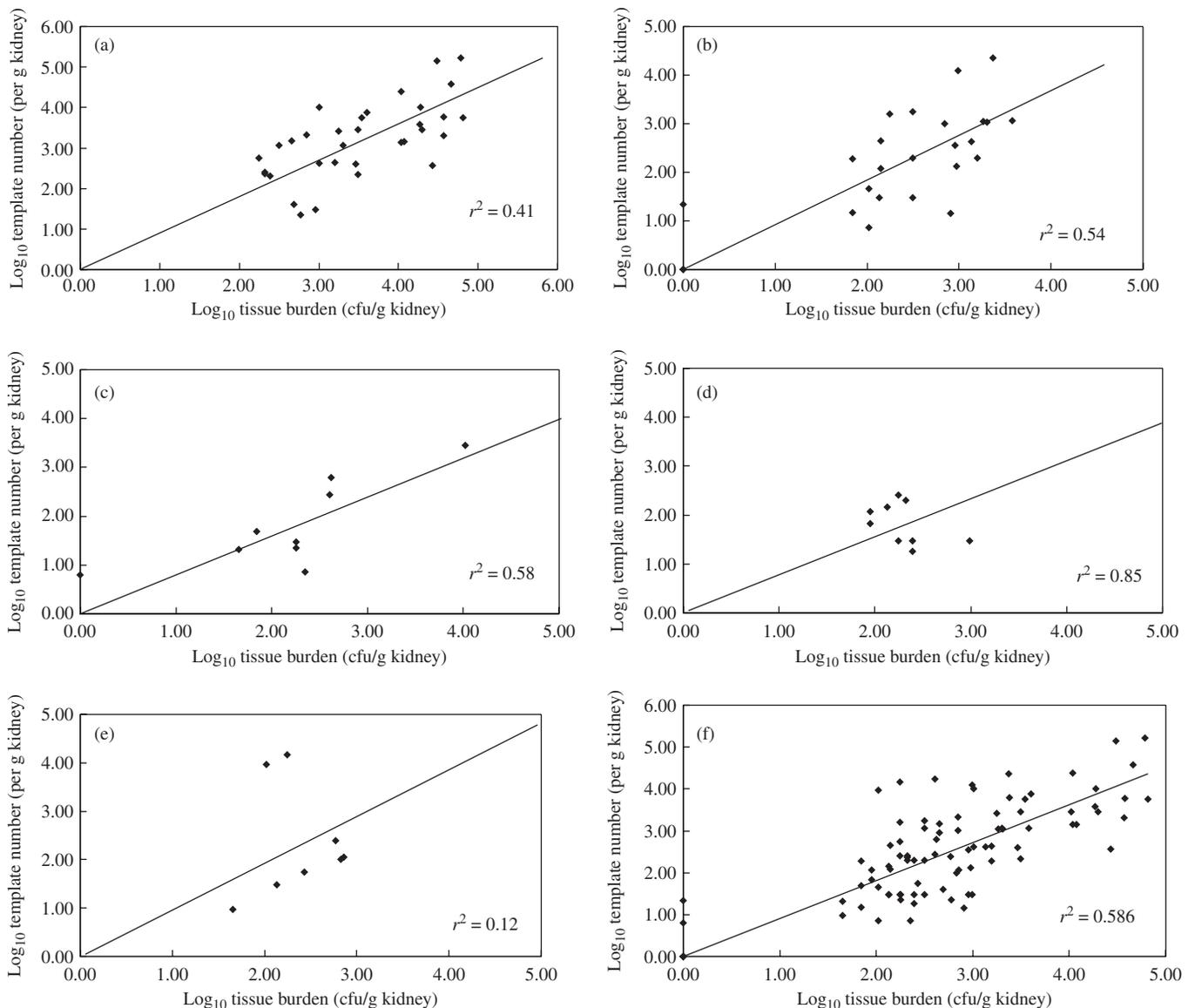
Figure 3. Scattergrams of the tissue burdens determined by conventional culture compared with tissue burdens determined by quantitative PCR. (a) Control mice treated with solvents. (b) Mice treated with WSA. (c) Mice treated with voriconazole. (d) Mice treated with itraconazole. (e) Mice treated with caspofungin. (f) Tissue burden and quantitative PCR results for all antifungals combined.

