

Activity of micafungin (FK463) against an itraconazole-resistant strain of *Aspergillus fumigatus* and a strain of *Aspergillus terreus* demonstrating *in vivo* resistance to amphotericin B

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We compared the activity of four doses of micafungin (FK463) with that of amphotericin B, liposomal amphotericin B and itraconazole in a murine model of disseminated aspergillosis. Temporarily neutropenic mice were infected with a lethal dose of either an itraconazole-resistant *Aspergillus fumigatus* isolate or *Aspergillus terreus*, a species that is less susceptible to amphotericin B. Treatment was started 24 h after infection and lasted for 7 days. Mice were treated with either amphotericin B (0.5 or 5 mg/kg), liposomal amphotericin (5 or 25 mg/kg), itraconazole (25 or 75 mg/kg) or FK463 (either 1, 2, 5 or 10 mg/kg). Treatment of the *A. fumigatus* model with either amphotericin B, liposomal amphotericin or FK463 prolonged survival. Doses of FK463 5 and 10 mg/kg had a 100% survival. Treatment of *A. terreus* infection with either itraconazole or FK463, but not amphotericin B, also prolonged survival. Doses of liposomal amphotericin of 5 and 25 mg/kg were ineffective against *A. terreus* infection. No treatment regime was able to totally clear the liver or kidneys in either model. The data indicate that FK463 may have a clinical role in the treatment of life-threatening invasive aspergillosis.

Keywords: micafungin, *Aspergillus*, murine, FK463, mouse

Introduction

Invasive aspergillosis represents a major threat to life in immunocompromised patients and is now one of the most common causes of infection in this group.¹ For many years, amphotericin B has been the drug of choice in the treatment of these cases.^{2,3} Concerns over its effectiveness,⁴ toxicity⁵⁻⁷ and reports of fungal resistance⁷⁻⁹ have highlighted the need for alternatives to be urgently sought. Itraconazole was until recently the only alternative licensed compound with useful clinical activity against invasive aspergillosis, but only moderate response rates⁴ and substantial interpatient variation in serum drug levels¹⁰ make this a less-than-ideal option.

Micafungin (FK463) is a new parenterally administered antifungal drug of the (echino)candin class of antifungal agents, which is currently undergoing clinical development.¹¹

It inhibits the synthesis of 1,3- β -D-glucan, an essential component of the fungal cell wall—a novel mode of action. It is likely that FK463 has activity against fungi resistant to other antifungal agents.¹²⁻¹⁴

FK463 has excellent *in vitro* activity against both *Candida* and *Aspergillus*¹¹ and promising *in vivo* activity in animal models of disseminated candidiasis^{15,16} and aspergillosis.¹⁶⁻²⁰ Preliminary clinical studies with the drug against oesophageal candidiasis in HIV-positive patients²¹ and febrile neutropenic paediatric patients²² have also shown good activity.

In this study, the activity of FK463 was compared with those of amphotericin B and itraconazole in a temporarily neutropenic murine model of aspergillosis caused by an itraconazole-resistant *A. fumigatus* strain or a strain of *A. terreus*, a species that is less susceptible to amphotericin B.

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Materials and methods

Test strains

One strain of *A. fumigatus* (AF91) and one strain of *A. terreus* (AT49) were used in this study. These strains have been deposited with the UK National Collection of Pathogenic Fungi, held at the Mycology Reference Laboratory, Bristol, UK, as NCPF 7100 and NCPF 7130, respectively. Strain AF91 was obtained from the sputum of a patient with AIDS who failed to respond to itraconazole therapy.²³ AT49 was recovered from the broncho-alveolar lavage of a patient who failed to respond to amphotericin B treatment.²⁴

The strains were maintained on slopes of Oxoid Sabouraud dextrose agar (Oxoid Ltd, Basingstoke, UK) supplemented with 0.5% (w/v) chloramphenicol and at -70°C in 15% glycerol for long-term storage.

In vitro susceptibility testing

In vitro susceptibility tests against amphotericin B and itraconazole were carried out as recommended by the NCCLS (using the microtitre variation).²⁵ In brief, susceptibility determinations were carried out in RPMI 1640 (Sigma, Poole, UK) buffered to pH 7.0 with MOPS. Deoxycholate amphotericin B (Fungizone, E.R. Squibb, Hounslow, UK) was initially reconstituted in distilled water then diluted in RPMI 1640 for use; itraconazole was prepared as described by Moore *et al.*²⁶ Conidia suspensions were adjusted to a final concentration of 2×10^5 conidia/mL. Plates were incubated at 37°C in a moist atmosphere and examined after 48 h incubation. The MICs were read visually and were defined as the concentration of the drug in the first well that showed no growth.

