Ibuprofen Potentiates the *In Vivo* Antifungal Activity of Fluconazole against *Candida albicans* Murine Infection

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Candida albicans is the most prevalent cause of fungemia worldwide. Its ability to develop resistance in patients receiving azole antifungal therapy is well documented. In a murine model of systemic infection, we show that ibuprofen potentiates fluconazole antifungal activity against a fluconazole-resistant strain, drastically reducing the fungal burden and morbidity. The therapeutic combination of fluconazole with ibuprofen may constitute a new approach for the management of antifungal therapeutics to reverse the resistance conferred by efflux pump overexpression.

*C*andida invasive infections represent an increasing challenge for clinicians, with an assigned mortality rate of around 40% (1–3). The extensive use of fluconazole in both prophylaxis and therapy has resulted in the emergence of resistance (4–6).

Knowledge of the mechanisms underlying resistance is of crucial importance, since it might support approaches to achieve reversion, leading to the development of new therapeutic strategies. Alterations in the *Candida albicans* transcriptome induced by azole exposure have been extensively reported (5, 7, 8). However, few studies have tackled antifungal resistance reversion.

Previously, we showed that the antifungal resistance conferred by increased efflux activity, due to overexpression of the *CDR1* and *CDR2* genes, in *Candida* clinical strains can be reversed by ibuprofen (9, 10). This effect is also observed with other drugs, such as pyrazinamide (11) and amphotericin B (12). Ibuprofen is a nonsteroidal anti-inflammatory drug used for its antipyretic, analgesic, and anti-inflammatory effects.

In this study, we aimed to examine the *in vivo* reversion of fluconazole resistance by ibuprofen using a *C. albicans* systemic model of infection.

A *C. albicans* blood culture strain (*Ca*S) susceptible to fluconazole, voriconazole, and posaconazole was used to induce an azole-resistant phenotype. For 60 days, a yeast suspension containing 10^6 cells in 10 ml of RPMI 1640 medium (Sigma) was incubated daily with fluconazole (Pfizer) at a final concentration of 16 μg/ml (the therapeutic serum level achieved during antifungal therapy) (13, 14). The MICs to azoles, fluconazole, voriconazole (Pfizer), and posaconazole (Schering-Plough) were determined for the parent strain and the successive fluconazole-exposed isolates, and the susceptibility profile was determined according to the CLSI M27-A3 protocol (15). For posaconazole, strains with an MIC of ≤0.06 μg/ml were considered susceptible (16). The repeated exposure of *C. albicans* to fluconazole resulted in the acquisition of an azole-cross-resistant phenotype (Table 1), achieved due to overexpression of efflux pump-encoding genes and the *ERG11* gene (data not shown) displayed by the *C. albicans* resistant (*Ca*R) strain.

The azole-resistant phenotype of the *Ca*R strain turned susceptible when the MICs to azoles were redetermined in the presence of 100 μg/ml ibuprofen, a concentration previously described to impair azole efflux (9, 10). These *in vitro* results demonstrate that ibuprofen potentiates azole fungistatic activity.

The pursuit of knowledge regarding the efflux pump mechanism in *Candida* arises from the homology between yeasts and human cells. In eukaryotic neoplasic cells, ATP-dependent drug efflux pumps, such as P-glycoprotein (P-gp), which is encoded by the *MDR1* gene, are important mediators of resistance, contributing to the failure of cancer therapy. In the human kidney, ibuprofen can inhibit methotrexate efflux transporters (17). A similar effect has been described for FK506 (tacrolimus), a potent immunosuppressive agent that shows a synergistic effect when combined with antineoplasic agents on tumor cells, decreasing or even suppressing multidrug resistance by competing with cytotoxic drugs for the P-glycoprotein (18–20). Thus, a similar approach could be applied to *C. albicans* cells.

To investigate the potential clinical application of fluconazole and ibuprofen, *in vivo* experiments were conducted in a murine candidosis model approved by the Directorate General of Food and Veterinary Medicine of the European Union (authorization no. 6411).