In all models, solvent-treated mice that survived to the end of the study had residual organ burdens.

Pharmacokinetics

Itraconazole levels (after 25 mg/kg/dose) peaked at 18.5 mg/L (Table 2). Voriconazole levels (after 25 mg/kg/dose) peaked at 16.4 mg/L (Table 2). BAL4815 peak levels were in the range 0.67–3.72 mg/L for doses ranging from 3 to 30 mg/kg (Table 2) with undetectable or extremely low levels of BAL4815 after 8 h. This demonstrates good absorbance of all azole drugs, and rapid transformation of the prodrug into the active compound BAL4815. The exposure of mice treated with WSA at the top dose of 30 mg/kg active compound was substantially less than voriconazole and itraconazole, with survival and culture clearance rates results in all three models similar to voriconazole and itraconazole.

Discussion

WSA demonstrated impressive antifungal activity against *A. flavus* in this temporarily neutropenic murine model. In the survival models at least 5/6 solvent-treated mice succumbed to infection within 14 days of infection demonstrating that untreated this *A. flavus* strain (with an inoculum of just 2.4×10^4 conidia) generated a severe lethal infection. In contrast the inoculum of different strains of *A. fumigatus* to achieve similarly severe infections is in the range $1.5–7.5 \times 10^5$ conidia per mouse. In this study all mice (PRE, 4POST and 24POST) were infected sequentially from a single suspension of *A. flavus* and overall
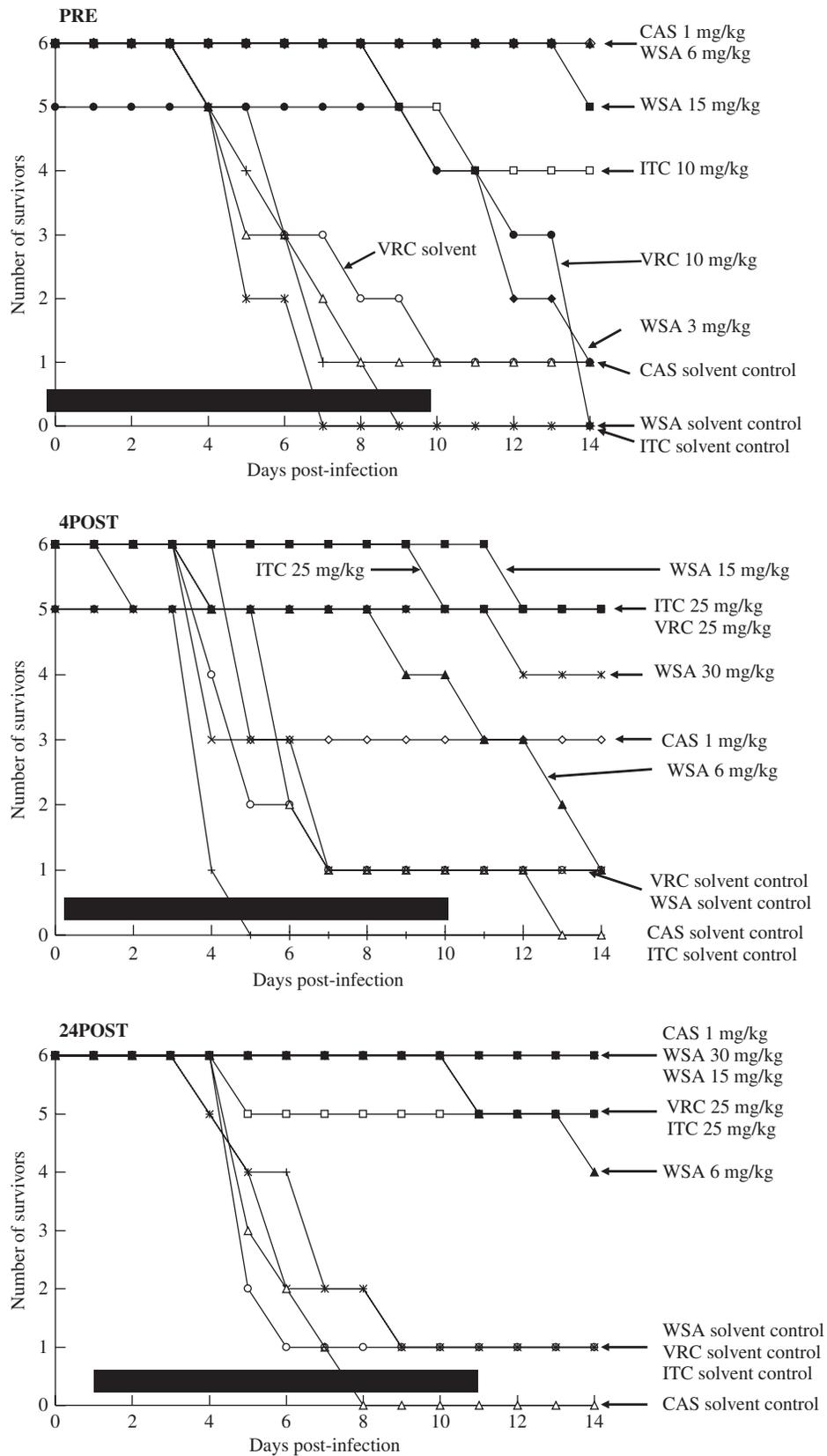
Figure 4. Plots of cumulative mortality against time in murine models of invasive aspergillosis. PRE, survival of mice treated 2 h pre-infection; 4POST, survival of mice treated 4 h post-infection; and 24POST, survival of mice treated 24 h post-infection. Solid black bar indicates duration of therapy. All WSA concentrations refer to the active compound. CAS, caspofungin; ITC, itraconazole; VRC, voriconazole.

