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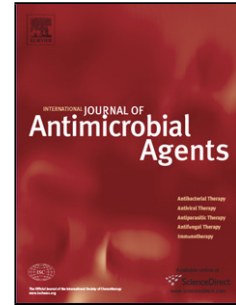
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## **Efficacy of anidulafungin against *Aspergillus niger* in vitro and in vivo**

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## ABSTRACT

In this study, anidulafungin (AFG) showed high in vitro activity against 10 isolates of *Aspergillus niger* by broth microdilution and disk diffusion methods. The efficacy of AFG at 1, 5 and 10 mg/kg was tested against six of the isolates in a murine model of disseminated infection. AFG was able to reduce mortality, showing survival rates of 70–100%, 60–100% and 30–60% in mice treated with AFG at 10, 5 and 1 mg/kg, respectively. AFG also showed a dose–response efficacy in reducing tissue burden in kidneys and spleen. A parallel experiment demonstrated that administration of AFG did not reduce serum concentrations of galactomannan in mice. Histopathological studies confirmed the efficacy of AFG.

## 1. Introduction

Invasive aspergillosis (IA) is a life-threatening fungal infection predominantly affecting immunocompromised patients. Although the majority of infections are caused by *Aspergillus fumigatus*, other species such as *Aspergillus flavus*, *Aspergillus niger* and *Aspergillus terreus* also have relevant clinical importance [1]. *Aspergillus niger* is mainly associated with otomycosis, although in immunocompromised patients it can produce severe infections such as peritonitis, endocarditis and disseminated infections, generally following major surgery [2].

Voriconazole (VCZ) is the drug of choice in the treatment of IA, and other azoles such as posaconazole and itraconazole are considered alternative therapies [1]. However, increasing resistance of *Aspergillus* isolates to azoles has been reported [3], making it necessary to develop new therapeutic approaches. The echinocandins are newer alternatives to VCZ, with caspofungin (CAS) having been approved as primary therapy and with micafungin recommended for prophylaxis and as salvage therapy for IA [1]. The efficacy of AFG against aspergillosis has only been demonstrated in experimental infections by *A. fumigatus* [4] and *A. flavus* [5]. It is not known whether this drug is effective against experimental *A. niger* infections.

In this study, the in vitro activity of AFG against 10 clinical isolates of *A. niger* was tested by two different methods and its in vivo efficacy was determined in a murine model of systemic infection using 6 randomly chosen isolates from the 10 isolates tested in vitro.

## 2. Materials and methods

The in vitro antifungal activity of AFG against 10 isolates of *A. niger* was determined by broth microdilution and disk diffusion methods. The broth microdilution method was carried out to determine the minimum effective concentration (MEC) (in mg/L), defined as the lowest antifungal concentration that produced morphological alterations of hyphal growth compared with that of the control well [6]. In the disk diffusion method, inhibition zone diameters (IZDs) (in mm) were determined using non-supplemented Mueller–Hinton agar and 6-mm diameter paper disks containing 5 µg of AFG [7]. To evaluate the in vitro susceptibility of *A. niger* isolates to AFG, the suggested breakpoints at 24 h for CAS against *Aspergillus* for MEC and IZD, respectively, were as follows: susceptible,  $\leq 1$  mg/L or  $\geq 17$  mm; intermediate, 2 mg/L or 14–16 mm; and resistant,  $\geq 4$  mg/L or  $\leq 13$  mm [8].

For the in vivo study, six isolates previously tested in vitro were randomly chosen. Two of these strains (FMR 7156 and FMR 11252) were environmental, whilst the other four had a clinical origin [FMR 11251 from cutaneous aspergillosis, FMR 11250 from sputum, FMR 11248 from perionyxis and FMR 11254 from sputum of a human immunodeficiency virus (HIV)-positive patient]. On the day of infection, cultures on potato dextrose agar (PDA) incubated at 30 °C for 7 days were suspended in sterile saline and were filtered through sterile gauze to remove clumps of spores or hyphae. The resulting suspensions were adjusted to the desired inoculum based on haemocytometer counts and by serial plating on PDA to confirm viability.

