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A novel zinc-chelating compound has antifungal activity against a wide range of *Candida* species, including multidrug-resistant *Candida auris*

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Objectives: In recent years, the incidence of invasive fungal infections has increased, resulting in considerable morbidity and mortality, particularly among immunocompromised individuals. Potential challenges in treating these infections with the few existing antifungal agents highlight the urgency of developing new ones. Here, we evaluated six alkyl polyamine compounds (APCs), not previously reported as antifungal drugs to our knowledge, that could deprive fungi of essential transition metals.

Methods: The APC with confirmed antifungal activity against *Candida* spp. was analysed by using transcriptomics, followed by metal-addition experiments, mass spectrometric analyses and intracellular zinc quantification with a fluorescent probe.

Results: A cyclic APC with three pyridylmethyl groups, APC6, had high antifungal activity against a wide range of *Candida* species, including MDR *Candida auris*. We conclusively demonstrated that APC6 was able to capture zinc within fungal cells. APC6 not only exhibited activity against *C. auris* as a single agent but also enhanced the efficacy of an azole antifungal agent, voriconazole, *in vitro* and *in vivo*. APC6 disrupted the biofilms formed by *Candida* species.

Conclusions: This zinc-chelating compound has potential as an antifungal agent, and the control of zinc levels in *Candida* species could be a powerful approach to treating drug-resistant candidiasis.

Introduction

In recent years, the incidence of invasive fungal infections has increased, leading to considerable morbidity and mortality, particularly among immunocompromised individuals.[1] The socioeconomic impact of these infections is substantial, with a burden exceeding $7.2 billion annually in the USA as of 2017.[2] Addressing these infections has become a global imperative. Treatment of invasive fungal infections is currently limited to three primary classes of drugs: polyenes, azole, and echinocandins. Polyenes target the membrane ergosterol itself, whereas azoles block ergosterol synthesis by inhibiting lanosterol 14-α-demethylase Erg11.[3,4] Echinocandins block the synthesis of 1,3-β-glucan of the fungal cell wall by inhibiting 1,3-β-D-glucan synthase.[3,4] However, the emergence of fungal resistance to these agents has become a widespread and serious problem worldwide. Bloodstream infections by MDR *Candida auris* have been reported in the USA, Europe, South America, South Africa and India. Therefore, since 2022 the WHO has designated *C. auris* as the priority fungal pathogen, emphasizing the need for its identification and management.[5] The *C. auris* strain AR-0389 is known for its high drug resistance due to an *ERG11* mutation (Y132F) and overexpression of the drug efflux transporter gene *CDR1*.[6,7] The potential difficulty in treating invasive fungal infections with existing antifungal agents makes the development of new antifungal agents an urgent matter.

Nutritional immunity is an inherent defensive system of the host designed to withhold essential micronutrients such as iron and zinc from microbes; the mechanism of nutritional immunity has recently been identified as a promising pharmacological target.[8] Zinc plays a crucial role in fungal pathogenicity.[9,10] It is an

indispensable trace metal, and about 9% of eukaryotic proteins are zinc metalloproteins; zinc confers structural stability on proteins and acts as an enzyme cofactor.[11] As various eukaryotic transcription factors require zinc,[12] the acquisition of zinc is vital for fungal proliferation and pathogenicity.[13–16] Zinc depletion leads to a decline in fungal growth and biofilm formation.[9,10] The potential efficacy of stringent zinc restriction by zinc-chelating compounds—even against pathogens resistant to current pharmaceutical agents—has been emphasized.[17,18]

In this study, we selected six alkyl polyamine compounds (APCs) that have not yet been evaluated as potential antifungal drugs, and we searched for compounds sequestering transition metals essential for fungal development. We identified a cyclic APC with three pyridylmethyl groups, APC6, that is highly effective against a wide range of *Candida* species [*Candida albicans*, *Candida glabrata* (*Nakaseomyces glabrata*), *Candida krusei* (*Pichia kudriavzevii*), *Candida parapsilosis* and even *C. auris*, which includes MDR strains].[19] Through transcriptomic analyses of *C. albicans* cells treated with APC6, along with experiments involving APC6 and transition metals, MS and intracellular zinc monitoring, we elucidated that APC6 exerted its antifungal activity by chelating zinc and depriving *Candida* cells of this essential metal. APC6 had low cytotoxicity to a human cell line, implying high specificity of the antifungal activity.

Materials and methods

MICs of antifungal compounds

The MICs were determined in accordance with the CLSI (M27-Ed4) methodology.[20] Antifungal susceptibility plates were prepared with a range of concentrations of each compound from 0.03125 to 2 mg/L in Roswell Park Memorial Institute (RPMI) 1640 medium buffered with MOPS. Growth inhibition was evaluated after 24 h of incubation at 35°C. The MIC was defined as the lowest concentration of the compound that resulted in at least 50% inhibition of yeast growth.

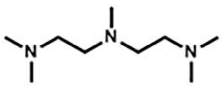
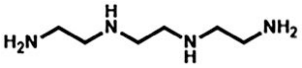
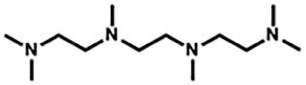
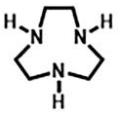
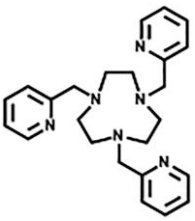
Quantitative real-time RT–PCR (RT–qPCR)

Total RNA was extracted by using beads and Isogen (Nippon Gene), an RNeasy Mini Kit (QIAGEN) and RNase-free DNase I (QIAGEN) in accordance with the manufacturers' instructions. Total RNA was reverse transcribed by using a ReverTraAce qPCR RT Master Mix kit (Toyobo). RT–qPCR was performed in an Mx3000P real-time PCR system (Agilent) by using SYBR Premix ExTaq (Takara Bio Inc.). Primer sequences are listed in Table S1 (available as Supplementary data at *JAC-AMR* Online).