Table 2. Drug exposure after treatment with WSA, voriconazole (VRC) or itraconazole (ITC)

	Frequency of administration	C_{max} (mg/L)	AUC_{0-24} (mg·h/L)	Trough level (mg/L)[a]
WSA 30 mg/kg/dose[b]	8 hourly for 2 days, then 12 hourly	3.72	16	0.03
WSA 15 mg/kg/dose[b]	8 hourly for 2 days, then 12 hourly	2.07	12	BLQ
WSA 6 mg/kg/dose[b]	8 hourly for 2 days, then 12 hourly	1.91	5	BLQ
WSA 3 mg/kg/dose[b]	8 hourly for 2 days, then 12 hourly	0.67	2	BLQ
VRC 25 mg/kg/day	once daily	16.4	69	6.4
ITC 25 mg/kg/dose	8 hourly for 2 days, then 12 hourly	18.5	406	0.28

BLQ, below level of quantification.
[a]On day 3 of therapy 8, 9 and 6 h post-dose for WSA, VRC and ITC, respectively.
[b]All WSA concentrations refer to the active compound.

the mortality was similar regardless of the delay in therapy; this finding is at variance from the situation in invasive candidiasis in which delayed therapy results in a much poorer outcome.[22]

In terms of survival WSA was at least equivalent to similar doses of itraconazole and voriconazole and superior to either drug administered at 10 mg/kg/dose. It is remarkable that there were no survivors after 10 mg/kg voriconazole in the PRE model whereas 5/6 mice survived in the delayed therapy models after treatment with 25 mg/kg daily; this is possibly due to the peak serum level in the PRE treatment occurring long before infection resulting in 22 h elapsing before effective therapy. There are concerns that voriconazole is metabolized in mice by the cytochrome P450 enzymes leading to very low plasma levels[18] but in this model all voriconazole-treated mice were co-administered grapefruit juice and adequate plasma levels were achieved (levels only measured after 25 mg/kg). It is possible that 10 mg/kg once daily is inadequate therapy for invasive murine *A. flavus* infection; 1–5 mg/kg twice daily voriconazole therapy in guinea pigs with disseminated murine *A. fumigatus*, whilst improving survival, was unable to achieve more than 80% efficacy in some studies.[23,24] WSA was at least as effective as itraconazole or voriconazole at reducing organ burden in all models and was able to clear all burden in 33–83% of mice treated with >15 mg/kg/dose. WSA 30 mg/kg/dose and caspofungin 1 mg/kg/dose were able to reduce the organ burden below detectable levels in the model in which treatment was delayed 24 h post-infection in contrast to lower doses of WSA or itraconazole and voriconazole. The excellent activity of WSA occurred despite much lower exposure as demonstrated by 4-fold lower AUCs.

