**ORIGINAL RESEARCH ARTICLE**

Candida albicans ergosterol disorders as a consequence of the new sulfone derivative action mode

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Abstract
A series of novel sulfone derivatives were synthesized and screened in vitro for their cytotoxicity and antifungal activity with annotated primary mechanism of action (MOA). We prioritized sulfones with high (4-(bromodichloromethylsulfonyl) benzoic acid **4**, 4-(difluoromethylsulfonyl)benzoic acid **12**), little (3-[4-(bromodichloromethylsulfonyl)phenyl]propanoic acid **8**, difluoromethyl 4-methylphenyl sulfone **11**, 4-(difluoromethylsulfonyl)benzoic acid **12**), or no cytotoxicity of 4-(4-(dichloromethylsulfonyl)benzoic acid **3**) and 3-[4-(dichloromethylsulfonyl)phenyl]propanoic acid **7** against mammalian cell lines. **3** was found to be the most potent sulfone against *Candida albicans* (R_{log}=7.25 at 128–256 μg/mL). The mutation in the *CNB1* gene (1) increased the sensitivity of the *C. albicans* biofilm to **3**; (2) reduced ergosterol production and therefore generated higher susceptibility to **4**. Sulfone **4** at 128 μg/mL increased cellular RH-123 fluorescence in the wild-type cells of *C. albicans*, except *CNB1/cnb1Δ*. Moreover, the uptake of sulfones into the cell was unaffected regardless of the presence or absence of RH-123, and the uptake of sulfones was strictly cell/strain dependent. Both RH123 and sulfones cumulatively competed with one another for access to transporters. Calcineurin played a role in this mechanism.

Sulfones attached to ZnO dots enter *Candida* sessile cells
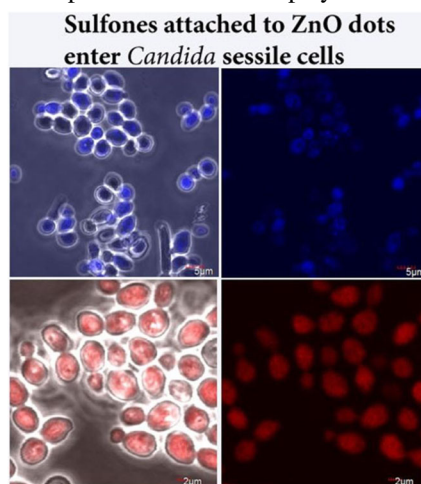
**Keywords** Antifungal activity · Sulfone · *Candida* · *CNB1* · Mutants · Ergosterol

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Scheme 1 Sulfone inhibitors of virulnce factors of *Candida*
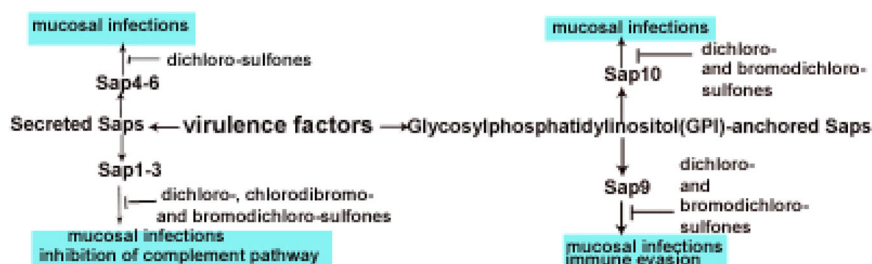
Introduction