Female specific-pathogen-free BALB/c mice (age, 6 to 8 weeks; weight, 17 to 20 g; Charles River Laboratories) were injected with 5×10^5 cells of the *Ca*S or the *Ca*R strain in 0.1 ml of sterile saline via the lateral tail vein (21). The fluconazole effective dose that reduced by 50% the pathological effects of intravenous (i.v.) *C. albicans* challenge relative to untreated mice (control) was defined as the 50% effective dose (ED_{50}) (8). Therapy was initiated 3 h after the yeast challenge and was administered daily for a total of 3 days (8, 21). The mice were treated intraperitoneally with fluconazole (8 to 60 mg/kg of body weight/day) (8, 21), ibuprofen (10 or 20 mg/kg/day) (11, 22), or fluconazole (8 to 60 mg/kg of body

TABLE 1 *In vitro* development of azole resistance and its reversion by ibuprofen

Strain	MIC (µg/ml)/phenotype for[a]:					
	FLC	FLC + Ibu	VRC	VRC + Ibu	PSC	PSC + Ibu
*Ca*S	1/S	1/S	0.06/S	0.06/S	0.03/S	0.06/S
*Ca*R	>64/R	2/S	>8/R	0.06/S	>8/R	0.125/R

[a] MICs to azoles, namely, fluconazole (FLC), voriconazole (VRC), and posaconazole (PSC), alone and in combination with subinhibitory concentration of ibuprofen (Ibu) (100 µg/ml). S, susceptible; R, resistant.

weight/day) plus ibuprofen (10 or 20 mg/kg/day). The weight of each mouse was registered daily, and at day 4 postinfection, the mice were euthanized and the kidneys aseptically removed. The fungal burden was calculated as the number of CFU per gram of tissue, and the isolates were preserved for later MIC determination. For histological studies, the kidneys were processed for periodic acid-Schiff (PAS) staining.

In mice infected with the *Ca*S strain and treated with 30 mg/kg of body weight/day (ED$_{50}$) of fluconazole, a significant reduction ($P <$ 0.001) in yeast colonization in the kidney was found compared with that in the untreated mice (Fig. 1). In mice infected with the *Ca*R strain, no significant reduction in fungal burden was achieved, even when treated with 60 mg/kg of fluconazole (data not shown). Mice infected with the *Ca*S and *Ca*R strains and treated with 10 or 20 mg/kg/day of ibuprofen did not show a reduction in fungal burden. However, when fluconazole was administered with ibuprofen, even at the lower concentration, a significant reduction in *Ca*R fungal burden ($P <$ 0.001) was observed (Fig. 1).

Mice infected with the *Ca*R strain and treated with fluconazole plus ibuprofen showed the lowest weight loss (Fig. 2).

Interestingly, yeasts recovered from mouse kidneys retained their susceptibility profile, i.e., susceptible or resistant for the *Ca*S or *Ca*R strain, respectively.

Histopathological sections of mouse kidneys confirmed the fungal burden quantitative analysis. Infection caused by *Ca*R was evident in untreated and fluconazole-treated mice (Fig. 3A to E). At day four postinfection, untreated mice infected with the *Ca*R strain revealed a dramatic increase in fungal colonization by different types of cells, yeasts and hypha, extensive tissue damage, and necrosis (Fig. 3A). An identical scenario was found in kidneys collected from mice treated with 30 mg/kg of fluconazole (Fig. 3B

to E). In Fig. 3E, fungal cells were predominantly in the hyphal form and were apparently intact, forming a clear barrier to the progression of inflammatory leukocytes. Notably, in mice treated with fluconazole plus ibuprofen, the kidney tissue architecture was preserved, and the fungal cells were rare, all displaying a yeast form (Fig. 3F and G).