In vitro susceptibility tests to FK463 were carried out as recommended in the NCCLS-38P document and described in brief for amphotericin and itraconazole. The interpretation of the MIC was modified to the lowest concentration at which a prominent decrease in turbidity occurred when compared with the growth control [the minimum effective concentration (MEC)].²⁷

In vivo studies

Animals. All mice included in this study were part of ongoing studies carried out under UK Home Office project licence PPL 40/1523 entitled Invasive Fungal Infections.

Male CD1 mice, 4–5 weeks old and weighing between 18 and 20 g, were purchased from Charles River UK Ltd (Margate, UK). The mice were virus-free and were allowed free access to food and water. Mice were randomized into groups of ten. Each cage was inspected at least twice daily and any infected animals with severely reduced mobility, unable to reach the drinker or otherwise in substantial distress were humanely terminated.

Immunosuppression. Cyclophosphamide (Sigma–Aldrich, Poole, UK) was administered intravenously (iv) via the lateral tail vein to all animals at a dose of 200 mg/kg. A state of profound neutropenia was achieved 3 days after administration of the drug. White cell counts began to recover 4 days after this nadir.²⁸

Inoculum. The isolate was grown in a vented tissue culture flask containing Sabouraud dextrose agar (Oxoid) for 10 days. The *Aspergillus* conidia were harvested in 25 mL of sterile phosphate-buffered saline (PBS) with 0.5% Tween-80 (Sigma). The stock solution was adjusted to an inoculum that would give 1 LD₉₀ (for AF91 5×10^6 conidia/mL and for AT49 1.1×10^7 conidia/mL), based on viability counts (0.2 mL of these inocula were administered intravenously). The LD₉₀ was defined as the inoculum of conidia that caused 90% mouse mortality within 10 days of infection. Three days after immunosuppression, all animals were infected with the LD₉₀ dose via the lateral tail vein. The inoculum was rechecked from the remaining conidia suspension after the animals were infected.

Antifungal therapy. Deoxycholate amphotericin B (Fungizone) was dissolved in 5% dextrose (Baxter Healthcare, Norfolk, UK) to a stock concentration of 5.0 mg/mL. Two doses of amphotericin B were used in the course of the experiment—0.5 mg/kg and 5.0 mg/kg (only the 5 mg/kg regime was used in the AT49 model). The stock solution of amphotericin B was diluted as necessary in 5% dextrose. All doses of amphotericin B (and a 5% dextrose control group of mice) were administered via intraperitoneal injection once daily after 24, 48, 96 h and 7 days post-infection.

Liposomal amphotericin B (L-amphotericin; AmBisome) (Nextar Pharmaceuticals, Cambridge, UK) was reconstituted as per the manufacturer's instruction with 12 mL of water. This was further diluted in 5% glucose for use. Two doses of L-amphotericin were used in the models, 5 and 25 mg/kg (only 5 mg/kg was used in the AF91 model). All doses of L-amphotericin (and a 5% dextrose control group of mice) were administered via iv injection once daily after 24, 48, 96 h and 7 days post-infection.

Itraconazole powder (Janssen, Beerse, Belgium) was solubilized in (2-hydroxypropyl)- β -cyclodextrin (Fluka, Poole, UK).²⁹ This produced a stock of 25 mg/mL, which was then further diluted in (2-hydroxypropyl)- β -cyclodextrin to provide doses of 25 and 75 mg/kg. All doses of itraconazole were administered by gavage three times daily on days 1 and 2 and twice daily on days 3–7.

FK463 powder (Fujisawa, Osaka, Japan) was reconstituted in 5% glucose to provide doses of 1, 2, 5 and 10 mg/kg. All drug dilutions were stored in the dark at room temperature until used; the stock solution and dilutions were used within

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48 h of reconstitution. FK463 was administered iv once daily for 7 days.

Control mice were treated with either 5% glucose given iv as for FK463, 5% glucose given intraperitoneally as for amphotericin B or (2-hydroxypropyl)- β -cyclodextrin by gavage as for itraconazole.

Each treatment or control group consisted of 10 mice.