Since many complex fungal mechanisms of resistance to antimycotics exist, new antifungal combating properties and harmless effects on the patient, are being sought. Novel non-toxic and broad-spectrum antifungals are urgently needed to combat life-threatening fungal diseases [1]. Sulfone derivatives represent a promising group of compounds that effectively combat candidiasis [1]. Their antitumor, anti-inflammatory, and antifungal activity were proven [2, 3]. They are heterocyclic compounds with various modifications, containing a sulfonyl group. Our group showed that sulfones containing bromine or chlorine in the halogenomethylsulfonyl group are fungicidal, and additional ring modifications increased its activity (Scheme 1) [4–6]. Also, the sulfone derivatives influenced the morphogenesis, adhesion, and expression of transcription factors (*EFG1*, *CPH1*) [7]. Based on our preliminary studies, a new group of aromatic sulfonyl derivatives was designed. Moreover, we included the calcineurin mutants in the study to understand whether the calcineurin signaling pathway is involved in antifungal sulfone resistance. Calcineurin is a calcium-calmodulin-dependent serine-threonine-specific phosphatase that controls fungal virulence pathways, such as invasive hyphal growth, drug resistance, and cell integrity [8]. Calcineurin is involved in forming infectious dimorphic transitions that play critical roles in pathogenesis [8]. Park et al. [8]. Reviewed that calcineurin-defective fungal strains cannot complete morphogenesis.

In the work, (1) we synthesized new sulfones, and (2) we screened their activity aiming to identify compounds that are fungicidal against the *Candida albicans* reference strain and calcineurin mutants growing in planktonic and sessile cells in vitro. Furthermore, the sulfone cytotoxicity against Vero E6 and MRC-5 was evaluated. We studied the *C. albicans* programmed cell death induced by sulfone derivatives. To visualize sulfones entering the cells in vitro the fluorescently marked sulfones were monitored in the *C. albicans* sessile cells. We analyzed the effect of sulfones on Rhod123 signals in a culture of fungal cells. We established whether sulfone uptake occurs by endocytosis and whether an efflux of agents from the cell by the ATP-dependent pumps exists.

We studied whether sulfones affected *Candida* wild-type's and calcineurin mutants' ergosterol levels. An efficient approach to control fungal infection could be the identification of agents that can increase existing antimycotics and synergize. Based on amphotericin B action mode [9], we undertook studies on exogenous ergosterol. Finally, by a combination of screening *C. albicans* calcineurin mutants, we were able to assign the sulfone synergism mechanism of action to the cell wall integrity (CWI) pathway.

Results

Sulfones against the *C. albicans* wild-type's and calcineurin mutants' planktonic and sessile growth

We screened the antifungal activity of new sulfones and summarized MFC and MIC in Table 1. According to the CLSI criteria [9], it was found that 4-(4-(dichloromethylsulfonyl) benzoic acid **3**) showed MFC $\geq 128 \, \mu g/mL$ (logR = 7.45) and $MIC_{100} \geq 128 \, \mu g/mL$ (Table 1). AmB was used as a licensed antifungal drug (Table 1). The antifungal screening revealed that six of seven tested sulfones: dichloromethyl-p-toluene sulfone **2**, 4-(bromodichloromethylsulfonyl)benzoic acid **4**, 3-[4-(dichloromethylsulfonyl)phenyl]propanoic acid **7** (and its labeled derivative **L1**), 3-[4-(bromodichloromethylsulfonyl) phenyl]propanoic acid **8** (and **L2**), difluoromethyl 4-methylphenyl sulfone **11**, and 4-(difluoromethylsulfonyl) benzoic acid **12** (**L3**) demonstrated moderate activity (Table 1 and Tables S1 and S2). Briefly, fungal blastoconidia showed MIC = $256 \, \mu g/mL$ for **2** (% = 57 ± 4) and **4** (% = 41 ± 1) after 48 h. The antifungal activity of the quantum-tagged sulfone derivatives **L1–L3** was similar to that of their unlabeled counterparts **7**, **8**, and **12** (Tables 1, S1–S3). Briefly, *C. albicans* had a MIC of $256 \, \mu g/mL$ respectively for **7** (% = 46 ± 5 after 48 h) and **L1** (47 ± 4 after 48 h); for **8** (% = 41 ± 3) and **L2** (% = 41 ± 4); for **12** (% = 41 ± 0) and **L3** (47 ± 1). Sulfone **11** (% = 16 ± 1) was less active than the remaining ones. Sulfones showed a lack of activity at concentrations of 1 and $0.5 \, \mu g/mL$.

