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Antifungal activity of azole compounds CPA18 and CPA109 against azole-susceptible and -resistant strains of *Candida albicans*

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Objectives: In this study we investigated the *in vitro* fungistatic and fungicidal activities of CPA18 and CPA109, two azole compounds with original structural features, alone and in combination with fluconazole against fluconazole-susceptible and -resistant *Candida albicans* strains.

Methods: Antifungal activities were measured by MIC evaluation and time–kill studies. Azole binding analysis was performed by UV-Vis spectroscopy. Hyphal growth inhibition and filipin and propidium iodide staining assays were used for morphological analysis. An analysis of membrane lipids was also performed to gauge alterations in membrane composition and integrity. Synergism was calculated using fractional inhibitory concentration indices (FICIs). Evaluation of cytotoxicity towards murine macrophages was performed to verify selective antifungal activity.

Results: Even though their binding affinity to *C. albicans* Erg11p is comparable to that of fluconazole, CPA compounds are active against resistant strains of *C. albicans* with a mutation in *ERG11* sequences and/or overexpressing the ABC transporter genes *CDR1* and *CDR2*, which encode ATP-dependent efflux pumps. Moreover, CPA18 is fungistatic, even against the two resistant strains, and was found to be synergistic with fluconazole. Differently from fluconazole and other related azoles, CPA compounds induced marked changes in membrane permeability and dramatic alterations in membrane lipid composition.

Conclusions: Our outcomes suggest that CPA compounds are able to overcome major mechanisms of resistance in *C. albicans*. Also, they are promising candidates for combination treatment that could reduce the toxicity caused by high fluconazole doses, particularly in immunocompromised patients.

Keywords: drug development, fungicidal, time–kill assays, synergism, resistance

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Introduction

Disseminated candidiasis is a serious life-threatening infection that causes significant mortality and morbidity in critically ill patients. In particular, oropharyngeal infection is very common in immunosuppressed individuals, particularly in patients with AIDS, while deep-seated infections are frequent in neutropenic patients. The incidence of candidiasis has increased 5-fold over the past 10 years due to the widespread use of broad-spectrum antibiotics and the growing population of immunocompromised patients as a consequence of the use of immunosuppressive agents, radiotherapy and antitumoral drugs.[1–3]

The current guidelines for treating invasive candidiasis by the Infectious Diseases Society of America recommend the use of an azole derivative (e.g. fluconazole, voriconazole or itraconazole), an echinocandin or amphotericin B, or a combination of these agents.[4] Amphotericin B exhibits broad-spectrum fungicidal activity, yet severe side effects limit its clinical utility. Echinocandins (e.g. caspofungin) exhibit potent fungicidal activities against *Candida* species and have, in general, a better safety profile than both azoles and polyenes since they are not appreciable substrates, inhibitors or inducers of the cytochrome P450 or P-glycoprotein systems. However, all echinocandins have low oral bioavailability and high protein binding capacity. Concentrations

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of these drugs (or active metabolites) in CSF and urine are minimal, and thus limit their clinical utility in treating urinary and CSF infections.[5] In addition, the high cost of these drugs cannot be ignored. Although not devoid of side effects and drug–drug interactions, azole antifungal agents, particularly fluconazole, exhibit a good profile of tolerance in the range of doses recommended in invasive candidiasis and are still considered the drugs of choice to treat most *Candida* infections due to their broad-spectrum activities and improved safety profiles. They competitively inhibit the activity of 14α-sterol demethylase (Erg11p/Cyp51p), the key enzyme in sterol biosynthesis in yeast and moulds,[6] thus causing depletion of ergosterol and accumulation of lanosterol and other 14-methyl sterols. Since ergosterol maintains the integrity and fluidity of cell membranes, inhibition of Erg11p/Cyp51p results in the growth inhibition of fungal cells.[7] Azole antifungals are generally not fungicidal but fungistatic; moreover, their intensive clinical use for both therapy and prophylaxis has favoured the emergence of resistant strains.[8] In *C. albicans* this resistance is governed by several mechanisms, the most significant and intensively studied of which are probably the overexpression of genes encoding efflux pumps and/or up-regulation of *ERG11* or amino acid substitutions in Erg11p.[9]

For the reasons mentioned above, there is an urgent need for new antifungal agents exhibiting broad-spectrum activities, low toxicity and different mechanisms of action. In particular, identification of new therapeutically useful azoles that have a synergic effect and that do not exhibit cross-resistance to current antifungals might overcome drug resistance and/or reduce side effects associated with high doses of drugs.[10,11]

After a preliminary screening of the compounds from our chemical library,[12] we ranked them on the basis of their *in vitro* antifungal potency. Then we selected the two highest ranking derivatives, namely 1-(1-(biphenyl-4-yl)-3-(4-fluorophenyl)propan-2-yl)-1*H*-imidazole (CPA18) and 1-(1-(biphenyl-4-yl)-3-(5-chlorothiophen-2-yl)propan-2-yl)-1*H*-imidazole (CPA109) (Figure 1). Although structurally related to known azole antifungal agents, CPA18 and CPA109 are characterized by the presence of a backbone [a central 2-(imidazol-1-yl) propane and two aromatic portions in positions 1 and 3] that provides extensive flexibility to the molecules. Most importantly, they possess a peculiar activity profile (see below).