On day 11 of the experiment, all surviving mice were humanely killed. The lungs, liver and kidneys were removed aseptically and transferred into 2 mL of sterile PBS (BDH, Poole, UK). The organs were homogenized in a tissue grinder (Polytron, Kinematica AG, Lucerne, Switzerland) and colony counts determined using serial 10-fold dilutions plated on the surface of the plates. The plates were incubated at 37°C in a moist atmosphere and examined daily for 5 days. Single colonies were accorded a negative result, because of the possibility of airborne contamination. This method detected *Aspergillus* at >30 cfu/organ.

Pharmacokinetics

Blood samples were collected from a separate group of immunosuppressed mice by cardiac puncture 2 h post-dose on day 5 to determine the pharmacokinetics of itraconazole. Serum samples were stored at -20°C until analysed.

For itraconazole measurement, samples were thawed and analysed as a batch in bioassays using RPMI MOPS agar (Sigma) and the *Candida kefyr* San Antonio strain.³⁰

Statistical analysis

Mortality and culture data were analysed using the Mann-Whitney *U*-test or the Kruskal-Wallis test if the Mann-Whitney test was not possible (i.e. when all values were identical in one group). Two-sided *P* values are given. Mice that died before day 10 were assumed to have organ counts at least as high as the highest counts in surviving mice in the calculation of culture result statistics. All data analysis was carried out using the computer package Arcus Quickstat (Addison Wesley Longman Ltd, Harlow, Essex, UK). Two-sided probability values are quoted in the text.

Results

In vitro susceptibility

AF91 is resistant *in vitro* to itraconazole (MIC > 16 mg/L) but susceptible to amphotericin B (0.5 mg/L) and FK463 (<0.015 mg/L). AT49 is susceptible *in vitro* to amphotericin B (1.0 mg/L), itraconazole (0.25 mg/L) and FK463 (<0.015 mg/L).

In vivo results

In both models, control groups showed 90–100% mortality (Figures 1 and 2), indicating the extremely high mortality of these models if left untreated.