Measurement of intracellular free zinc levels by using Zinbo-5

Zinbo-5 (Santa Cruz Biotechnology) is a fluorescent probe that specifically binds to Zn^{2+} and is used to measure intracellular Zn^{2+} content. *C. auris* AR-0389 cells were grown overnight in yeast extract-peptone-dextrose (YPD) broth [1% Bacto yeast extract (Difco), 2% Bacto peptone (Difco) and 2% glucose; pH 6.8], pelleted, washed three times with PBS, and resuspended in PBS to obtain an OD_{600} of 2.0. The cells were incubated with various concentrations of APC6 or *N*,*N*,*N*′,*N*′-tetrakis(2-pyridylmethyl) ethylenediamine (TPEN; TCI) in 96-well plates at 30°C for 6 h; this was followed by incubation with 5 μmol/L Zinbo-5 for 30 min. Fluorescence was measured in a microplate reader (excitation at a wavelength of 355 nm, emission at 485 nm).

Table 1. MICs of APC compounds (APC1–6) for *C. albicans* SC5314

Compound	Structure	MIC (mg/L)
APC1		>10
APC2		>10
APC3		>10
APC4		>10
APC5		>10
APC6		0.5

Growth-inhibition assay

C. auris AR-0389 cells (1×10^3 cfu/mL) were incubated in 5 mL of RPMI-MOPS medium at 35°C with gentle agitation in the presence of various concentrations of voriconazole (TCI), APC6 or both. At each timepoint, a 15 μL aliquot was removed, serially diluted (5-fold) in PBS, and each dilution was plated on YPD agar plates. The plates were incubated at 30°C for 24 h and colonies were counted. Cells treated with DMSO (1%, vol/vol) served as a control.

Galleria mellonella survival assay

A total of 72 *G. mellonella* larvae were randomly allocated to four groups. The last right foreleg ventral aspect of each larva was injected with 10 μL of *C. auris* AR-0389 suspension (2×10^5 cfu/larva) containing a drug or vehicle: APC6 (1.5 μg/larva), voriconazole (2 μg/larva), APC6 and voriconazole (1.5 μg/larva and 2 μg/larva, respectively) or control (PBS). The number of surviving larvae in each group was calculated at the same time every day for 7 days.

Results

An APC is a potent broad-spectrum antifungal agent

APCs have tandemly repeated ethylenediamine units and are able to chelate divalent metal ions. They can be cyclized to provide rigid geometries, leading to the formation of more stable metal complexes. Pyridylmethyl groups of APCs can be modified at the nitrogen atoms to enhance their metal-binding abilities. The positive charge of APCs might interact with the negative charge of fungal membrane surfaces. On the basis of our knowledge of coordination chemistry and mycology,