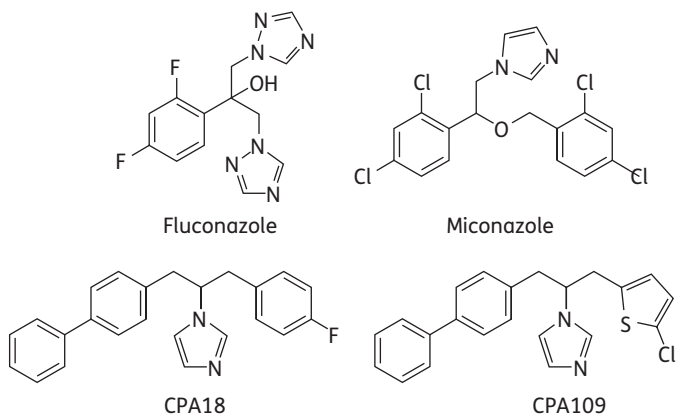

Figure 1. Chemical structures of fluconazole, miconazole, CPA18 and CPA109.

In this study we investigated the *in vitro* fungistatic and fungicidal activities of CPA compounds against fluconazole-susceptible and -resistant *C. albicans* strains in comparison with those of fluconazole and miconazole. As representative fluconazole-resistant strains, we selected the Darlington strain (ATCC 64124), in which the sensitivity of sterol biosynthesis to inhibition by fluconazole is greatly reduced due to mutations in *ERG11* sequences and which shows cross-resistance to other azoles,[13,14] and *C. albicans* ATCC MYA-574, overexpressing the ABC transporter genes *CDR1* and *CDR2*, which encode ATP-dependent efflux pumps.[15] The effects of tested compounds on membrane lipid composition and fungal morphology were also analysed in order to explain the different biological activities of CPA compounds with respect to the reference azole drugs. Finally, we also examined the antifungal activity of CPA compounds in combination with fluconazole.

Materials and methods

Chemicals

CPA compounds were prepared according to previously reported procedures.[12] Fluconazole and miconazole were purchased from Sigma–Aldrich (Milan, Italy). Stock solutions were prepared in pure water (for fluconazole) or DMSO (for miconazole and CPA compounds). Filipin and propidium iodide were obtained from Sigma–Aldrich (Milan, Italy) and dissolved in DMSO and water, respectively.

Fungal strains

The Darlington strain (ATCC 64124) and *C. albicans* ATCC MYA-574 were obtained from the ATCC-LGC Standards partnership, while the strain SC5314 was kindly provided by Professor W. A. Fonzi (Georgetown University, Washington, DC, USA). All strains were maintained on yeast extract (1%)/peptone (2%)/dextrose (2%) (YEPD) agar.

In vitro antifungal activity

The *in vitro* MICs of tested compounds were determined in 96-well microtest plates according to the guidelines suggested by the CLSI document M27-A3[16] using three separate plates each containing the same batch of antifungal compound. Microtitre plates containing 100 μL of 2-fold serial dilutions of the antifungal drugs in Roswell Park Memorial Institute (RPMI) 1640 medium (with L-glutamine, without glucose and $NaHCO_3$, buffered to pH 7.0 with 0.165 M MOPS) were inoculated with 100 μL of cells containing 0.5×10^3 to 2.5×10^3 yeast/mL. Antifungal concentrations, when reconstituted with the yeast suspensions, ranged from 0.03 to 16 mg/L for miconazole and CPA compounds, and from 0.125 to 64 mg/L for fluconazole. After inoculation, plates were incubated at 37°C in a non-CO_2 incubator and observed for the presence or absence of growth after 24 h. The MIC of azoles was read as the lowest concentration that produced a prominent decrease in growth (MIC_2: inhibition ≥50%) or an absence of growth (MIC_0: optically clear) compared with control cells containing only medium and vehicle.

Time–kill assay

Time–kill curves[17] were obtained for the susceptible strain SC5314 and the two azole-resistant strains, Darlington ATCC 64124 and *C. albicans* ATCC MYA-574. The starting inoculum was 10^6 cells/mL in liquid RPMI 1640 medium at 35°C into tubes (5 mL volume) without shaking. A concentration of 10 mg/L of each tested compound was used against all strains. Aliquots of 0.1 mL were removed from each test tube at

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defined timepoints (0, 2, 4, 6, 8 and 24 h) and the cfu count was determined by the serial dilution and plating method on YEPD plates in triplicate. Each experiment was performed at least three times.

Azole binding spectra determinations

Binding of azole antifungal agents to purified *C. albicans* Erg11p was performed as previously described[18] using split-cuvettes of 4.5 mm path length. Stock 100 mg/L solutions of CPA18, CPA109 and fluconazole were prepared in dimethylformamide (DMF) and were progressively titrated against 3 μM *C. albicans* Erg11p in 0.1 M Tris-HCl (pH 8.1) and 25% (w/v) glycerol at 22°C. Equivalent amounts of DMF were added to the P450-containing compartment of the reference cuvette. Difference spectra between 500 and 350 nm were determined after each incremental addition of azole. Each azole/Erg11p titration was performed in triplicate. Binding saturation curves were constructed from the change in absorbance between the spectral peak and the trough against the azole concentration. A rearrangement of the Morrison equation for tight ligand binding[19,20] was used to fit the data and calculate the dissociation constant (K_d). Tight binding is observed when the K_d for the ligand is similar to or less than the concentration of the Erg11p present.[21] Curve fittings of azole binding data were performed using the computer program ProFit (version 6.2; QuantumSoft, Zurich, Switzerland).