The *C. albicans* sessile cell metabolic activity reduced by the most active sulfones **3** and **4** is summarized in Table 2. Sulfon **3** at $256 \, \mu g/mL$ reduced the metabolic activity of

Table 1 Fungistatic and fungicidal activity of sulfone derivatives against the *C. albicans* SC5314 planktonic cells

Conc. µg/mL	2	3	4	7	8	11	12	AmB
Log reduction (R) of cells								
256	0.01	7.45	2.1	0	0	0.03	0.34	7.45
128	0.06	7.45	2.1	0.02	0.19	0.03	0.08	7.45
64	0.11	0	0	0	0	0.16	0	7.45
Cell inhibition (CI%)								
256	57 ± 4**	100 ± 2	41 ± 1	46 ± 5	41 ± 3	16 ± 1	41 ± 0	100 ± 0.1
128	44 ± 4	100 ± 2*	37 ± 1	44 ± 5	33 ± 1	11 ± 4	20 ± 4	100 ± 0.02
64	44 ± 5	7 ± 1	36 ± 2	43 ± 5	31 ± 1	10 ± 4	6 ± 3	100 ± 0.01
32	20 ± 3	1 ± 4	22 ± 3	29 ± 4	27 ± 1	7 ± 4	0	100 ± 0.03
16	6 ± 5	0	21 ± 0.1	18 ± 3	9 ± 2	6 ± 4	0	100 ± 0.03
8	2 ± 1	0	19 ± 0.2	9 ± 4	2 ± 0.1	1 ± 0.1	0	100 ± 0.02
4	0	0	14 ± 4	7 ± 1	2 ± 0.1		0	100 ± 0.03
2	0	0	14 ± 0.2	0	1 ± 0.1		0	100 ± 0.01

Data are expressed as cell metabolic activity % (means ± SD). AmB notes amphotericin B, * means MIC = 100, ** means MIC = 50. Cell inhibition (%) was assessed using the MTS (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay) (MTS, Promega, USA)

sessile cells of SC5314 and mutants. Hetero- and homozygote (deleted in *CNB1*) in biofilm displayed no metabolic activity (reduced cell proliferation, CI% = 100) for **4** at the range from 2 to 256 µg/mL. Contrariwise, SC5314, and CAI4 displayed metabolic activity of 39–60% after treatment with **4** at 256 µg/mL. The CAI4 sessile cell metabolic activity was reduced to 10% ± 0.5 for **4** at 2 µg/mL. Contrariwise, **4** at 2 µg/mL was not active against the SC5314 biofilm.

Sulfone approaches to control *C. albicans* programmed cell death

In Fig. 1, **4** at 128 µg/mL induced the late apoptosis of planktonic cells (~42%) after 18 h. Contrariwise, **4** at 16 µg/mL prompted early (10.6%) and late apoptosis (6.0%) of planktonic cells compared to the untreated counterparts. Sulfones (**7**, **8**, and **12**) are predisposed to apoptosis in the *C. albicans* planktonic cells. All sulfones favored necroptosis ranging from 0.3% (**8** at 16 µg/mL) to 4.3% (**4** at 128 µg/mL) in *C. albicans*. **3** at 128 µg/mL or 16 µg/mL induced necroptosis of 1% or 0.83% of planktonic blastoconidia. Planktonic cells treated with sulfones stay alive in the range from 47.9% (**4** at 128 µg/mL) to 99.4% (**11** at 16 µg/mL). Taken together, we identified sulfones that display the activity against *Candida*. Sulfones displayed very different mechanisms of programmed cell death action. The gating strategy is included in the Supplementary data.

In vitro cytotoxicity of sulfone derivatives

As shown in Fig. 2, the tested sulfones at 256 µg/mL were toxic and reduced the viability of the Vero E6 cells by 100%, excluding **3** and **7**. **4** at 80 µg/mL displayed IC_{50} against the Vero E6 monolayer. **7** was not toxic against the Vero E6 cells. **11** at 60 µg/mL displayed IC_{50} against the Vero E6 cells. The sulfone derivatives: **4** and **12** showed 100% toxicity against the MRC-5 cells at < 20 µg/mL. **3** was not toxic against MRC5 at the range from 0.12 to 256 µg/mL.