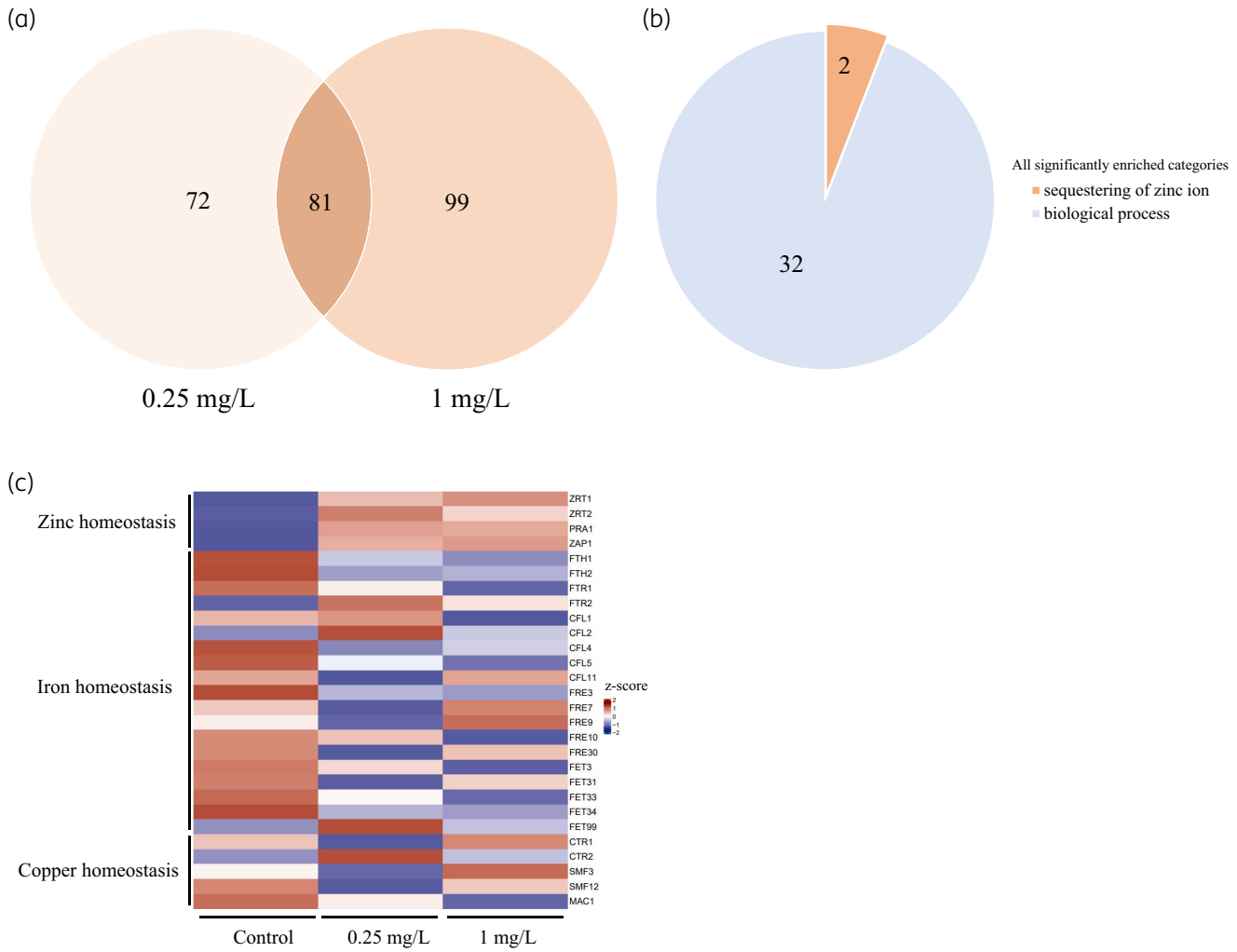


Figure 1. Transcriptional analysis of APC6-treated *C. albicans*. Cells at an initial concentration of 1×10^7 cells/mL were left untreated or were treated with APC6 (0.25 or 1.0 mg/L) in RPMI-MOPS medium at 35°C for 6 h. RNA was extracted, and genes that showed at least a 2-fold difference in transcript levels compared with controls were defined as DEGs. (a) DEGs up-regulated by APC6 at 0.25 mg/L (0.5× MIC; left circle) and 1 mg/L (2× MIC; right circle). (b) GO analysis of the 81 DEGs up-regulated by both concentrations of APC6. Treatment with APC6 significantly increased two GO terms (total of 34 genes), one of which was 'sequestering of zinc ion.' (c) Heatmap of the transcript levels of genes associated with the maintenance of zinc, iron and copper homeostasis under APC6 treatment.

we selected a series of linear and cyclic APCs with basic structures (Table 1) and assessed their antifungal activities. We measured the MIC of each APC for *C. albicans* SC5314 (Table 1). Linear APC1–APC3 and cyclic APC4 and APC5 did not inhibit *C. albicans* growth. To enhance both metal-binding ability and hydrophobic interactions with the fungal lipophilic membrane, we chemically added three pyridylmethyl groups to the backbone of APC4, which had the most rigid structure among these APCs, and obtained APC6. APC6 had the lowest MIC (0.5 mg/L; 1.25 μmol/L). We assessed the APC6 antifungal activity against 19 strains of *C. auris* and one strain each of *C. glabrata*, *C. krusei* and *C. parapsilosis* (Table S2). APC6 was effective across a wide range of *Candida* species and strains, with MICs of 0.25 mg/L (*C. glabrata*) or 0.5 mg/L (other species tested), including *C. auris* AR-0389 strain (Table S3).

APC6 up-regulates expression of a Zn transporter gene

To elucidate the effect of APC6 on fungal cells at the transcriptional level, we analysed the transcriptome of *C. albicans* SC5314. We obtained a total of 279457074 reads. We compared the transcripts per million (TPM) of SC5314 treated with APC6 at 0.25 mg/L (0.5× MIC) or 1 mg/L (2× MIC) against a control group treated with PBS (Table S4). Genes showing at least a 2-fold difference in expression (up or down) were designated as differentially expressed genes (DEGs). At 0.5× MIC, 153 DEGs (left circle, Figure 1a) were up-regulated and 218 were down-regulated (371 DEGs in total). At 2× MIC, 180 DEGs (right circle, Figure 1a) were up-regulated and 2 were down-regulated. We identified 81 DEGs up-regulated in both groups (Figure 1a) and applied gene ontology (GO) analysis to these genes. APC6 significantly increased two GO terms (Figure 1b, Table S5), one of

which included genes encoding a Zn transporter (*ZRT1*) and a Zn scavenger (*PRA1*).

The heatmap shows the gene groups involved in the homeostasis of zinc, iron and copper, under control or APC6 treatment (Figure 1c); these genes have been extensively studied for their importance in fungal growth. To validate the transcriptome data (Table S4), we used RT–qPCR. *C. albicans* SC5314 and *C. auris* AR-0389 were cultured in RPMI-MOPS medium and treated with APC6 at 0.5× MIC or 2× MIC. Gene expression levels were normalized to that of *ACT-1* (*C. albicans*) or *B9J08_000486* (*C. auris*). The transcript levels of *ZRT1*, *ZRT2* and *PRA1* were significantly up-regulated by APC6, whereas those of iron- and copper-related genes were not altered (Figure 2a). Among the orthologous genes in *C. auris* corresponding to *ZRT1* (*B9J08_000004*), *ZRT2* (*B9J08_000003* and *B9J08_003657*) and *PRA1* (*B9J08_002992*), the genes corresponding to *ZRT1* and *PRA1* were significantly up-regulated (Figure 2b). The up-regulation of zinc transporter expression is reported to occur due to zinc starvation,[21–23] and considering these results, it was suggested that APC6 treatment caused defects in zinc utilization in *Candida* species.