There are concerns that conventional quantitative culture is an imprecise measure of organ burden in filamentous fungus infections.[25–27] *Aspergillus* spp. grow in tissue as an open hyphal structure; it has been proposed that homogenization of tissues leads to inconsistent fracturing of single fungal microcolonies in the tissue that results in variable and in the case of caspofungin multiple cfu on culture plates. Quantitative real-time PCR has been proposed as a superior measure of tissue burden as the genome equivalents determined are consistent regardless of the microcolony structure and extraction; additionally enhanced sensitivity leading to detection in culture negative samples is possible. In this study we examined the tissue burden by both conventional culture and quantitative real-time PCR using fluorescence resonance energy transfer (FRET) technology. The primers and probes were designed targeting the ITS1 region of the rDNA gene. We attempted to generate species-specific primers

without success (the specific primer and probes sets produced weak fluorescent signals). The probe and primer sets ultimately used in this study whilst not specific to *A. flavus* had weak signals for other *Aspergillus* species and did not cross-react with mouse DNA. After solvent therapy or treatment with triazoles the quantitative real-time PCR burden correlated strongly with conventional culture with no increase in sensitivity. Correlation was poor in mice treated with caspofungin; in most cases lower burdens were detected using quantitative PCR. These findings are consistent with others and is probably due to the fragmentation of filamentous fungi after echinocandin therapy.[25,27,28] The enhanced sensitivity of quantitative PCR compared with conventional therapy should be regarded with caution as the technology detects fungal DNA and is equally effective at detecting dead or viable fungi. In this study quantitative PCR is a useful and sensitive marker of fungal burden in murine kidneys that showed similar results to conventional culture for all treatments other than the echinocandins.

BAL8557 the prodrug of BAL4815 is the first broad-spectrum triazole antifungal agent that is water soluble. After administration either orally or parenterally BAL8557 is rapidly metabolized to BAL4815 plus a small inactive cleavage product BAL8728. The water solubility of the prodrug and rapid and complete cleavage into an active moiety provides a significant advantage over other triazoles as potentially toxic excipients such as cyclodextrins are not required. In single and multiple-dose pharmacokinetics in humans[29,30] and mice serum levels are dose proportional after multiple doses with no indication of induction or inhibition of its own metabolism and excellent bioavailability. Treatment results in a high plasma concentration that is maintained in the circulation for prolonged periods; the half-life after oral administration in immunocompromised and infected mice is ~3.8 h, much less than the 55–77 h observed in humans.

The pharmacodynamic driver of the triazoles during invasive aspergillosis has not been fully established but in candidiasis the AUC/MIC appears to be the most predictive indicator of outcome.[31,32] The MIC of BAL4815 for the *A. flavus* isolate used in this study was 1 mg/L; this results in a ratio of unbound 24 h AUC/MIC of less than 1 after treatment with 6 mg/kg/dose WSA active compound but ratios in excess of 1 were obtained after treatment with 15 and 30 mg/kg/dose WSA. It therefore seems likely that AUC/MIC ratios of >1 are an indicator of satisfactory response in the treatment of invasive aspergillosis. Though it is not yet clear, it is likely that trough levels maintained

above the MIC will predict a good outcome, either because time over MIC is the primary pharmacodynamic driver of response, or because it is a proxy for an adequate AUC/MIC ratio. In contrast, during treatment of invasive candidiasis free drug AUC/MIC ratios of 20–25 are required to achieve an ED_{50}.[32] During triazole therapy of invasive aspergillosis free drug AUC/MIC ratios of this level are very unlikely to be met as triazole MIC_{50}s for *Aspergillus* are generally at least 10-fold higher than for *Candida* (in the range 0.25–1 mg/L).[33] The isolate of *A. flavus* selected for this study had an MIC of BAL4815 greater than the MIC_{90} for all *A. fumigatus* and *Aspergillus terreus* strains[34] and therefore if an AUC/MIC ratio of >1 is an indicator of likely success most strains of *Aspergillus* should respond to therapy.