Sulfones compared with rhodamine 123 (RH123) dye efflux properties of *C. albicans*

The SC5314 cells treated with sulfones (**4**, **7**, **8**, **12**) at 128 µg/mL in the presence of RH-123 (15 µM) for 15 min-18 h showed that the fluorescence intensity of the supernatant increased vs. untreated control (Fig. 3). In the same staining pattern, the dye efflux properties of *cnb1Δ/cnb1Δ*, CAF2-1, and CAI4 indicated **4** at 16 µg/mL for 18 h (Fig. S1). Contrariwise, the follow of this protocol with *cnb1Δ/cnb1Δ* treated with **4** at 128 µg/mL showed no statistically significant changes in supernatant staining intensity. The dye efflux properties of *CNB1/cnb1Δ* were dependent on **4** at 128 µg/mL.

Sulfone derivative influence on endogenous ergosterol

2 at 0.5 or 16 µg/mL decreased the quantity of ergosterol extracted from SC5314 (Fig. 4A). Contrariwise, **4** at 16 µg/mL increased the quantity of ergosterol extracted from SC5314 (Fig. 4B). The quantified ergosterol in CAI4 and *cnb1Δ/cnb1Δ* treated with **4** at 16 µg/mL indicated low concentration of ergosterol (Fig. 4B). Contrariwise, **4** at 16 µg/mL produced greater concentration of ergosterol

Table 2 The sulfone derivatives against the *C. albicans* pre-formed biofilm

Comp.	*C. albicans*	Cell inhibition (%)							
		256	128	64	32	16	8	4	2
3	SC5314	100 ± 2	94 ± 2	50 ± 3	45 ± 7	40 ± 1	20 ± 1	1 ± 0.1	0
	CAI4	100 ± 3	95 ± 2	95 ± 3	94 ± 2	93 ± 3	93 ± 2	94 ± 3	93 ± 3
	CNB1/cnb1Δ	100 ± 3	91 ± 3	75 ± 2	54 ± 1	92 ± 3	93 ± 3	97 ± 3	96 ± 3
	cnb1Δ/cnb1Δ	100 ± 3	98 ± 2	91 ± 2	94 ± 3	93 ± 2	99 ± 3	96 ± 2	59 ± 2
4	SC5314	40 ± 4	31 ± 6	22 ± 1	21 ± 1	19 ± 5	14 ± 3	14 ± 4	0
	CAI4	61 ± 2	30 ± 2	34 ± 2	33 ± 3	30 ± 1	26 ± 2	28 ± 2	10 ± 0.5
	CNB1/cnb1Δ	100 ± 5	100 ± 8	100 ± 6	100 ± 3	100 ± 1	100 ± 2	100 ± 4	100 ± 2
	cnb1Δ/cnb1Δ	100 ± 3	100 ± 2	100 ± 2	100 ± 3	100 ± 2	100 ± 1	100 ± 1	100 ± 7

Data are expressed as cell metabolic activity % (Means ± SD). Sessile cell inhibition was assessed using MTS (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay) (MTS, Promega, USA)