The phenotypic switching between yeast and hypha in *C. albicans* is often described as the major virulence factor, as hyphal formation is associated with elevated secretion of hydrolytic enzymes, direct tissue invasion, and adherence to host surfaces (23). Hyphal morphotypes are more invasive, and their extension is essential for dissemination and the subsequent events responsible for the gross damage of tissues, which are commonly observed in the kidneys of infected mice (24, 25).

Mice treated with fluconazole and ibuprofen experienced clearance of infection, recovery of body weight, and the conservation of tissue architecture, having scarce fungal cells in the kidneys, being predominantly yeast forms. The hypothesis that the presence of ibuprofen may target the regulation of the morphological switch from yeast to hypha thus deserves more attention and should be a subject of future in-depth research.

C. albicans cells recovered from mice treated with fluconazole plus ibuprofen still displayed a fluconazole-resistant phenotype. Consequently, we can conclude that the presence of ibuprofen is crucial and mandatory for the reversion of azole resistance. The *in vivo* assays clearly demonstrate that ibuprofen potentiates the antifungal activity of fluconazole and reduces the virulence of *C. albicans*. Since it is not immunosuppressive, its anti-inflammatory activity has advantages over FK506 (26). Further studies are being addressed to uncover the mechanism of ibuprofen on yeast cell physiology and to assess its influence on the dynamics of antifun-
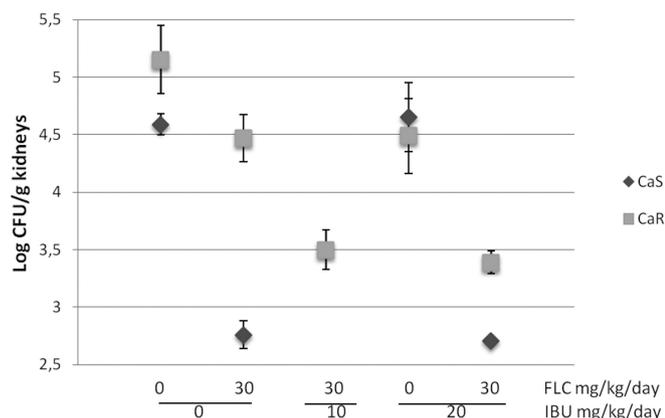

FIG 1 *In vivo* antifungal potentiating effect between fluconazole and ibuprofen against *C. albicans* systemic infection. The log CFU per gram of kidney values are plotted as the mean and standard error. *Ca*S, susceptible strain; *Ca*R, resistant strain; FLC, fluconazole; Ibu, ibuprofen.
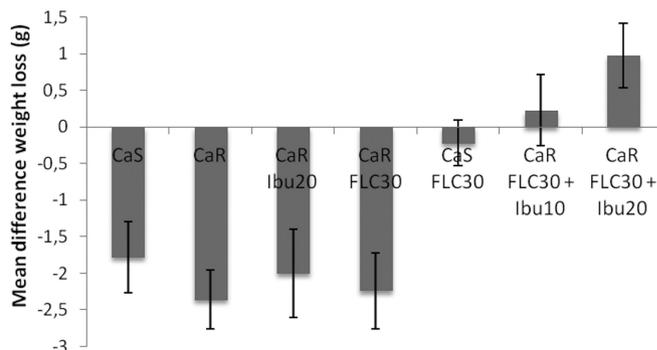