Azole interaction

The interaction[22] of tested azole compounds with fluconazole (CPA18 plus fluconazole and CPA109 plus fluconazole) was studied using a broth microdilution method in 96-well microtitre plates according to CLSI M27-A3 guidelines.[16] The initial concentration of yeast suspension in RPMI 1640 medium was 10^3 cfu/mL, and the final azole concentrations ranged from 0.005 to 0.08 mg/L. Plates were incubated at 35°C for 24 h. Experiments were performed in triplicate. To assess the interaction of combinations of drugs, the data obtained by visual reading were further analysed using the fractional inhibitory concentration index (FICI).[23] Synergy and antagonism were defined by an FICI of ≤0.5 and >4, respectively. An FICI >0.5 but ≤4 was considered to be the result of indifference.[24]

Morphological analysis

Hyphal growth of *C. albicans* SC5314 treated cells was induced using RPMI 1640 medium, supplemented with 2.5% heat-inactivated fetal calf serum, 20 mM HEPES, 2 mM L-glutamine and 16 mM sodium hydrogen carbonate (pH 7.0, Gibco-BRL). Stationary yeast cells were inoculated into a fresh pre-warmed medium at a density of 6×10^6 cells/mL in 96-well microtitre plates. Different concentrations of azoles (0.08, 0.04, 0.1 and 0.2 mg/L) were added to each well. After incubation at 37°C for 24 h, each microtitre plate was examined using an inverted microscope to monitor phenotypic modification and hyphae formation. To determine the extent of hyphal growth, a crystal violet staining assay was performed as previously described.[25]

Phase-contrast and fluorescence microscopy

Cells were examined with a Zeiss fluorescence microscope using a Zeiss Axiovert 200M. Images were acquired using the Axio-vision software package and processed using Adobe Photoshop 5.0.

Filipin staining assay

For visualization of sterol-rich domains, 10^4 cells/mL were grown in RPMI 1640 medium with or without 0.15 mg/L CPA18, CPA109 and fluconazole at 35°C for 8 h in disposable plastic tubes. Cells were harvested, washed with water, stained with 15.3 μM filipin in the dark for 30 s, washed three times in medium, mounted for microscopy and observed immediately by fluorescence microscopy using a 4′-6-diamidino-2-phenylindole ('DAPI') filter set.[26]

Staining with propidium iodide

Experimental cultures (10^4 cells/mL) were grown with or without 0.15 mg/L CPA18, CPA109 and fluconazole at 35°C for 24 h in disposable plastic tubes. Cells were harvested and washed with water and stained with 5 mg/L propidium iodide for 20 min at room temperature in the dark. Cells were then examined under phase-contrast and fluorescence microscopy with a ×20 lens using a rhodamine filter.

Microsomal membrane isolation

An overnight culture of *Candida* SC5314 was diluted to 1.5×10^4 cells/mL in yeast nitrogen base ('YNB') containing 0.05 mg/L CPA109, 0.05 mg/L CPA18, 0.05 mg/L fluconazole or only vehicle (as a control) and incubated with shaking (160 rpm) at 28°C for 10–14 h (exponential phase).

Spheroplasts from *Candida* SC5314 were prepared following a slight modification of the procedure reported by Li and Cutler.[27] Exponential-phase yeast cells were washed with 50 mM Tris-HCl pH 7.5 containing 0.25 M sucrose and 10 mM EDTA (disodium salt). Cells were pelleted by centrifugation at 1500 **g** for 10 min and suspended with the same buffer (2 mL/g of wet weight) containing Zymolase 20T at 80 U/g of wet weight of yeast cells. After incubation at 36°C for 60 min under continuous agitation, cells were pelleted by centrifugation at 1500 **g** for 30 min. Cells were osmotically shocked by resuspending them in 50 mM Tris-HCl pH 7.5 and cell debris was removed by centrifugation at 3000 **g** for 10 min. The mitochondrial pellet was eliminated by centrifugation at 15000 **g** for 20 min and the microsomal fraction was collected after centrifugation at 100000 **g** for 45 min.[28]

Lipid extraction and separation

Lipids were extracted following the procedure described by Quartacci *et al.*[29] Briefly, after the addition to cells of boiling isopropanol followed by chloroform:methanol (2:1, v/v) containing butylhydroxytoluol (50 mg/L) as an antioxidant, the solvent mixture was washed with 0.88% KCl and total lipids were fractionated into neutral lipid, glycolipid and phospholipid (PL) fractions on Sep-Pack cartridges (Waters, Milford, MA, USA). Cartridges were sequentially eluted with 20 mL of chloroform:acetic acid (100:1, v/v) for neutral lipids [sterol esters and free sterols (FSs)], 10 mL acetone and 10 mL of acetone:acetic acid (100:1, v/v) for glycolipids and 7.5 mL of methanol:chloroform:water (100:50:40, by volume) for PLs. Separation of individual lipids was performed by thin layer chromatography (TLC; Silica Gel 60, 0.25 mm thickness; Merck, Germany) with the following solvent mixtures: petrol ether:ethyl ether:acetic acid (80:35:4, by volume) for neutral lipids and chloroform:methanol:acetic acid:water (85:15:10:3.5, by volume) for PLs. After development, bands were located with iodine vapour and individual PLs were identified by chromatography with authentic standards. Quantitative analyses of PLs were performed using KH_2PO_4 as a standard in the presence of silica gel from TLC.