Fig. 1 Flow cytometry analysis. Sulfones trigger *C. albicans* programmed cell death (PCD)
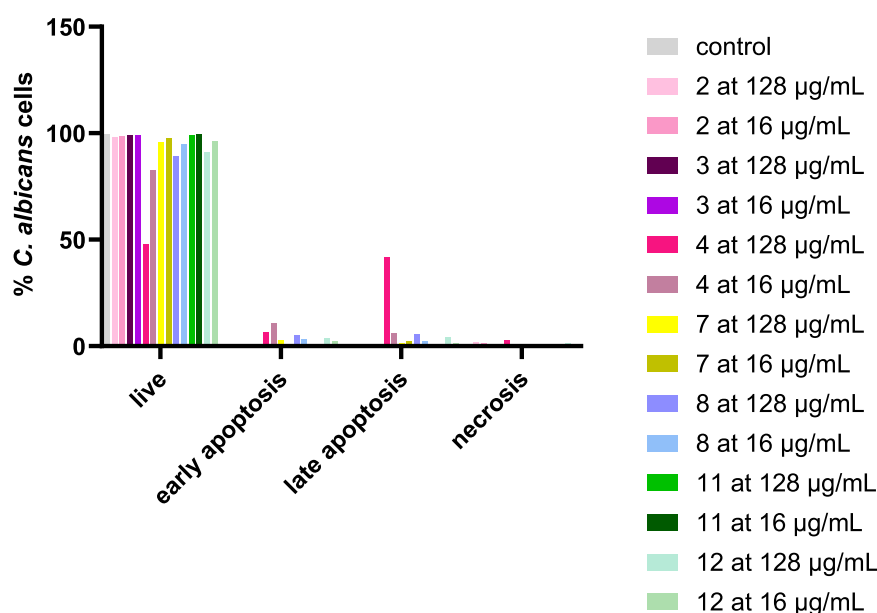
extracted from *CNB1/cnb1Δ* compared to untreated control (Fig. 4B). Insignificant differences were noted between the concentration of ergosterol extracted from SC5314 treated with **3, 11** or **12** at 0.5 or 16 μg/mL compared with the untreated control.

Antifungal activity of sulfone derivatives against exogenous ergosterol

In the ergosterol binding assay, we considered the exposure of sulfones to exogenous sterols (Fig. 5A–D, Tables S5–S7). Sulfone (**2, 3, 7, 11**) affinity for sterols led to the rapid formation of complex, thereby impeding complexation with the sterols of the cell membrane resulting in a MIC increase (almost 100% growth reduction) after 96 h (Fig. 5C). Sulfones **3, 4, 11**, and **12** used in combination with AmB in the medium with ergosterol had a significant effect

at the range of concentration tested from 0.5 to 16 μg/mL (Fig. 5D).

Inhibitory concentration (IC%) and fractional inhibitory concentration index (FICI) were used to confirm antifungal susceptibility and indifferent effects. Reduced activity was observed relative to the effect of individual sulfones or AmB. An indifferent interaction between sulfone and AmB treatments occurred at 16 μg/mL. Compound joint effects were noted at the range of concentration from 0.5 to 8 μg/mL.

Entry of the sulfone derivatives into the *C. albicans* cells. Influence of the sulfone derivatives on the biofilm morphology and the activity of ATP-dependent drug efflux pumps in *C. albicans*

Sulfones **L1, L2**, and **L3** at 128 μg/mL, after 18 h of treatment affected the *C. albicans* SC5314 biofilm (Fig. 6). Sulfones

Fig. 2 Viability of the Vero E6 and MRC5 cells treated with the sulfone derivatives. The analysis of cell viability was assayed after a 20-h treatment with sulfones. Data are expressed as the mean ± SD of three independent experiments. All samples were tested in duplicate
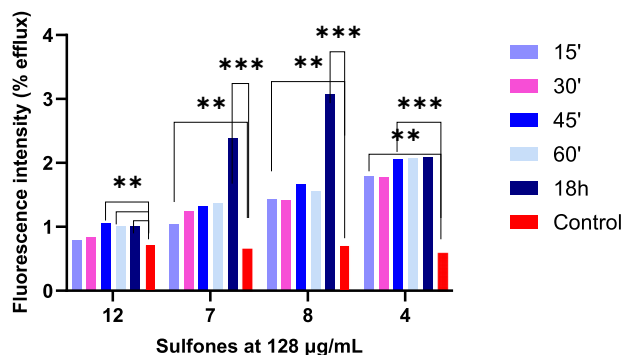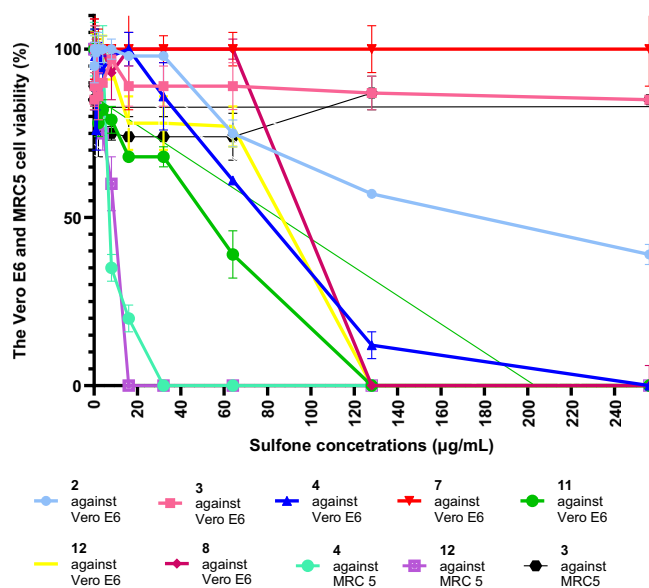