FIG 2 Effect of the combined therapeutic fluconazole plus ibuprofen on mouse weight during systemic infection. Doses of drug are in milligrams per kilogram of body weight per day. Differences in weight loss between the first and the fourth day of infection are plotted as the mean values plus the respective standard errors. *Ca*S, susceptible strain; *Ca*R, resistant strain; FLC, fluconazole; Ibu, ibuprofen.
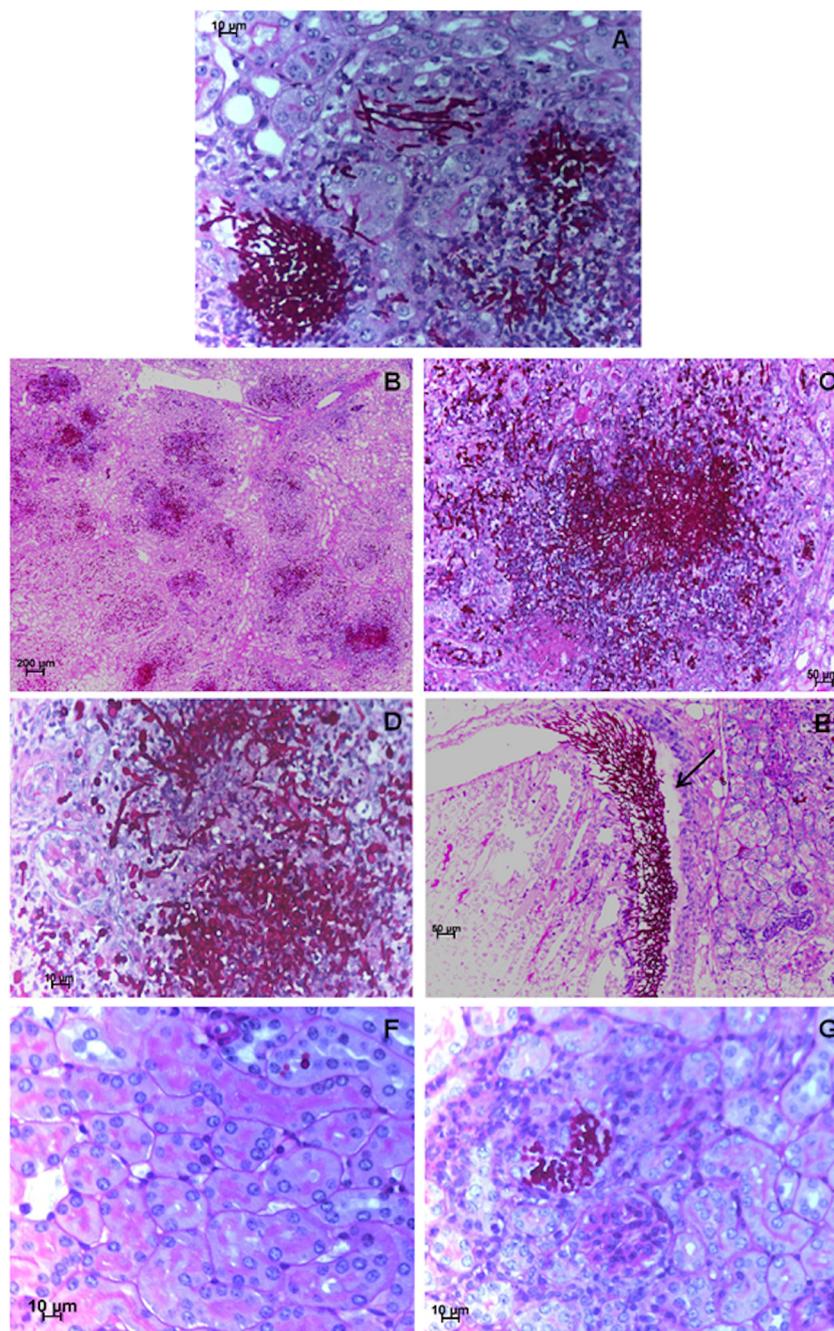
FIG 3 *C. albicans* mouse kidney colonization. Representative example of kidney histology slides of PAS-stained paraffin sections of kidneys recovered from mice infected with a *C. albicans*-resistant (*Ca*R) strain at day four postinfection and untreated (A) (×40 magnification), treated with 30 mg/kg/day of fluconazole (B to E) (×10, ×20, ×40 and ×20 magnification for the four panels, respectively), or treated with 30 mg/kg/day of fluconazole plus 20 mg/kg/day of ibuprofen (F and G) (×40 magnification). (E) Arrow shows a clear barrier to the progression of inflammatory leukocytes.

gal resistance induction and reversion. Ibuprofen in combination with fluconazole might play a relevant role in a therapeutic strategy for severe fungal infections.

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