APC6 exerts its antifungal activity by preferentially capturing zinc

We added metal ions (Fe^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Mg^{2+} or Ca^{2+}) that constituted the yeast nitrogen base medium considered essential for fungal growth. We then tracked changes in APC6 MIC in the SC5314 and AR-0389 strains and determined the concentration of metal ions at which the MIC increased from 1.25 μmol/L (0.5 mg/L) to at least 5 μmol/L (2 mg/L) (Table 2). Upon the addition of various metals in 10-fold serial dilutions to the medium, a sharp increase in the APC6 MIC was observed in the presence of zinc or copper at low concentrations (1.25–12.5 μmol/L); the amount of the next ion, Mn^{2+}, that had the same effect was about 10 times those of Zn^{2+} and Cu^{2+}. These data suggested that APC6 interacted with Zn^{2+}, Cu^{2+} or both. Considering the aforementioned details, the effect of APC6 on the expression of genes for zinc transporters, but not copper transporters, indicated that APC6 exerted its antifungal activity by preferentially capturing zinc, rather than copper, in fungal cells.

To assess the binding of APC6 to zinc ions *in vitro*, we mixed APC6 and $ZnSO_4$ and analysed the mixture by electrospray ionization (ESI) MS. We detected a positive ion peak cluster at *m/z* 233.09 (Figure S1a and b), the calculated isotope pattern of which was identical to that of a 1:1 Zn^{2+}:APC6 complex (Figure S1b). The ESI mass spectra indicated the formation of a stable zinc complex formulated as $[Zn(APC6)]^{2+}$ (Figure S1c). The electron-donating nitrogen atoms of three pyridines and three alkyl amines of APC6 can cooperatively form a six-coordinate geometry around a Zn ion. The stability constant, log*K*, of the Zn complex with APC6 is 17.25,[24] which is higher than the 15.94 of the Zn complex with EDTA, a metal-chelating hexadentate compound.[25] The high stability constant in ESI mass spectral measurements strongly suggests that APC6 stably coordinates a Zn ion in solution.

Antifungal activity of APC6 is mediated by capturing intracellular zinc ions in fungi

The *C. auris* AR-0389 strain was exposed to APC6, and intracellular free zinc levels were monitored with the fluorescent probe Zinbo-5, which specifically binds to free zinc ions. A concentration-dependent decrease in intracellular zinc levels was observed upon administration of APC6: by 8.3% at 0.5× MIC (0.625 μmol/L), 9.5% at MIC (1.25 μmol/L) and 15% at 2× MIC (2.5 μmol/L) (Figure 3). As a positive control, we used TPEN, a chelating agent that sequesters intracellular zinc ions.[26] TPEN tended to sequester zinc ions more robustly than APC6, yet the antifungal activities (MICs) of APC6 and TPEN against *C. auris* AR-0389 were equivalent. The MIC of TPEN for both *C. albicans* SC5314 and *C. auris* AR-0389 was 1.25 μmol/L.

Antifungal activity of APC6 is not decreased by antioxidants

The thiosemicarbazone derivative NSC319726 with metal-chelating ability exerts its fungicidal action by inhibiting ribosome synthesis and amplifying the generation of reactive oxygen species.[27] Therefore, we tested the effects of antioxidants (glutathione reduced form, *N*-acetylcysteine and α-thioglycerol) on the antifungal activity of APC6 against the AR-0389 strain. We found no changes, even with high concentrations of antioxidants (Figure S2).

APC6 inhibits Candida proliferation alone and in combination with voriconazole

Because the mechanism of action of APC6 differed from those of conventional antifungal agents,[3,4] we expected APC6 to be efficacious against fungi currently deemed drug resistant. We compared the effect of APC6 on growth kinetics with those of voriconazole and amphotericin B, and found that APC6 acted as a fungistatic agent, similar to voriconazole (Figure S3). Next, we investigated the effects of APC6 alone or in combination with voriconazole against AR-0389. The growth kinetics of the AR-0389 strain are shown in Figure 4. APC6 at 0.25 mg/L inhibited growth compared with the control. APC6 at 0.25 mg/L and voriconazole at 2 mg/L (0.5× MIC) inhibited growth more strongly than either APC6 or voriconazole alone. The additive effect of APC6 was still observed at 72 h with voriconazole at 2× MIC. In a standard microdilution chequerboard assay, the combination of voriconazole and APC6 had an FIC index of 1.0, with no explicit synergistic effect.

APC6 has a therapeutic effect against C. auris invasive candidiasis in G. mellonella larvae

To evaluate fungal virulence and the effectiveness of antifungal drugs, invasive infection models involving the lepidopteran *G. mellonella* infected with *Candida*, *Cryptococcus*, *Trichosporon*, *Aspergillus* or *Mucorales* are routinely used.[28] We infected *G. mellonella* larvae with *C. auris* AR-0389 (2×10^5 cfu/larva) together with APC6, voriconazole or both, and we monitored larval survival. At 7 days after infection, the survival rate was a mere 11% in the control group but 44% in the group treated with APC6 (1.5 μg/larva) or voriconazole (2 μg/larva). A combination of both agents at the same concentrations increased the survival rate to 67% (Figure 5).