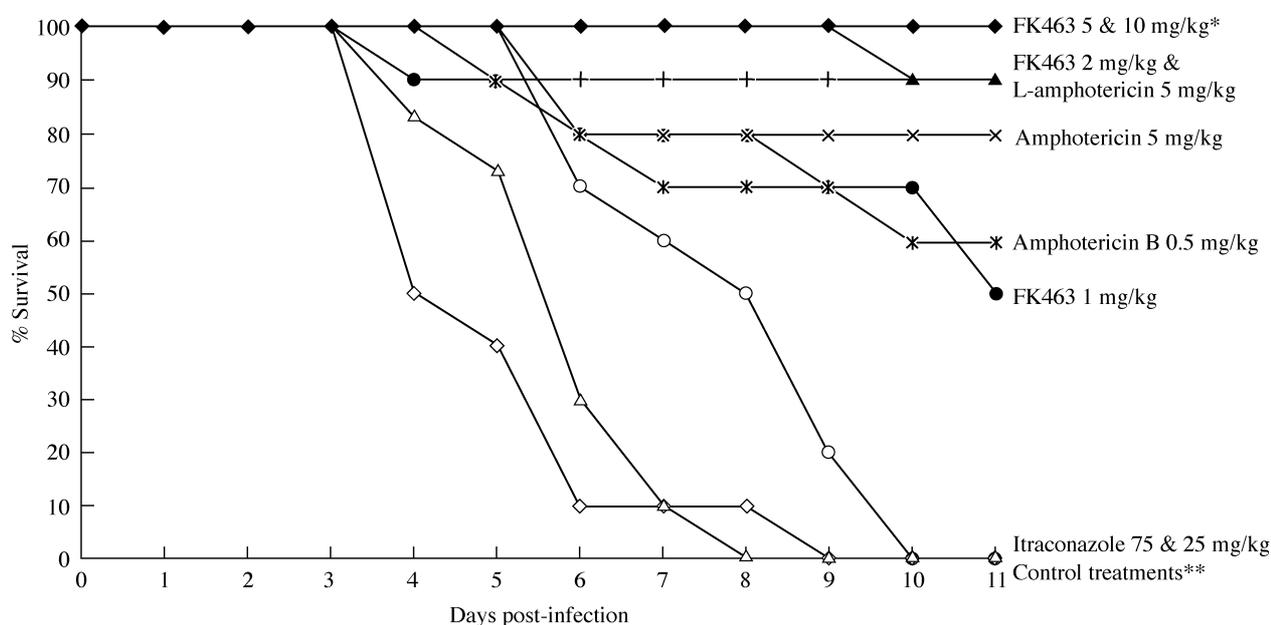


Figure 1. Plot of cumulative mortality against time in a murine model against *Aspergillus fumigatus* AF91. Black diamonds, FK463 10 mg/kg; black squares, FK463 5 mg/kg; black triangles, FK463 2 mg/kg; black circles, FK463 1 mg/kg; upright cross, L-amphotericin 5 mg/kg; diagonal cross, amphotericin B 5 mg/kg; star, amphotericin 0.5 mg/kg; white diamonds, itraconazole 75 mg/kg; white circles, itraconazole 25 mg/kg; white triangles, controls; *Treatment with 10 or 5 mg/kg per day FK463 superior to 1 mg/kg per day FK463, *P*=0.02; **Treatment with FK463 (all doses), amphotericin B and liposomal amphotericin superior to itraconazole and controls, *P*<0.0001.

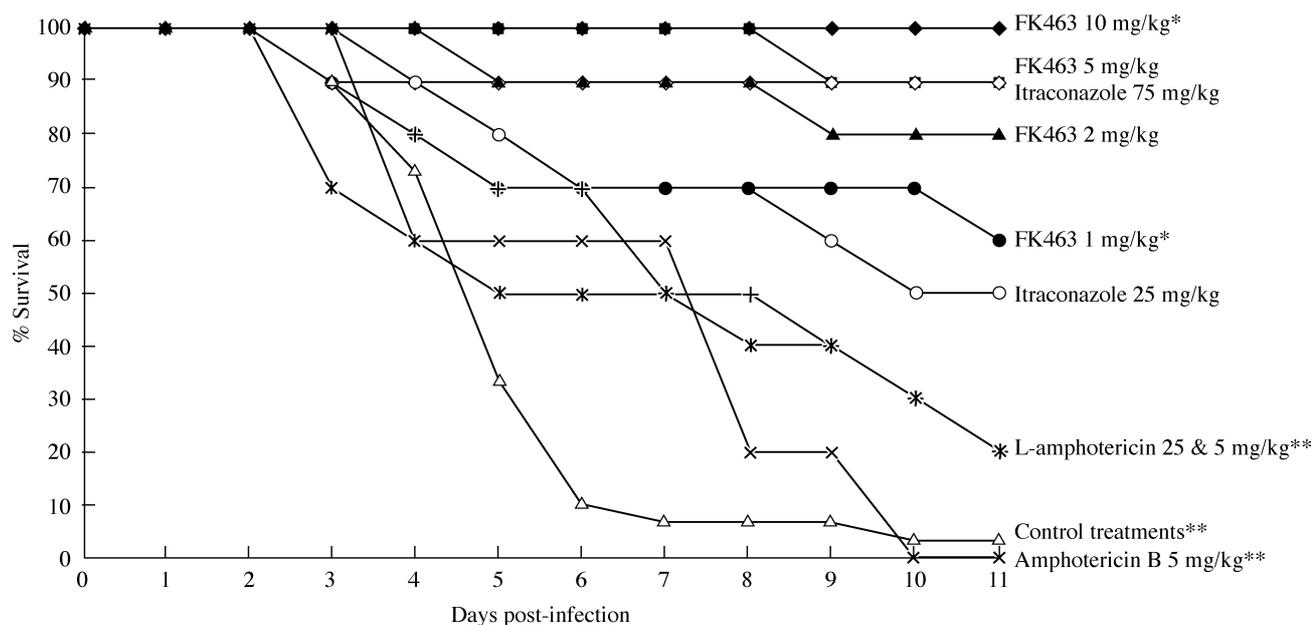


Figure 2. Plot of cumulative mortality against time in a murine model against *Aspergillus terreus* AT49. Black diamonds, FK463 10 mg/kg; black squares, FK463 5 mg/kg; black triangles, FK463 2 mg/kg; black circles, FK463 1 mg/kg; upright cross, L-amphotericin 5 mg/kg; diagonal cross, amphotericin B 5 mg/kg; star, L-amphotericin 25 mg/kg; white diamonds, itraconazole 75 mg/kg; white circles, itraconazole 25 mg/kg; white triangles, controls; *10 mg/kg per day FK463 was superior to 1 mg/kg per day FK463, $P=0.03$; **2, 5 and 10 mg/kg per day FK463 and itraconazole were superior to amphotericin B, liposomal amphotericin B and controls, $P<0.009$