Male OF1 mice weighing 30 g (Charles River, Criffa S.A., Barcelona, Spain) were used. Animals were immunosuppressed 1 day prior to infection by administering a

single intraperitoneal dose of 200 mg/kg cyclophosphamide plus a single intravenous dose of 150 mg/kg 5-fluorouracil. To prevent bacterial infection, the mice received ceftazidime (5 mg/day subcutaneously) from Days 1–7 after challenge. Mice were infected with a conidial suspension of  $1 \times 10^5$  colony-forming units (CFU) in 0.2 mL of sterile normal saline injected intravenously via a lateral tail vein. Previous tests with the six strains have shown that this inoculum was suitable for producing an acute infection, with all the animals dying within 10 days of challenge (data not shown).

AFG (Ecalta; Pfizer Inc., Madrid, Spain) was administered in 0.1 mL of sterile saline at 1, 5 or 10 mg/kg body weight intraperitoneally once a day. All treatments began 24 h after challenge and lasted for 7 days. Groups of 20 mice were established for each strain and each treatment. Control animals received no treatment. The efficacy of the drug was evaluated through prolongation of survival and reduction of tissue fungal burden. For each strain and dose, groups of 10 mice were randomly assigned to the survival study and were checked daily for 30 days. For tissue burden studies, the other 10 mice from each group were sacrificed on Day 5 after infection. The kidneys and spleen were aseptically removed and approximately one-half of each organ was weighed and homogenised in 2 mL of sterile saline. Serial 10-fold dilutions of the homogenates were plated on PDA and were incubated for 48 h at 30 °C.

Mean survival time was estimated by the Kaplan–Meier method and was compared among groups using the log-rank test. Colony counts in tissue burden studies were analysed by the Mann–Whitney *U*-test. A *P*-value of  $\leq 0.05$  was considered statistically significant.

For the histopathological study, the remaining half of each organ was fixed with 10% buffered formalin. Samples were dehydrated, paraffin-embedded and sliced into 2  $\mu\text{m}$  sections that were stained with haematoxylin–eosin, Periodic acid Schiff and Grocott's methenamine silver and were examined in blinded fashion by light microscopy.

In a parallel study, groups of five immunosuppressed mice were challenged with  $2 \times 10^2$  CFU of *A. niger* strain FMR 11254. These mice were treated for 7 days with the same doses of AFG to determine the galactomannan serum levels by enzyme immunoassay (Platelia™ *Aspergillus* EIA; Bio-Rad, Marnes-la-Coquette, France) on the last day of therapy as an additional marker of treatment efficacy. Serum was obtained from centrifuged blood samples extracted by cardiac puncture with the mice anaesthetised by sevoflurane inhalation. Following the procedure described by the manufacturer, 100  $\mu\text{L}$  of treatment solution [4% ethylene diamine tetra-acetic acid (EDTA)] was added to 300  $\mu\text{L}$  of each serum sample. The mixture was heated for 6 min at 120 °C and the samples were centrifuged. Then, 50  $\mu\text{L}$  of the supernatant and 50  $\mu\text{L}$  of conjugate were added to each well of an anti-galactomannan antibody-coated plate. Plates were incubated at 37 °C for 90 min and were washed five times. Finally, 200  $\mu\text{L}$  of chromogen solution was added to each well and was incubated for 30 min at room temperature in darkness. After stopping the reaction, the optical density was measured at 450 nm and 620 nm. Data were expressed as a serum galactomannan index (GMI), with results expressed as mean  $\pm$  standard deviation. Results were compared using a *t*-test.



### 3. Results

The MECs were  $\leq 0.06$  mg/L for all strains tested. The disk diffusion test showed a IZD range of 23–25 mm (23 mm for strains FMR 11251 and FMR 11254, 24 mm for strains FMR 7156, FMR 11252 and FMR 11250 and 25 mm for strain FMR 11248). All the MEC and IZD values for AFG were within the suggested ranges of susceptibility, i.e. all the strains showed MECs  $\leq 1$  mg/L and IZDs  $\geq 17$  mm.