Sterol analysis

Sterols were separated and quantified by gas liquid chromatography as underivatized residue.[29] The sterol moiety dissolved in ethyl acetate was analysed with a Perkin-Elmer Sigma 2B gas chromatograph using a flame ionization detector and a 30 m×0.32 mm SPB-5 fused silica capillary column (Supelco). The operating conditions were a column

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temperature of 250°C, injector and detector temperatures of 280°C and N_2 was the carrier gas at 1 mL/min (split ratio 1:70). Ergosterol and lanosterol identification was made on the basis of the retention time relative to commercial standards. Cholestane was used as an internal standard and correction was made for differences in detector response.

Statistical analysis

Data were analysed using Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Results are expressed as mean ± SD. Statistical differences were evaluated by means of two-way ANOVA. A P value <0.05 was considered statistically significant. Each experiment was performed at least three times as stated. Treatments were performed in duplicate in each experiment.

Murine macrophage infection

Murine macrophage cell line J774A.1 was grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L D-glucose supplemented with 10% heat-inactivated fetal bovine serum, 4 mM L-glutamine, 50 U/mL penicillin G sodium and 50 mg/L streptomycin sulphate at 37°C under 5% CO_2 in 75 cm^3 flasks.

Aliquots of macrophages ($\sim 3\times10^4$ cells/mL) were dispensed into 175 cm^2 Falcon tissue culture flasks (and incubated at 37°C under 5% CO_2). After 3–4 days, yeast cells of *C. albicans* were resuspended in DMEM and added to the monolayer of macrophages (ratio 5:1) in the presence of azoles (MIC_2). The flasks were then incubated at 37°C under 5% CO_2. After 60 min non-ingested *Candida* were removed by several washes with sterile PBS. Infection was followed by time-lapse microscopy using an inverted phase-contrast microscope (Leica CTR6500). Images were captured every 10 min in an 8 h period. To monitor morphological modifications of *Candida* during macrophage infection, co-cultures with non-confluent macrophages were incubated for 5 h in Lab-Tek Chamber Slides (Nalge Nunc International) at 37°C with 5% CO_2 and then stained using the Hochtkiss–McManus method as reported previously.[30] Briefly, cells were stained with fuchsine and diluted light green dye.

Results

In vitro antifungal activity

The MICs of the four antifungal agents for the three reference strains of *C. albicans*, as determined by broth microdilution, are shown in Table 1. When tested against the fluconazole-susceptible strain (*C. albicans* SC5314), all the azole compounds showed strong antifungal activity. As expected, both *C. albicans* 64124 (strain with mutations in *ERG11* resistance mechanism) and *C. albicans* MYA-574 (strain with efflux pump overexpression) were resistant to fluconazole ($MIC_0>32.0$) and only scarcely susceptible to miconazole ($MIC_0>16.0$ and $MIC_0=5.0$, respectively). However, it should be noted that CPA compounds maintained their antifungal activity against both the fluconazole-resistant strains of *C. albicans*. In particular, CPA18 exhibited an MIC_0 of 2.4 for *C. albicans* 64124 and an MIC_0 of 0.4 for *C. albicans* MYA-574, while CPA109 exhibited an MIC_0 of 3.5 for *C. albicans* 64124 and an MIC_0 of 0.6 for *C. albicans* MYA-574.

Time–kill curves

The killing activity of the four antifungal agents against the three reference strains of *C. albicans* over a 24 h period is shown in Figure 2. When *C. albicans* SC5314 was treated with 10 mg/L CPA109, a fungistatic activity was observed and maintained throughout the 24 h period (Figure 2a), whereas a 10 mg/L fluconazole treatment exhibited fungistatic activity only during the first 6 h of growth, after which cells entered exponential growth phase. In contrast, both CPA18 (10 mg/L) and miconazole (10 mg/L) showed fungicidal activity against the same strain (after 24 h of incubation there was a 3 $\log_{10}$ decrease in cfu/mL).

As expected, the growth of both azole-resistant strains was unaffected by 10 mg/L fluconazole, while miconazole, CPA18 and CPA109 (10 mg/L) yielded a decrease of approximately 1 $\log_{10}$ cfu/mL of the growth of *C. albicans* 64124 throughout the first 6 h. However, after 24 h only the fungistatic activity of CPA18 was maintained, with a 0.5 $\log_{10}$ cfu/mL decrease (Figure 2c). When *C. albicans* MYA-574 was treated with either CPA109 (10 mg/L) or CPA18 (10 mg/L), a 1.3 $\log_{10}$ cfu/mL decrease was observed, whereas miconazole induced a decrease of 1.1 $\log_{10}$ cfu/mL. Surprisingly, after 24 h of treatment, CPA18 still gave a 1.3 $\log_{10}$ cfu/mL decrease and CPA109 a 0.3 $\log_{10}$ cfu/mL decrease (Figure 2b). In contrast, after 24 h of incubation with fluconazole or miconazole both *C. albicans* MYA-574 and *C. albicans* 64124 reached a growth comparable to the control (*Candida* with only vehicle).