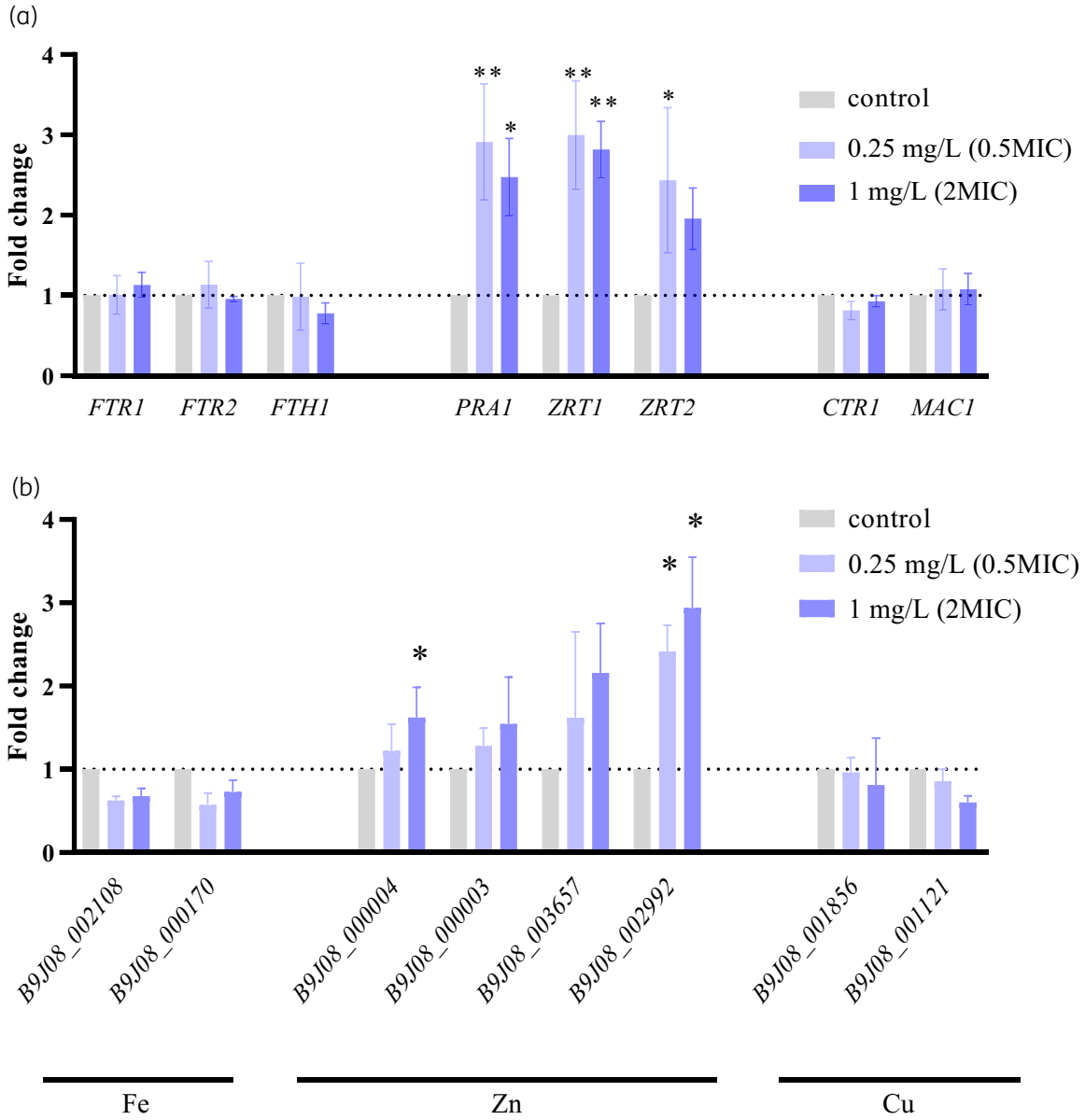


Figure 2. RT–qPCR analysis of the expression of genes associated with iron, zinc and copper homeostasis in APC6-treated *C. albicans* and *C. auris*. (a) *C. albicans* SC5314 and (b) *C. auris* AR-0389 were cultured in RPMI-MOPS medium and treated with 0.25 mg/L (0.5× MIC) or 1 mg/L (2× MIC) APC6 for 6 h at 35°C. Gene expression levels were normalized to that of *ACT-1* (*C. albicans*) or *B9J08_000486* (*C. auris*). *FTR1*, *FTR2* and *FTH1* are iron transporter-associated genes in *C. albicans*, with their orthologues in *C. auris* being *B9J08_002108* (*FTR1*) and *B9J08_000170* (*FTH1*). *PRA1*, *ZRT1* and *ZRT2* are zinc transporter-associated genes in *C. albicans*, with their orthologues in *C. auris* being *B9J08_002992* (*PRA1*), *B9J08_000004* (*ZRT1*), *B9J08_000003* and *B9J08_003657* (*ZRT2*). *CTR1* is associated with copper transportation, and *MAC1* is associated with a transcription factor that becomes up-regulated during copper deficiency, with their orthologues in *C. auris* being *B9J08_001856* and *B9J08_001121*, respectively. Primer sequences are listed in Table S1. Three replicates were performed. Bars represent mean ± SD. * $P < 0.05$; ** $P < 0.01$ versus control.