Fig. 1 shows the results of the survival study. For all the strains tested, AFG at 5 mg/kg and 10 mg/kg significantly prolonged the survival of mice with respect to the control group. AFG at 1 mg/kg prolonged the survival for all the strains with the exception of FMR 11248 and FMR 11250. In addition, AFG at 10 mg/kg for strains FMR 11251 and FMR 11254 and AFG at 5 mg/kg for strain FMR 11254 also prolonged survival with respect to mice treated with AFG at 1 mg/kg. Table 1 shows the results of the tissue burden study. AFG at 10 mg/kg reduced the fungal load in comparison with the control group for all strains tested in the kidney, and for all the strains except FMR 11251 and FMR 11250 in spleen tissue. AFG at 10 mg/kg reduced the spleen fungal recovery in comparison with mice treated with AFG at 1 mg/kg for strain FMR 11252, and in kidney tissue for strains FMR 7156, FMR 11252, FMR 11250 and FMR 11248. For strain FMR 11252, AFG at 10 mg/kg also reduced the fungal recovery in spleen and kidney with respect to mice treated with AFG at 5 mg/kg. AFG at 5 mg/kg reduced the fungal load with respect to the control group in the spleen for strains FMR 7156, FMR 11252, FMR 11248 and FMR 11254, and for all strains except FMR 11252 in kidney tissue. Such a dose also reduced the fungal load in comparison with mice treated with AFG at 1 mg/kg for strain FMR 11252 in the spleen, and for strains FMR 11250 and FMR 11248 in kidney tissue. AFG at 1

mg/kg reduced the fungal load in comparison with the control group for strain FMR 11254 in the spleen, and for strains FMR 7156, FMR 11248 and FMR 11254 in the kidney.

None of the doses of AFG was able to reduce significantly the GMI levels in comparison with the control group. After 7 days of therapy, GMI values were  $6.67 \pm 0.19$  for control mice and  $6.49 \pm 0.16$ ,  $6.22 \pm 0.33$  and  $6.26 \pm 0.27$  for mice treated with AFG at 1, 5 and 10 mg/kg, respectively.

In untreated mice and in those treated with AFG at 1 mg/kg, the histopathological study revealed fungal invasion in renal parenchyma and blood vessels. Kidneys of mice treated with the higher doses of AFG did not show fungal elements. These findings were observed in the spleen and kidney but were more evident in the latter (Fig. 2).

#### 4. Discussion

Infections by *A. niger*, although relatively rare, are usually fatal in immunosuppressed patients [9–11]. In most infections, patients receive amphotericin B as the sole antifungal therapy, with poor clinical efficacy. Although VCZ remains the first option against IA, strains of *A. niger* have been reported that are resistant in vitro to azoles [3]. In addition, at least one case of invasive infection by *A. niger* did not respond to the VCZ therapy [12].

Both in vitro methods used in this study showed good activity of AFG against *A. niger*. The good correlation between the two in vitro methods used suggests a

potential use, due to its simplicity, of the disk diffusion method in the clinical laboratory for testing susceptibility to echinocandins.

The murine study confirmed the high efficacy of AFG against experimental invasive infections by *A. niger*. This drug had already shown efficacy in experimental infections by *A. fumigatus* [4] and *A. flavus* [5]. In a previous study [5], the three doses tested in the current study showed AFG serum concentrations above the MECs of the *A. niger* isolates tested. Despite this, and in agreement with a previous animal study that tested CAS on *A. fumigatus* murine infections [13], the higher doses of AFG performed better than the lowest one. This fact was especially evident in the histopathological study, where no fungal elements were found in kidneys of mice treated with the higher doses, in contrast to the abundant cells observed in kidneys of untreated mice or mice treated with 1 mg/kg AFG. Despite the good efficacy of AFG in the histopathological, survival and tissue burden studies, AFG was not able to reduce the galactomannan serum concentrations after 7 days of treatment in mice infected with a low inoculum of *A. niger*. A previous study demonstrated that using a similar murine model of invasive aspergillosis, GMI values started to decline after 5 days of therapy [14]. For this reason, a new group of animals was infected with a lower fungal dose to ensure that all animals survived for the 7 days of therapy. In control mice, galactomannan levels were high (mean value  $6.67 \pm 0.19$ ) and fell very slightly following AFG therapy, although the changes were not significant. In previous studies, we have demonstrated that successful treatment of *A. terreus* with posaconazole produces highly significant reductions in galactomannan levels [15]. Our result agrees with other experimental studies with *A. fumigatus* and *A. flavus* in which treatment with echinocandins did not lead to a

decrease in galactomannan levels [5,14]. The fungistatic activity of echinocandins has been suggested as the probable cause of maintained galactomannan levels, in contrast to the fungicidal drugs like the azoles that reduce the concentration of this antigen [14].