In vitro data demonstrate that BAL4815 has fungicidal activity against *Aspergillus* with MFCs just 2.5-fold higher than MICs (geometric mean MFC of 1.68 mg/L). Human pharmacokinetic data demonstrate that 100 mg daily would lead to plasma levels of >1.7 mg/L for the entire dosing interval,[29] which is in excess of the MFC for the vast majority of *Aspergillus* clinical isolates tested. Thus WSA demonstrates excellent *in vivo* activity and warrants further clinical development in invasive aspergillosis.

Acknowledgements

Transparency declarations

References

1. Denning DW. Invasive aspergillosis. *Clin Infect Dis* 1998; **26**: 781–803.

2. Groll AH, Kurz M, Schneider W *et al.* Five-year-survey of invasive aspergillosis in a paediatric cancer centre. Epidemiology, management and long-term survival. *Mycoses* 1999; **42**: 431–42.

3. Morgan J, Wannemuehler KA, Marr KA *et al.* Incidence of invasive aspergillosis following hematopoietic stem cell and solid organ transplantation: interim results of a prospective multicenter surveillance program. *Med Mycol* 2005; **43** Suppl 1: S49–58.

4. Herbrecht R, Denning DW, Patterson TF *et al.* Voriconazole versus amphotericin B for primary therapy of invasive aspergillosis. *N Engl J Med* 2002; **347**: 408–15.

5. Perfect JR, Cox GM, Lee JY *et al.* The impact of culture isolation of *Aspergillus* species: a hospital-based survey of aspergillosis. *Clin Infect Dis* 2001; **33**: 1824–33.

6. Abbasi S, Shenep JL, Hughes WT *et al.* Aspergillosis in children with cancer: a 34-year experience. *Clin Infect Dis* 1999; **29**: 1210–9.

7. Steinbach WJ. Pediatric aspergillosis: disease and treatment differences in children. *Pediatr Infect Dis J* 2005; **24**: 358–64.

8. Walmsley S, Devi S, King S *et al.* Invasive *Aspergillus* infections in a pediatric hospital: a ten-year review. *Pediatr Infect Dis J* 1993; **12**: 673–82.

9. Heinemann S, Symoens F, Gordts B *et al.* Environmental investigations and molecular typing of *Aspergillus flavus* during an outbreak of postoperative infections. *J Hosp Infect* 2004; **57**: 149–55.

10. Chakrabarti A, Gupta V, Biswas G *et al.* Primary cutaneous aspergillosis: our experience in 10 years. *J Infect* 1998; **37**: 24–7.

11. Alrajhi AA, Enani M, Mahasin Z *et al.* Chronic invasive aspergillosis of the paranasal sinuses in immunocompetent hosts from Saudi Arabia. *Am J Trop Med Hyg* 2001; **65**: 83–6.

12. Bowden R, Chandrasekar P, White MH *et al.* A double-blind, randomized, controlled trial of amphotericin B colloidal dispersion versus amphotericin B for treatment of invasive aspergillosis in immunocompromised patients. *Clin Infect Dis* 2002; **35**: 359–66.

13. Maertens J, Raad I, Petrikkos G *et al.* Efficacy and safety of caspofungin for treatment of invasive aspergillosis in patients refractory to or intolerant of conventional antifungal therapy. *Clin Infect Dis* 2004; **39**: 1563–71.

14. Mosquera J, Warn PA, Morrissey J *et al.* Susceptibility testing of *Aspergillus flavus*: inoculum dependence with itraconazole and lack of correlation between susceptibility to amphotericin B *in vitro* and outcome *in vivo*. *Antimicrob Agents Chemother* 2001; **45**: 1456–62.

15. National Committee for Clinical Laboratory Standards. *Reference Method for Broth Dilution Antifungal Susceptibility Testing of Conidium-Forming Filamentous Fungi: Approved Standard M38-A.* NCCLS, Wayne, PA, USA, 2002.

16. Warn PA, Sharp A, Guinea J *et al.* Effect of hypoxic conditions on *in vitro* susceptibility testing of amphotericin B, itraconazole and micafungin against *Aspergillus* and *Candida*. *J Antimicrob Chemother* 2004; **53**: 743–9.

17. Denning DW, Hall L, Jackson M *et al.* Efficacy of D0870 compared with those of itraconazole and amphotericin B in two murine models of invasive aspergillosis. *Antimicrob Agents Chemother* 1995; **39**: 1809–14.