Table 2. Concentrations of metal ions (μmol/L) that inhibited the antifungal activity of APC6 (MIC increased to at least 2 mg/L) against *C. albicans* SC5314 and *C. auris* AR-0389

Candida strain	Fe^{2+}	Fe^{3+}	Mn^{2+}	Zn^{2+}	Cu^{2+}	Mg^{2+}	Ca^{2+}
SC5314	500	>1000	12.5–125	1.25–12.5	1.25–12.5	>1000	>1000
AR-0389	>1000	>1000	12.5–125	1.25–12.5	1.25–12.5	>1000	>1000

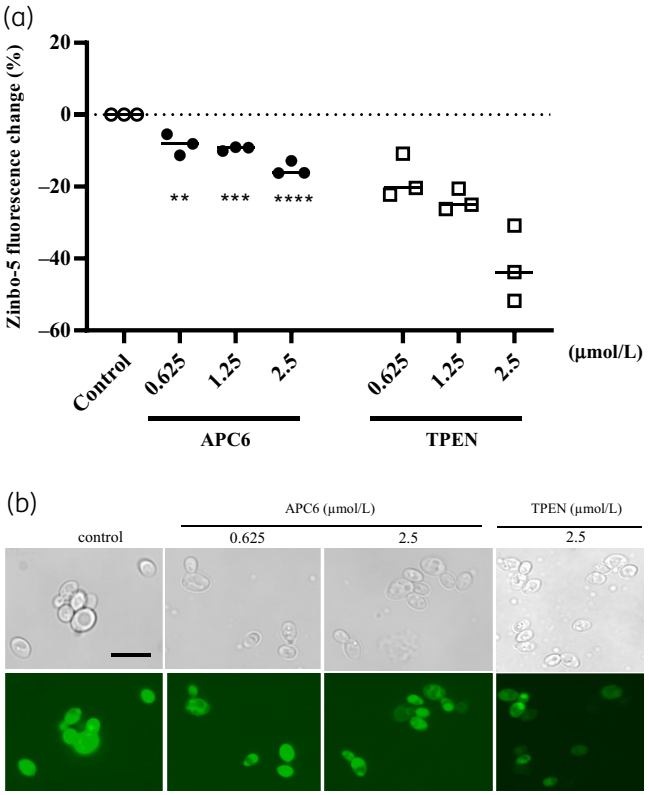


Figure 3. Zinbo-5 assay. A decrease in the fluorescence signal of Zinbo-5 indicates the chelation of intracellular zinc ions by APC6 or TPEN. *C. auris* AR-0389 cells (OD_{600} = 2.0) were treated with the indicated concentrations of APC6 or TPEN for 6 h at 35°C in 96-well plates and stained with 5 μmol/L Zinbo-5. (a) Fluorescence intensity measured with a plate reader (excitation at a wavelength of 360 nm, emission at 460 nm). Horizontal bars represent means of three replicates. ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. (b) Zinbo-5-stained cells observed under a fluorescence microscope. Scale bar = 10 μm.

APC6 disrupts biofilms of C. albicans and C. auris

The AR-0382 strain of *C. auris* has prominent biofilm-producing capacity, which underlies its high pathogenicity.[29] We used the crystal violet method to evaluate the effect of APC6 on biofilms of *C. albicans* SC5314 and *C. auris* AR-0382. Both biofilms were significantly disrupted at 0.25 mg/L APC6, akin to the effect of amphotericin B. (Figure S4).

APC6 has low cytotoxicity to HepG2 human liver cancer cells

To assess the potential toxicity of APC6 to the HepG2 human liver cancer cells, we used an MTT assay. The value of 50% cytotoxic concentration (CC_{50}) was 11.9 mg/L for APC6 and 0.91 mg/L for TPEN (Figure 6). The selectivity index (the ratio of CC_{50} against a mammalian cell line to MIC for *C. auris* AR-0389) was 23.66 for APC6 and 1.71 for TPEN (Table S6). These data indicated that APC6 was safer than TPEN, implying high specificity of the antifungal activity of APC6.

Discussion

In this study, we found that a cyclic APC with three pyridylmethyl groups, APC6, had high growth-inhibitory activity against a wide range of *Candida* species and strains, including the MDR pathogen *C. auris* (Table S2). APC6 antifungal activity was decreased by the addition of zinc or copper to the culture medium (Table 2). In *Candida* cells, APC6 increased the transcript level of the zinc transporter genes, but it did not affect those of copper transporter genes (Figures 1 and 2). These results suggest that, despite APC6's potential to bind copper, it preferentially interacts with zinc within *Candida* cells. Proteins encoded by *ZRT1* and *PRA1*, along with *ZRT2*, form a 'zincophore' system similar to the siderophore system for iron transport.[30] When zinc is restricted or depleted, *C. albicans* increases the expression of these genes; both are regulated by the Zn-responsive transcription factor