A. fumigatus AF91 had 100% mortality in all control treatments, which was identical to both itraconazole treatments (itraconazole treatment outcomes were not statistically different), confirming the *in vitro* resistance of this strain to itraconazole (Figure 1). Survival rates after amphotericin B therapy demonstrated a tendency towards a dose-response with 5 mg/kg being numerically superior to 0.5 mg/kg. Treatment with L-amphotericin had a similar survival rate to amphotericin B at the same dose. Survival rates after FK463 treatment showed a clear dose-related response: both the 5 and 10 mg/kg having 100% survival, 90% survival was seen after 2 mg/kg and 50% survival after 1 mg/kg. Treatment with 5 or 10 mg/kg FK463 was superior to FK463 1 mg/kg ($P=0.02$). FK463, amphotericin B (both doses) and L-amphotericin were superior to itraconazole and control ($P<0.0001$).

A. terreus AT49 had 90–100% mortality in all control treatments, which was equivalent to treatment with amphotericin B 5 mg/kg (Figure 2). Treatment with L-amphotericin did not show a dose-dependent response over the five-fold range examined (5 and 25 mg/kg) with both treatments having a 20% survival. Survival rates after itraconazole demonstrated a tendency towards a dose-dependent response with 90% survival after 75 mg/kg but only 50% survival after 25 mg/kg ($P=0.15$). Survival rates after FK463 treatment showed a clear dose-dependent response with 100% survival after 10 mg/kg and 90%, 80% and 60% survival after 1, 2 and

5 mg/kg, respectively (FK463 10 mg/kg was superior to 1 mg/kg, $P=0.03$). Treatments with FK463 (2, 5 or 10 mg/kg) and itraconazole 75 mg/kg were superior to amphotericin B, L-amphotericin and controls ($P<0.009$).

Colony counts were calculated from the organ culture results and are summarized in Tables 1 and 2. After infection with AF91 and treatment, organ counts in both the liver and kidneys were statistically equivalent for FK463 2, 5 and 10 mg/kg, L-amphotericin 5 mg/kg and amphotericin B 5 mg/kg. The counts were statistically significantly lower after treatment with these regimens than amphotericin B 0.5 mg/kg or FK463 1 mg/kg in both the liver and kidneys ($P\leq 0.02$ and $P\leq 0.002$, respectively).

After infection with AT49, the counts were much higher in the liver and lungs than after infection with AF91 (>100-fold higher). There was also no dose-dependent reduction in organ burden with escalating doses of either FK463 (10-fold range) or L-amphotericin (five-fold range). There was a trend towards a dose-related reduction after itraconazole treatment but this did not reach statistical significance.

Itraconazole plasma concentrations

Itraconazole plasma concentrations in the uninfected treatment groups were a mean of 1.7 mg/L (25 mg/kg dosing) and 10.5 mg/L (75 mg/kg dosing) in samples collected 2 h post-dose on day 5 of treatment.

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Table 1. Means of organ counts for *A. fumigatus* AF91

Treatment	Survivors/no. in group (%)	Mean count (cfu/mL) ^a		
		lungs	liver	kidneys
FK463 10 mg/kg	10/10 (100)	30	2.5 × 10 ^{2*}	1.9 × 10 ^{3**}
FK463 5 mg/kg	10/10 (100)	40	2.8 × 10 ^{2*}	3.4 × 10 ^{2**}
FK463 2 mg/kg	9/10 (90)	10	7.2 × 10 ^{2*}	1.8 × 10 ^{3**}
FK463 1 mg/kg	5/10 (50)	40	1 × 10 ³	8 × 10 ³
L-amphotericin 5 mg/kg	9/10 (90)	10	2.6 × 10 ^{2*}	1.8 × 10 ^{3**}
Amphotericin B 5 mg/kg	8/10 (80)	10	3.8 × 10 ^{2*}	3.3 × 10 ^{3**}
Amphotericin B 0.5 mg/kg	6/10 (60)	10	1.2 × 10 ³	6.8 × 10 ³
Itraconazole 75 mg/kg	0/10 (0)			
Itraconazole 25 mg/kg	0/10 (0)			
Control groups ^b	0/30 (0)			

^aAll counts are expressed as cfu/mL of organ homogenate.

^bControl groups combined.

*Superior to amphotericin B 0.5 mg/kg and FK463 1 mg/kg *P* < 0.02.