18. Sugar AM, Liu XP. Effect of grapefruit juice on serum voriconazole concentrations in the mouse. *Med Mycol* 2000; **38**: 209–12.

19. Hostetler JS, Hanson LH, Stevens DA. Effect of cyclodextrin on the pharmacology of antifungal oral azoles. *Antimicrob Agents Chemother* 1992; **36**: 477–80.

20. Warn PA, Brampton MW, Sharp A *et al.* Infrared body temperature measurement of mice as an early predictor of death in experimental fungal infections. *Lab Anim* 2003; **37**: 126–31.

21. Denning DW, Radford SA, Oakley KL *et al.* Correlation between *in-vitro* susceptibility testing to itraconazole and *in-vivo* outcome of *Aspergillus fumigatus* infection. *J Antimicrob Chemother* 1997; **40**: 401–14.

22. MacCallum DM, Odds FC. Need for early antifungal treatment confirmed in experimental disseminated *Candida albicans* infection. *Antimicrob Agents Chemother* 2004; **48**: 4911–4.

23. MacCallum DM, Whyte JA, Odds FC. Efficacy of caspofungin and voriconazole combinations in experimental aspergillosis. *Antimicrob Agents Chemother* 2005; **49**: 3697–701.

24. Chandrasekar PH, Cutright JL, Manavathu EK. Efficacy of voriconazole plus amphotericin B or micafungin in a guinea-pig model of invasive pulmonary aspergillosis. *Clin Microbiol Infect* 2004; **10**: 925–8.

25. Bowman JC, Abruzzo GK, Anderson JW *et al.* Quantitative PCR assay to measure *Aspergillus fumigatus* burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. *Antimicrob Agents Chemother* 2001; **45**: 3474–81.

26. Singh G, Imai J, Clemons KV *et al.* Efficacy of caspofungin against central nervous system *Aspergillus fumigatus* infection in mice determined by TaqMan PCR and CFU methods. *Antimicrob Agents Chemother* 2005; **49**: 1369–76.

27. O'Sullivan CE, Kasai M, Francesconi A *et al.* Development and validation of a quantitative real-time PCR assay using fluorescence

resonance energy transfer technology for detection of *Aspergillus fumigatus* in experimental invasive pulmonary aspergillosis. *J Clin Microbiol* 2003; **41**: 676–82.

28. Bowman JC, Hicks PS, Kurtz MB *et al.* The antifungal echinocandin caspofungin acetate kills growing cells of *Aspergillus fumigatus in vitro*. *Antimicrob Agents Chemother* 2002; **46**: 3001–12.

29. Schmitt-Hoffmann A, Roos B, Maares J *et al.* Multiple-dose pharmacokinetics and safety of the new antifungal triazole BAL4815 after intravenous infusion and oral administration of its prodrug, BAL8557, in healthy volunteers. *Antimicrob Agents Chemother* 2006; **50**: 286–93.

30. Schmitt-Hoffmann A, Roos B, Heep M *et al.* Single-ascending-dose pharmacokinetics and safety of the novel broad-spectrum antifungal triazole BAL4815 after intravenous infusions (50, 100, and 200 milligrams) and oral administrations (100, 200, and 400 milligrams) of its prodrug, BAL8557, in healthy volunteers. *Antimicrob Agents Chemother* 2006; **50**: 279–85.

31. Andes D, Marchillo K, Conklin R *et al.* Pharmacodynamics of a new triazole, posaconazole, in a murine model of disseminated candidiasis. *Antimicrob Agents Chemother* 2004; **48**: 137–42.

32. Andes D, Marchillo K, Stamstad T *et al. In vivo* pharmacodynamics of a new triazole, ravuconazole, in a murine candidiasis model. *Antimicrob Agents Chemother* 2003; **47**: 1193–9.

33. Pfaller MA, Diekema DJ, Messer SA *et al. In vitro* activities of voriconazole, posaconazole, and four licensed systemic antifungal agents against *Candida* species infrequently isolated from blood. *J Clin Microbiol* 2003; **41**: 78–83.

34. Warn PA, Sharp A, Denning DW. *In vitro* activity of a new triazole BAL4815, the active component of BAL8557 (the water-soluble prodrug), against *Aspergillus* spp. *J Antimicrob Chemother* 2006; **57**: 135–8.