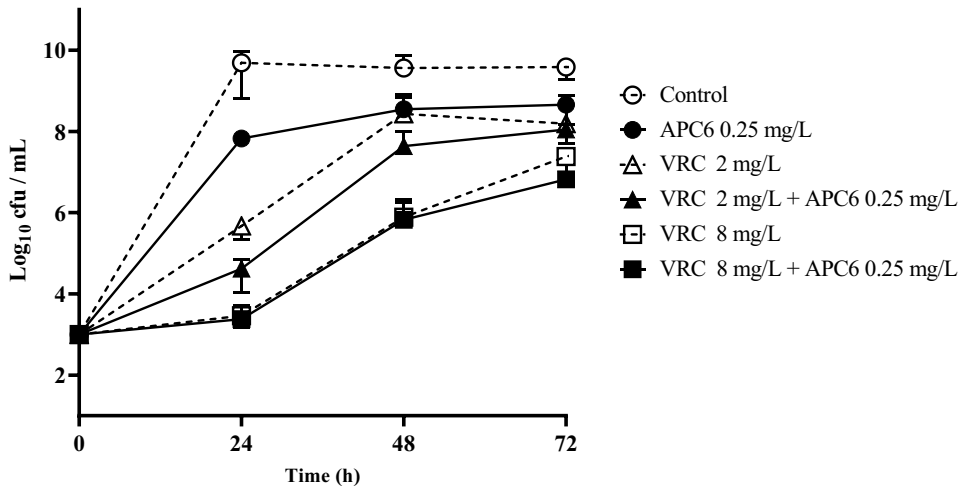


Figure 4. Inhibition of *C. auris* AR-0389 growth by APC6, voriconazole (VRC), or their combination. The cells were grown and treated in RPMI-MOPS medium. Three replicates were performed.

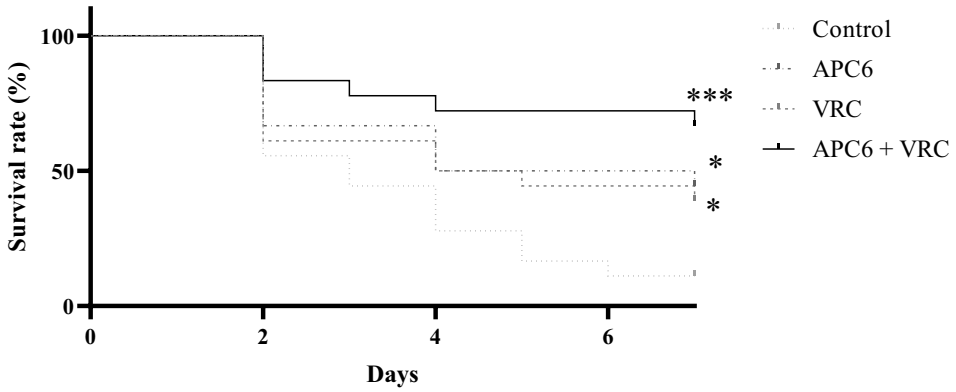


Figure 5. Therapeutic antifungal efficacy of APC6, voriconazole (VRC) or their combination in a *G. mellonella* infection model. Larvae ($n = 18$ per group) were infected with *C. auris* AR-0389 (2×10^5 cfu/larva) and treated with PBS (control), APC6 (1.5 μg/larva), VRC (2 μg/larva) or their combination. Kaplan–Meier survival curves of *G. mellonella* infected with *C. auris* AR-0389 under different drug treatments ($n = 18$ per group) were assessed for significance by log-rank test. * $P < 0.05$; *** $P < 0.001$ versus control.

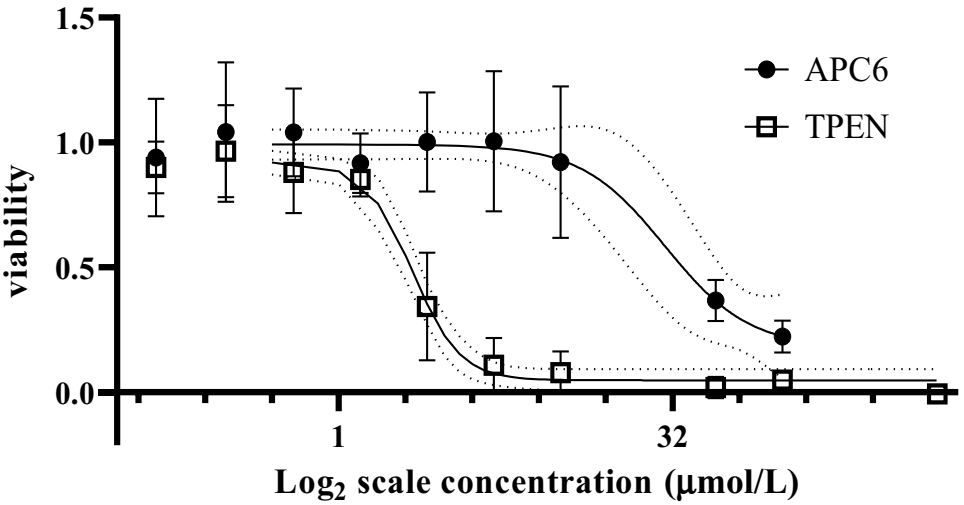


Figure 6. Cytotoxicity of APC6 and TPEN against HepG2 cells. Cytotoxicity was evaluated in an MTT assay. HepG2 is a human hepatocellular carcinoma cell line.

Zap1.[21–23] As zinc starvation up-regulates the expression of zinc transporters,[21–23] APC6 appears to decrease zinc availability in *Candida* species. An increase in APC6 concentration decreased zinc levels in *Candida* cells (Figure 3), and APC6 and zinc formed a stable complex (Figure S1). APC6 had antifungal activity as a standalone agent, and its combination with a known antifungal agent (voriconazole) was more effective (Figure 4). In the survival assay of *G. mellonella* infected with *C. auris* AR-0389, APC6 significantly decreased the mortality rate, and its therapeutic effect was increased in combination with voriconazole as an antifungal drug (Figure 5). APC6 had promising activity in disrupting the biofilms of *C. auris* and *C. albicans* (Figure S4); this activity was similar to that of amphotericin B. Our findings indicate that APC6 has potential as a therapeutic agent against a wide range of *Candida* species and strains, including MDR *C. auris*.