In summary, we have found a correlation between in vitro activity and in vivo efficacy of AFG in experimental infections by *A. niger*, suggesting a potential role of this drug in the treatment of IA caused by *A. niger*.

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None.

**Competing interests**

None declared.

**Ethical approved**

All animal care procedures were supervised and approved by the Universitat Rovira i Virgili Animal Welfare and Ethics Committee (Reus, Spain).

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**Fig. 1.** Cumulative mortality of mice infected with  $1 \times 10^5$  colony-forming units (CFU) of *Aspergillus niger* strains (A) FMR 7156, (B) FMR 11252, (C) FMR 11251, (D) FMR 11250, (E) FMR 11248 and (F) FMR 11254. AFG 1, anidulafungin at 1 mg/kg; AFG 5, anidulafungin at 5 mg/kg; AFG 10, anidulafungin at 10 mg/kg. <sup>a</sup>  $P < 0.05$  vs. control; <sup>b</sup>  $P < 0.05$  vs. AFG 1.

**Fig. 2.** (A) Kidney section of a control mouse showing invasion of fungal elements in parenchyma and in a blood vessel. (B) Kidney section of a mouse treated with anidulafungin (AFG) at 1 mg/kg, showing infiltration of hyphae (arrows). (C,D) Kidney sections of mice treated with AFG at 5 mg/kg (C) and 10 mg/kg (D), showing no fungal invasion. Grocott's stain.

**Table 1**

Effects of antifungal treatment on colony counts of *Aspergillus niger* in spleen and kidney tissues of mice

Isolate	Dose	Mean log <sub>10</sub> CFU/g (95% CI)	
		Spleen	Kidney
FMR 7156	None	4.53 (4.43–4.62)	3.21 (3.11–3.31)
	AFG 1	4.38 (4.25–4.50)	2.71 (2.58–2.84) *
	AFG 5	4.24 (4.16–4.32) *	2.48 (2.36–2.60) *
	AFG 10	4.15 (4.03–4.26) *	2.14 (1.95–2.34) *,**
FMR 11252	None	4.73 (4.62–4.85)	2.94 (2.75–3.14)
	AFG 1	4.63 (4.53–4.72)	2.72 (2.61–2.83)
	AFG 5	4.33 (4.20–4.45) *,**	2.69 (2.51–2.86)
	AFG 10	3.73 (3.57–3.90) *,**,†	2.08 (1.96–2.21) *,**,†
FMR 11251	None	4.33 (4.19–4.47)	3.15 (2.99–3.31)
	AFG 1	4.14 (4.01–4.27)	2.76 (2.64–2.88)
	AFG 5	4.16 (4.03–4.28)	2.39 (2.14–2.64) *
	AFG 10	4.19 (4.05–4.32)	2.44 (2.23–2.64) *
FMR 11250	None	4.43 (4.34–4.53)	3.17 (3.04–3.29)
	AFG 1	4.30 (4.23–4.38)	2.87 (2.72–3.01)
	AFG 5	4.29 (4.17–4.40)	2.49 (2.31–2.66) *,**
	AFG 10	4.20 (4.07–4.34)	2.37 (2.22–2.53) *,**
FMR 11248	None	4.52 (4.42–4.63)	3.32 (3.20–3.43)
	AFG 1	4.41 (4.33–4.49)	2.86 (2.75–2.98) *
	AFG 5	4.29 (4.19–4.38) *	2.58 (2.44–2.73) *,**
	AFG 10	4.30 (4.18–4.42) *	2.41 (2.23–2.59) *,**
FMR 11254	None	4.05 (3.96–4.13)	3.17 (3.07–3.26)
	AFG 1	3.78 (3.61–3.94) *	2.31 (2.07–2.55) *
	AFG 5	3.74 (3.64–3.84) *	2.18 (2.02–2.34) *
	AFG 10	3.60 (3.46–3.73) *	1.95 (1.77–2.12) *

CFU, colony-forming units; CI, confidence interval; AFG 1, anidulafungin at 1 mg/kg;

AFG 5, anidulafungin at 5 mg/kg; AFG 10, anidulafungin at 10 mg/kg.

\*  $P < 0.05$  vs. control.



\*\*  $P < 0.05$  vs. AFG 1.

†  $P < 0.05$  vs. AFG 5.

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