Fig. 3 Energy-dependent efflux of rhodamine 123 (RH-123) by the *C. albicans* SC5314 cells treated with sulfones (**4, 7, 8** and **12**) at 128 µg/mL. The fluorescence of RH-123 released from the cells treated with the sulfones was compared with the untreated control and recorded as % efflux. Significant differences between control and treated groups are indicated: **, ***$p < 0.05$ (unpaired *t*-test)

(**L1–3**) marked with ZnO quantum dots, were visible inside sessile cells (Fig. 6A–F) as blue fluorescence. The ZnO quantum dots (negative control) alone did not enter the fungal sessile cells (Fig. 6G, H). Sulfones induced RH-123 efflux (Fig. 6A–F), while red fluorescence was developed inside the sulfone-untreated cells (Fig. 6I, J). Generally, sulfones at 128 µg/mL for 18 h of treatment of *C. albicans* inhibited hyphae formation in biofilm conglomerate.

Discussion

We summarized our current studies on new sulfone action modes against emerging *Candida* pathogens. The presence of chlorine halogens in halogenomethylsulfonyl group increases the antifungal action (sulfone **3** in Table 1)

[4, 5, 9, 10]. We defined **3** at 128–256 µg/mL inhibits *C. albicans* metabolic activity (Table 2). It was shown that the antifungal properties of sulfones decreased when the addition of bromine/ fluorine halogen to the sulfonyl group is considered (**4** and **12** in Tables 1 and 2). Khode et al. [11]. Showed that electrophilic addition of halogen (Cl$_2$) to sulfone showed promising antifungal activity. The role of halogens (particularly chlorine) in the recent antifungal drug discovery processes was described and approved by the FDA in 2021 [10]. We presented the profiling of the cytotoxicity of sulfones with annotated primary mechanism of action (MOA): anti-biofilm, anti-hyphae formation, disturbance of the cell wall and plasma content, blastoconidia late apoptosis and necroptosis. In the study, we provided a reliable way to prioritize sulfones with high (**4, 12**), little (**8, 11, 12**), or no cytotoxicity (**3, 7**) against mammalian cell lines. **12** and **2** at 256 µg/mL, **4** and **8** at 128 µg/mL reduced the viability of Vero cells by 100% (Fig. 2). Specifically, in contrast to MRC-5, **4, 7**, and **12** showed no toxicity against Vero E6 up to 80 µg/mL.