C. auris readily colonizes itself on the skin and can survive in medical environments for extended periods, leading to high infectivity among patients and numerous reported outbreak cases.[31–33] Additionally, its tendency towards drug resistance exacerbates the issue. When it causes bloodstream infections, the high mortality rate of 30%–60% classifies it as a priority fungal pathogen, constituting a significant public health threat.[31–33] Therefore, there is growing anticipation that APC6, with its potent antifungal activity, could emerge as an effective strategy against *C. auris*.

Zinc depletion leads to a decline in fungal growth and biofilm formation,[9,10] but the mechanisms of this decline have not been fully investigated. The antifungal effects of the thiosemicarbazone derivative NSC319726 with metal-binding affinity have been attributed to its inhibition of ribosome biogenesis and to an increase in the generation of reactive oxygen species.[27]

However, none of the antioxidants tested affected the antifungal activity of APC6 (Figure S2), suggesting that it is not associated with the generation of reactive oxygen species.

Fungus-specific zinc-chelating compounds could be used to develop antifungal therapies, but their effects at the cellular and organismal levels need to be thoroughly investigated. Increasing evidence suggests that zinc levels inside or outside the cell can control apoptosis.[34] In many patients, a decrease in intracellular zinc concentrations by the anti-TB drug ethambutol results in visual impairment or irreversible vision loss;[35] it has been proposed that a decrease in zinc levels triggers apoptosis of retinal ganglion cells via caspase activation.[36] Decreased intracellular zinc concentrations alter zinc-dependent protein processing, rendering neurons more susceptible to glutamate excitotoxicity and triggering apoptosis cascades in response to non-toxic levels of glutamate.[36,37] The zinc chelator TPEN depletes intracellular zinc and induces apoptosis in human cells.[34] In comparison with TPEN, APC6 induced a more moderate decrease in intracellular zinc levels (Figure 3), but its activity against fungal cells was equivalent. At the concentrations effective against fungi (with the MIC of both APC6 and TPEN against *C. albicans* SC5314 and *C. auris* AR-0389 being 1.25 μmol/L), the adverse effects of APC6 on human HepG2 cells were negligible, whereas TPEN caused approximately 10%–20% cell death (Figure 6). Thus, APC6 might be safer than TPEN. Generally, drugs undergo metabolism in the liver; accordingly, HepG2 cells are used in cytotoxicity tests.[17,18] However, it is possible that organs other than the liver could be affected by the administration of APC6 *in vivo*, and detailed tests are needed to confirm its safety before clinical application. The difference between the concentrations effective against fungi and those cytotoxic to human cells is highly important in pharmacological development.[38–41]

At the physiological level, zinc deficiency leads to multiple pathologies.[42] Initial symptoms comprise dermatitis, diarrhoea, alopecia and anorexia.[42] In individuals with zinc deficiency who undergo zinc supplementation therapy, the effectiveness of APC6 could be compromised. Although it may not be straightforward to confer selective toxicity on zinc-chelating agents, the potent antifungal activity of APC6 warrants continued investigation. Further modification of APC6 can be expected to produce a safe antifungal drug.

The drug resistance mechanisms of *C. auris* AR-0389 are thought to include a Y132F substitution in the ergosterol synthesis enzyme encoded by *ERG11*, as well as overexpression of the drug efflux transporter gene *CDR1*.[6,7] APC6 alone, or in combination with voriconazole, could effectively circumvent these drug resistance mechanisms. Azole antifungal agents decrease fungal ergosterol levels, leading to the up-regulation of *ERG* genes;[43] our RNA-seq data indicated no effect of APC6 on *ERG* genes (Table S7), suggesting a minimal impact of APC6 on the ergosterol synthesis pathway. Zinc is an essential micronutrient for eukaryotes, involved in numerous metabolic pathways and cellular responses.[11,12] APC6 can suppress fungal proliferation by reducing the availability of intracellular zinc, and it has an additive antifungal effect in conjunction with voriconazole. Given that no antagonistic interaction was observed with azoles, including voriconazole (this study) and fluconazole (data not shown), and that targeting zinc regulation is suggested to have reduced propensity for inducing drug resistance,[16,17] we have high hopes for novel derivatives based on APC6 and combination therapies with existing drugs.

In conclusion, our findings can be of great value in meeting the recent need for treatments for drug-resistant fungal infections, because we found that APC6 (i) inhibits the growth of a wide range of *Candida* species, including *C. auris*; (ii) is active alone or in combination with voriconazole; (iii) is able to disrupt biofilms; and (iv) is significantly less toxic than TPEN to a human cell line. The data on the basic structure and function of APC6 provide valuable information for the development of novel antifungal agents with greater efficacy and safety.

In the future, we plan to include *Aspergillus* and *Cryptococcus*, as well as *Fusarium*, *Scedosporium* and *Scopulariopsis*, which are frequently resistant to the available antifungals, and to assess the antifungal activity of other compounds based on the APC6 backbone.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S4 and Tables S1 to S7 are available as Supplementary data at *JAC-AMR* Online.

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