Table 1. *In vitro* antifungal activity of CPA compounds, fluconazole (FLC) and miconazole (MCZ) against *C. albicans* strains

		MIC (mg/L)	
	Compound	MIC_2	MIC_0
C. albicans SC5314	CPA18	0.035	0.09
	CPA109	0.095	0.19
	FLC	0.080	0.24
	MCZ	0.030	0.15
C. albicans MYA-574	CPA18	0.07	0.40
	CPA109	0.12	0.60
	FLC	>16.0	>32.0
	MCZ	1.75	5.0
C. albicans 64124	CPA18	0.14	2.4
	CPA109	0.24	3.5
	FLC	>16.0	>32.0
	MCZ	5.5	>16.0

Azole binding affinities to C. albicans Erg11p

CPA18, CPA109 and fluconazole bind to *C. albicans* Erg11p producing type II difference spectra (Figure 3a), characteristic of azole-inhibiting drugs. This was characterized by a peak at 432 nm and a trough at 412 nm for CPA18 and CPA109, and a peak at 430 nm and a trough at 412 nm for fluconazole, indicative of the triazole ring N-4 nitrogen coordinating as the sixth ligand of the haem ferric ion.[31,32] Azole-binding saturation curves (Figure 3b) indicate that all three azoles tightly bind to *C. albicans* Erg11p, as the Morrison equation gave the best fit to the data,[19,20] whereas both the Michaelis-Menten and Hill equations exhibited poor fits. The Morrison equation can accurately determine K_d values as low as one-hundredth of the

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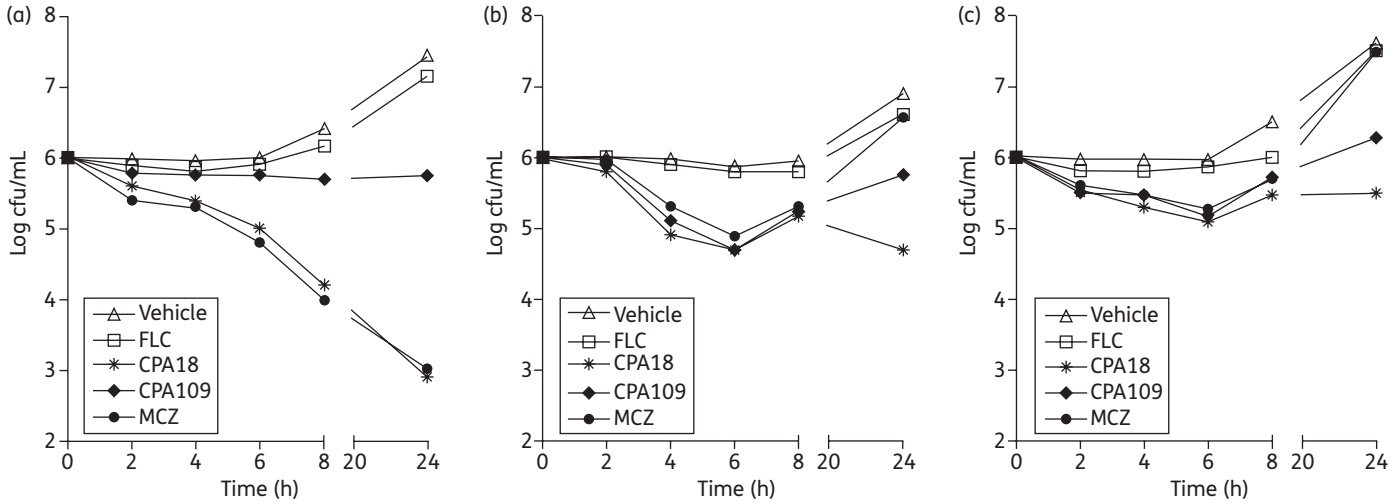

Figure 2. Killing curves. Comparison of the fungicidal or fungistatic activities of CPA18 (asterisks), CPA109 (filled diamonds), fluconazole (FLC; open squares) and miconazole (MCZ; filled circles), all at 10 mg/L, against (a) *C. albicans* SC5314, (b) *C. albicans* MYA-574 and (c) *C. albicans* 64124.

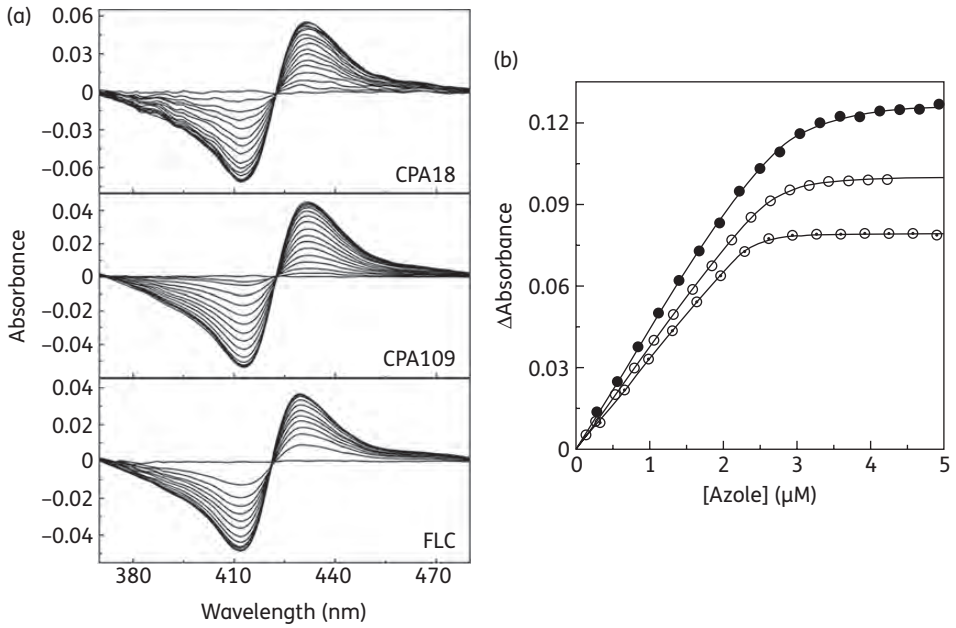

Figure 3. Binding properties of CPA18, CPA109 and fluconazole (FLC) with *C. albicans* Erg11p. (a) Type II difference spectra produced when CPA18, CPA109 and fluconazole were titrated against 3 μM *C. albicans* Erg11p. (b) Azole saturation curves constructed from the type II difference spectra for CPA18 (filled circles), CPA109 (open circles) and fluconazole (open circles with bullet points). One representative determination (from three replicates) is shown for each azole.

enzyme concentration, in this instance as low as 0.03 μM. CPA18, CPA109 and fluconazole have K_d values close to this limit at 56 ± 4, 36 ± 12 and 27 ± 6 nM, respectively. This suggests that all three azoles bind unimpeded to the haem ferric ion through the azole ring nitrogen atom.