Halogenation of sulfones increased anti-biofilm activity [5]. The electrophilic addition of halogen (Cl$_2$ in **3** or Br$_2$ in **4**) provided the activity against the *C. albicans* SC5314 biofilm. **3** inhibited the sessile growth independently on *CNB1* expression accounting for increased drug resistance of biofilms (Table 2). **4** in the highest tested concentration caused a 100% reduction of biofilm metabolic activity of *cnb1Δ/cnb1Δ* (Table 2). The mutation in the *CNB1* gene increased the sensitivity of biofilm to **3**. Thus *CNB1* regulates the *Candida* tolerance to osmotic stress induced by sulfone. In the presence of sulfones, the phosphatase calcineurin activates several stress pathways, including chitin synthase expression resulting in the overproduction of chitin [6].
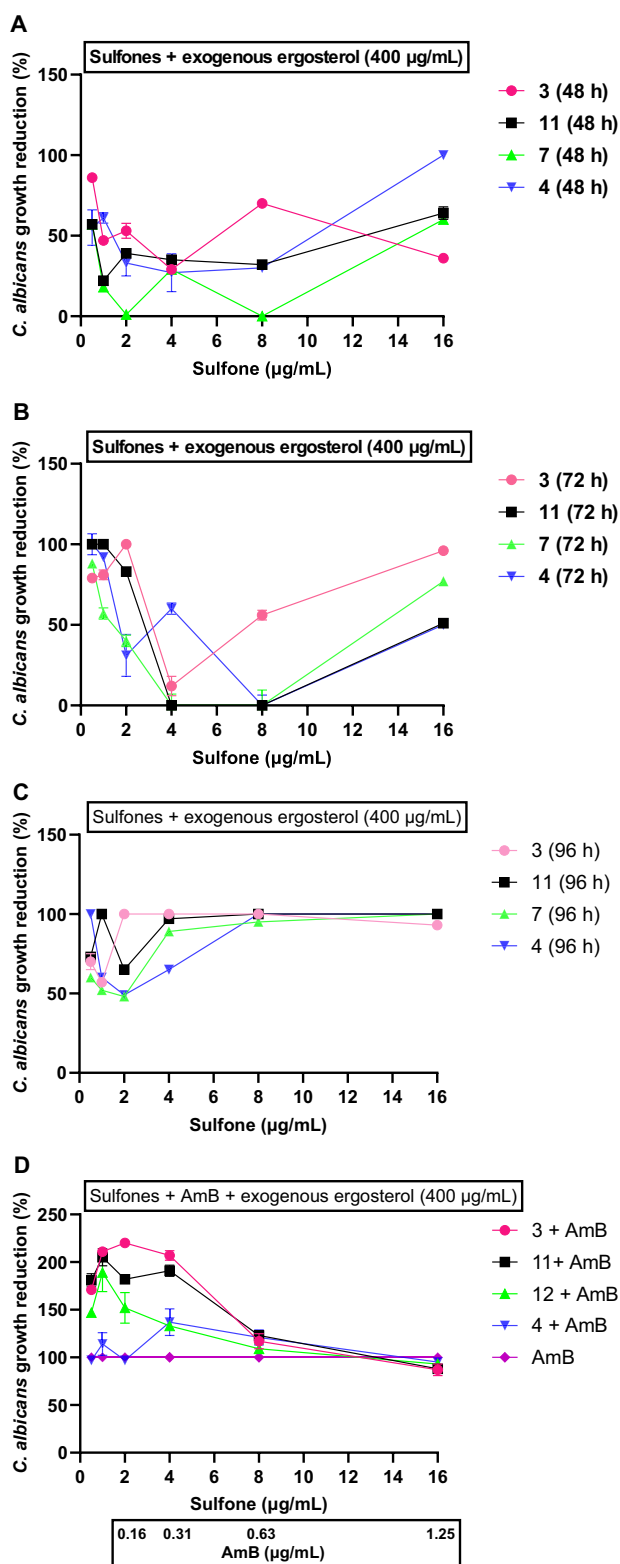
Fig. 4 Endogenous ergosterol content in the *C. albicans* wild-type cells and *CNB1* mutants treated with sulfones. Endogenous ergosterol content (µg/mg) in **A** *C. albicans* SC5314 wild-type cells treated with the sulfones **3**, **11**, **12**, and **2** at 0.5 µg/mL or 16 µg/mL and in **B** SC5314, CAI4 wild-type cells, and *CNB1* mutants treated with **4** at 16 µg/mL for 24 h vs. untreated control. Extracts were analyzed via high-performance liquid chromatography HPLC, based on a standard curve of the relationship between the peak area and the positive control. The test was performed in 3 independent replications, the graph shows the average results and error bars. Significant differences between control and treated groups are indicated: **, ***$p < 0.05$ (unpaired *t*-test); ns means no significant differences
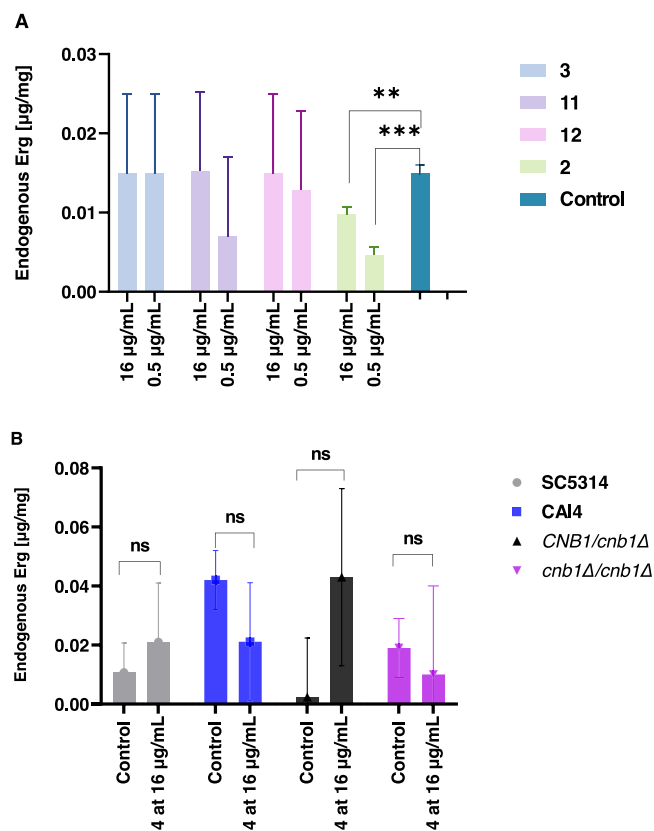
Testing sulfones under standardized conditions, we observed a sulfone-specific phenomenon called the "paradoxical effect" – the ability to grow in the presence of higher sulfone concentrations while being fully susceptible in lower ones (**4**, **7**, **8**, and **12** in Tables S1–S3). Consequently, the activity of these