**Superior to amphotericin B 0.5 mg/kg and FK463 1 mg/kg *P* < 0.002.

Table 2. Means of organ counts for *A. terreus* AT49

Treatment	Survivors/no. in group (%)	Mean count (cfu/mL) ^a		
		lungs	liver	kidneys
FK463 10 mg/kg	10/10 (100)	50	5.8 × 10 ⁴	4.2 × 10 ²
FK463 5 mg/kg	9/10 (90)	75	5.1 × 10 ⁴	2.9 × 10 ²
FK463 2 mg/kg	8/10 (80)	1.3 × 10 ²	4.9 × 10 ⁴	8 × 10 ²
FK463 1 mg/kg	6/10 (60)	2.1 × 10 ²	5.1 × 10 ⁴	1.4 × 10 ³
L-amphotericin 25 mg/kg	2/10 (20)	3.2 × 10 ²	7.1 × 10 ⁴	6.6 × 10 ³
L-amphotericin 5 mg/kg	2/10 (20)	3.3 × 10 ²	7.7 × 10 ⁴	8.7 × 10 ³
Amphotericin B 5 mg/kg	0/10 (0)			
Itraconazole 75 mg/kg	9/10 (90)	90	2.4 × 10 ⁴	3.2 × 10 ²
Itraconazole 25 mg/kg	5/10 (50)	3 × 10 ²	6.2 × 10 ⁴	4.2 × 10 ³
Control groups ^b	1/30 (3)			

NB. All comparisons non-significant.

^aAll counts are expressed as cfu/mL of organ homogenate.

^bControl groups combined.

Discussion

As expected from previous work, AF91 was itraconazole resistant and AT49 was amphotericin B resistant *in vivo*.^{23,24} FK463 was highly active against both isolates *in vitro* and *in vivo*, indicating no cross-resistance with azoles or polyenes. Given emerging resistance in *Aspergillus* to amphotericin B and itraconazole,^{7,8,23,31} and the lack of alternative therapies for diseases caused by these organisms, the development of new drugs is essential. The high level *in vitro* and *in vivo* activity of FK463 is an encouraging addition to our antifungal strategies.

FK463 showed a dose–response in both models. In terms of mortality, 1 mg/kg FK463 was less effective than doses of ≥2 mg/kg. Doses of ≥2 mg/kg FK463 were slightly better than 1 mg/kg in reducing tissue burden in both models but no FK463 dose was effective at sterilizing any organs examined. Above 2 mg/kg FK463, there was no suggestion of a dose-related effect. No discernible toxicity was noted in any of the FK463-treated mice.

Liposomal amphotericin was as effective as the same dose of conventional amphotericin B with AF91 and superior to a 10-fold lower dose of conventional amphotericin B. With respect to AT49, a dose of 25 mg/kg L-amphotericin was

ineffective. This is disappointing from a clinical perspective, but indicates the need to use alternative agents to amphotericin B in the treatment of *A. terreus* infections. It is important to note that the *in vitro* MIC measurement of the susceptibility of AT49 to amphotericin B was 1 mg/L, which would normally be regarded as fully susceptible. In view of the high mortality after treatment with amphotericin B 5 mg/kg and liposomal amphotericin 25 mg/kg per day, we have again demonstrated a lack of correlation of the *in vivo* response and *in vitro* susceptibility measurement of these drugs.²⁴

FK463 has previously been used in trials against murine models of both pulmonary and disseminated *A. fumigatus*^{15,17} and in both cases was highly effective both in terms of prolonging survival and reducing the organ burden. FK463 has also been used in the treatment of experimental pulmonary *A. fumigatus* in rabbits but was ineffective at reducing tissue counts of *Aspergillus* at doses up to 2 mg/kg.^{16,20} In all previous models the *A. fumigatus* isolates were fully susceptible to the comparator used. This paper is the first report of the activity of FK463 against an itraconazole-resistant *A. fumigatus* and also the first report of its use against a non-*fumigatus* *Aspergillus* demonstrating *in vivo* resistance to amphotericin B (even though the *in vitro* MIC was 1 mg/L).

FK463 is active against experimental *Aspergillus* infections and oesophageal candidiasis in HIV-positive patients.²¹ Another echinocandin, caspofungin, had a 40% response rate as salvage therapy in patients with invasive aspergillosis.³² These data, together with the data presented in this paper, indicate a clinical role for the echinocandins in the treatment of life-threatening invasive aspergillosis. Doses >1 mg/kg might be more effective than the daily doses of 50 mg daily being used clinically at present.

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