Inhibition of hyphae formation

The effect of CPA18, CPA109 and fluconazole on *C. albicans* SC5314 hyphae formation is displayed in Figure 4. The vehicle-treated control showed extensive hyphae formation, whereas when azole compounds were present, the formation of hyphae was severely inhibited in a concentration-dependent manner. However, even though the MIC_2 values of CPA18, CPA109 and fluconazole are comparable, there is a significant difference in their ability to inhibit hyphae elongation. Hyphae formation was more severely inhibited by CPA18 and CPA109 than fluconazole. Within the range of drug used, this inhibition was particularly significant in cells treated with CPA18 (28% and 83% at 0.04 and 0.20 mg/L, respectively; Figure 4).

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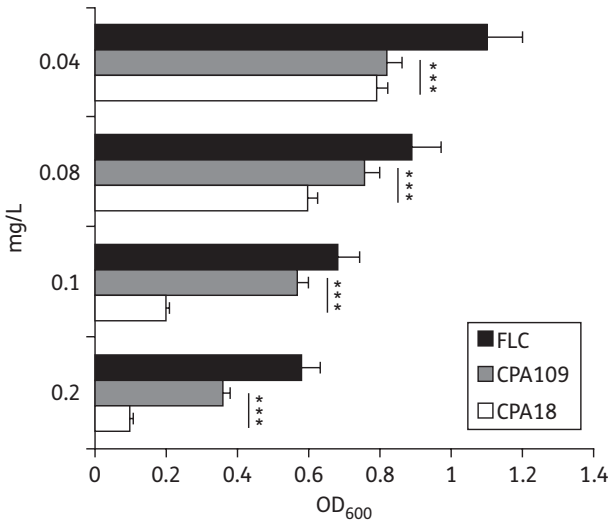

Figure 4. Hyphal formation. Inhibition of hyphal development of *C. albicans* SC5314 induced by CPA compounds was statistically significant at all concentrations tested compared with fluconazole (FLC) (***$P<0.001$, as determined by two-way ANOVA). OD_{600}, optical density at 600 nm.

Effect of azoles on membrane polarization during hyphal growth

Morphological differences of *Candida* induced by CPA compounds with respect to fluconazole prompted us to investigate their effect on the localization of sterols by using filipin, a fluorescent polyene antibiotic that specifically binds membrane sterols.[26] In untreated *C. albicans* SC5314 (controls), a strong staining with filipin was detected at the tip of hyphal growth. *Candida* cells treated with 0.15 mg/L fluconazole displayed an intense filipin staining at the actively growing hyphal tips of most of the cells that were still forming germ tubes, indicating that fluconazole does not prevent the formation of sterol-rich domains (SRDs) in *Candida*. In contrast, both CPA18 and CPA109 (0.15 mg/L) prevented SRD formation and cells showed uniform filipin staining throughout the membrane, suggesting that sterol composition and/or sterol distribution is perturbed differently in the presence of CPA compounds or fluconazole (Figure S1, available as Supplementary data at *JAC* Online).

Effect of azoles on membrane integrity

To detect the increased permeability of cell membrane after antifungal treatment, *Candida* SC5314 cells were incubated with propidium iodide, a membrane-impermeable dye. After a 24 h treatment with 0.15 mg/L fluconazole, *C. albicans* initiated germination and remained impermeable to propidium iodide, while CPA18-treated cells (0.15 mg/L) did not germinate and most of yeasts became completely permeable to propidium iodide. Cells treated with CPA109 (0.15 mg/L), though showing an intense red colour, exhibited abnormal germ tube formation. Data not shown.

Effect of azoles on membrane lipid composition

In order to determine the effects of azole treatments on the lipid composition of *Candida* SC5314 membranes, cells were grown in YEPD containing 0.05 mg/L CPA109, 0.05 mg/L CPA18, 0.05 mg/L fluconazole or only vehicle (as a control), and FSs and PLs were analysed in microsomal membranes extracted from control and treated cells. All the azole treatments resulted in a dramatic reduction in the ergosterol content of *C. albicans* membranes (Table 2); indeed, ergosterol represented ~13% of total lipids in control membranes, whereas ergosterol represented only ~1% and 0.3% after CPA and fluconazole treatments, respectively. Correspondingly, an increase in lanosterol content was found in *C. albicans* membranes after all azole treatments, the highest level being observed with fluconazole (Table 2). Overall, changes in lipid composition induced by CPA drug treatments resulted in a remarkable reduction of the FS/PL molar ratio, whereas this ratio remained almost unaltered after the treatment of cells with fluconazole (Table 2). Moreover, all azole treatments caused a general rearrangement of PL composition of *C. albicans* membranes (Table 3). Indeed, all treatments resulted in a decrease in the two main (zwitterionic) PLs, namely phosphatidylcholine (PC) and phosphatidylethanolamine (PE), and in an increase in all negatively charged PLs [i.e. phosphatidic acid (PA), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidylglycerol (PG)], with the only exception being PG in fluconazole-treated cells, which did not change with respect to

Table 2. FS/PL molar ratio and ergosterol and lanosterol content (% of total lipids) of microsomal membranes of *C. albicans* untreated and treated with CPA18, CPA109 and fluconazole (FLC)

Parameter	Control	CPA18	CPA109	FLC
FS/PL	1.21	0.81	0.57	1.31
Ergosterol	13.1 ± 0.9	1.3 ± 0.02	0.9 ± 0.03	0.3 ± 0.04
Lanosterol	traces	2.6 ± 0.28	3.1 ± 0.21	3.9 ± 0.39

Results are the means of three independent experiments each analysed twice ± SD ($n=3$).

Table 3. PL composition (mol%) and PC/PE molar ratio of microsomal membranes of *C. albicans* untreated and treated with CPA18, CPA109 and fluconazole (FLC)

PL	Control	CPA18	CPA109	FLC
PC	52.5 ± 0.8	35.5 ± 2.1	32.5 ± 2.5	39.1 ± 1.4
PE	30.8 ± 0.9	27.3 ± 3.1	23.8 ± 0.9	26.0 ± 2.3
PS	6.2 ± 0.7	10.6 ± 0.7	11.7 ± 0.3	12.8 ± 0.9
PI	3.8 ± 0.2	9.2 ± 0.9	14.8 ± 1.7	11.1 ± 1.0
PG	3.3 ± 0.9	7.9 ± 0.4	8.2 ± 0.6	3.1 ± 0.7
PA	3.4 ± 0.9	9.4 ± 1.2	9.1 ± 0.9	8.0 ± 1.8
PC/PE	1.70	1.30	1.37	1.50

Results are the means of three independent experiments each analysed twice ± SD ($n=3$).

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the control. Moreover, similar increases were observed for PA and PS levels after the different treatments, whereas a much greater increase in PI content was found when cells were treated with CPA109. Taken together, changes in membrane lipid composition in azole-treated *C. albicans* cells resulted in lowered PC/PE molar ratios, with CPA treatments inducing a more severe reduction.

Effect of antifungal agents on macrophage infection

The ability of *C. albicans* SC5314 treated with azoles to grow inside host cells was tested by following macrophage infection for 8 h by time-lapse microscopy. To determine whether the different azoles had an effect on yeast cell morphology during macrophage infection, co-cultures were fixed at different time-points and stained (Figure S2, available as Supplementary data at *JAC* Online). CPA18 had no cytotoxic effect towards murine macrophages up to a concentration corresponding to MIC_0 (Figure S2A, available as Supplementary data at *JAC* Online). *Candida* hyphae started to disrupt macrophages 3 h after infection (Figure S2B, available as Supplementary data at *JAC* Online), while *Candida* treated with fluconazole at MIC_2 still produced germ tubes that disrupted macrophages (Figure S2C, available as Supplementary data at *JAC* Online). In contrast, yeast cells treated with CPA18 at MIC_2 did not produce germ tubes and were phagocytized and killed by macrophages (Figure S2D, available as Supplementary data at *JAC* Online).

Interaction of CPA18 and CPA109 with fluconazole

An MIC-like assay was then performed on *Candida* SC5314 for the two CPA azoles in the presence of subinhibitory concentrations of fluconazole. As shown in Table 4, the median MICs of CPA18 and CPA109 decreased significantly when used in combination with 0.015, 0.020 or 0.030 mg/L fluconazole with FICIs ranging from 0.23 to 0.47.

Positive interactions were confirmed by time–kill curves (data not shown). While fluconazole (10 mg/L) did not affect the growth of *C. albicans* SC5314 over 24 h of incubation, treatment with 2.5 mg/L CPA18 in combination with 3 mg/L fluconazole resulted in a 1.6 $\log_{10}$ cfu/mL decrease, comparable to that of 10 mg/L CPA18 alone.

Discussion

Therapy of fungal infections, particularly in the expanding population of immunocompromised persons, is a challenging task and there is a pressing need for new antifungal agents or therapies endowed with broad-spectrum activities and able to overcome drug resistance and/or reduce side effects associated with high doses of drugs. As a matter of fact, numerous attempts have been made to develop novel effective and safer antifungal agents with original mechanisms of action.

After screening a number of compounds from our in-house chemical library, we selected two novel azole derivatives, CPA18 and CPA109, and examined their potential antifungal activity, alone and in combination with fluconazole.

Although MICs of CPA compounds against the fluconazole-susceptible strain *C. albicans* SC5314 are comparable to those of known azole antifungal drugs, namely fluconazole and miconazole, their activity was maintained even against two selected azole-resistant strains (*C. albicans* 64124 and *C. albicans* MYA-574).

The fungicidal activity is another noteworthy feature of CPA compounds. In fact, azoles commonly used to treat systemic infections are known to be only fungistatic against *Candida* species,[33] since their inhibitory activity of yeast 14α-sterol demethylase is reversible. In contrast, the killing activity of CPA18 was remarkable and fast on azole-susceptible as well as -resistant strains. Miconazole, a representative of first-generation antifungal azoles, displayed a comparable action on the azole-susceptible strain, but its activity was completely lost in both azole-resistant strains. Also, CPA18 did not affect murine macrophage viability even at high concentrations (MIC_0), thus exhibiting a selective antifungal activity.

As mentioned above, CPA compounds share common structural features with the azole class of antifungal agents, yet possess novel and peculiar characteristics. In fact, different from fluconazole, the antifungal properties exhibited by CPA compounds are not ascribable to intrinsic differences in binding affinity to *C. albicans* Erg11p, as all three azoles had K_d values <0.06 μM. Instead, the increased antifungal potency of CPA compounds could be due to secondary activities/actions within *C. albicans* cells not shared by fluconazole. To gain insight into the potential mechanism of action of CPA compounds, we examined whether CPA18- and CPA109-treated cells displayed distinctive morphological features compared with fluconazole-treated cells. Since the morphological switch from yeast cells to hyphae is an important virulence factor in *C. albicans*,[34] we evaluated the proficiency of tested derivatives to inhibit hyphae formation. Untreated yeast *C. albicans* cells were able to form extensive hyphal branching, whereas the treatment of yeast cells with different concentrations of azole compounds hampered or completely blocked germ tube formation in a concentration-dependent manner. This inhibition was more evident in the case of CPA18 treatment, followed by CPA109 and fluconazole, notwithstanding the nearly identical MIC values.

To determine whether the different effect on morphology correlated with different membrane properties, we investigated the likely presence in the membrane of sterol-rich domains by filipin staining[26] and analysed the content of FSs, in particular ergosterol and lanosterol, in isolated membranes. Although azole-treated *Candida* had a lower ergosterol and a higher lanosterol concentration, the FS/PL ratios and the distribution of sterols within the membranes were notably different depending on the azole used. Indeed, in fluconazole-treated *Candida* cells there is a polarization of sterols at the actively growing hyphal

Table 4. *In vitro* azole (mg/L) interactions as determined by non-parametric methods

	FLC+CPA18	FICI	FLC+CPA109	FICI
MIC_2	0.015+0.010	0.47	0.020+0.015	0.38
MIC_0	0.030+0.020	0.40	0.030+0.020	0.23

FICI values are shown as arithmetic means of three independent experiments. Synergy was defined as an FICI ≤0.5, antagonism was defined as an FICI >4.0 and indifference was defined as an FICI >0.5 to 4.

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tips that does not inhibit the formation of SRDs. In contrast, CPA compounds, especially CPA18, disrupted this polarization effect, with uniform filipin staining observed throughout the cell membrane. Moreover, the FS/PL ratio remained almost unaltered after fluconazole treatment, whereas it remarkably decreased after CPA treatments. These data suggest that CPA compounds and fluconazole affect sterol biosynthesis and distribution differentially in *C. albicans*. Moreover, the proportion of different PLs in isolated *Candida* membranes dramatically changed after azole treatments, with all the treated samples showing a strong decrease in the zwitterionic PLs, PC and PE, and a concomitant increase in the negatively charged ones. In particular, a greater decrease in the PC/PE ratio was observed for CPA-treated samples than for the fluconazole-treated one and a significant increase in PG content was observed only for the CPA-treated samples (Table 3). As far as permeability is concerned, *Candida* did not incorporate propidium iodide inside the cell after treatment with fluconazole, as previously reported.[35] In contrast, most of the yeast cells became completely permeable to propidium iodide after exposure to CPA18.

The data regarding the different effects of CPA compounds on *Candida* cell morphology and permeability compared with those observed with fluconazole could be correlated to the observed different changes in membrane lipid composition. Indeed, membrane properties and functions are regulated by PL composition, in particular by an appropriate balance between bilayer-forming and non-bilayer-forming lipids and between anionic and zwitterionic lipids, and by the ratio between FSs and PLs, as different sterols specifically influence packing of PLs in membrane bilayers.[36–40] The status of membrane lipids, regulating intracellular accumulation of azoles by influencing both passive diffusion and efflux pump activity,[41] has been correlated to the azole resistance of *Candida*, although no typical lipid composition could be established that could be directly linked to azole susceptibility or resistance.[42] In addition, sterol modifications were found to mechanically influence membrane properties and hence fungal viability in yeasts.[43–45] In the present case, the remarkable decrease in the FS/PL and PC/PE ratios and the stronger increase in the proportion of charged lipids, particularly of PG, observed in membranes of CPA-treated cells with respect to those of fluconazole-treated cells could determine an increased disorder of the membrane lipid bilayers or phase separation and therefore altered membrane properties and functioning.

Finally, our experiments evidenced a significant *in vitro* interaction between CPA18 and fluconazole against *C. albicans* as well as a potent fungicidal synergism.

In conclusion, the current study reports on the antifungal properties of two novel azole compounds, CPA18 and CPA109, whose mechanism of action goes beyond the binding affinity to *C. albicans* Erg11p. As a matter of fact, different from known antifungal azoles, CPA compounds are active against resistant strains of *C. albicans* with a mutation in *ERG11* sequences and/or overexpressing the ABC transporter genes *CDR1* and *CDR2*, which encode ATP-dependent efflux pumps. Moreover, CPA18 is fungistatic, even against the two resistant strains.

These outcomes, together with the observation that the activity of fluconazole can be enhanced by CPA18, suggest an important alternative approach to overcome the high incidence of resistance developed during therapy and the toxicity induced by high fluconazole doses.[46,47] However, the possibility of using CPA18 alone or in combination with other azole antifungals *in vivo* requires further investigation, as well as a thorough elucidation of the underlying mechanism of action.

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Transparency declarations

None to declare.

Supplementary data

Figure S1 and Figure S2 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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