sulfones is considered fungistatic. The effect depends on the derivative and time of incubation of blastoconidia with sulfones. The phenomenon was described previously for echinocandins [12]. The in vitro phenomenon relevance in a clinical setting for the treatment of fungal infection still lacks conclusive answers. We presented the "paradoxical effect" based on removing sulfones from the cell by activating the membrane pumps where drug transporters bind to the drug sensor motifs of the sulfone regulators, resulting in either activation or repression of transporter genes [13, 14], Sulfones application induced an increase in cellular RH-123 fluorescence after 18 h in cells of SC5314, CAI4, and CAF2-1 treated with **4** at 128 µg/mL but not in cells of *CNB1/cnb1Δ* (Figs. 3 and S1). This indicates possible mitochondrial RH123 dequenching, which indicates Δψ depolarization. Moreover, this is because the diffusion of sulfones into the cell is unaffected regardless of the presence or absence of RH-123, and the uptake of sulfones is strictly cell/strain dependent (Figs. 3 and S1). The SC5314 cells treated with sulfones (**4**, **7**, **8**, **12**) at 128 µg/mL in the presence of RH-123 (15 µM)

for 15 min-18 h showed that the fluorescence intensity of the supernatant increased vs. untreated control (Fig. 3). It was due to the efflux-mediated function of the ABC transporters in the presence of RH-123 inhibitor. Since every cell has a finite number of ABC transporters, both RH123 and sulfones cumulatively compete with one another for access to transporters. Calcineurin plays a role in this mechanism. Calcineurin is essential for the *Candida* cells to survive membrane stress [15]. It was hypothesized that reduced expression or mutation of the *CNB1* gene leads to reduced production of ergosterol and therefore higher susceptibility to **4**. Treatment of the *C. albicans* CAI4 cells with **4** at 16 µg/mL resulted in a significant decrease in the endogenous ergosterol compared to the untreated cells (Fig. 4B). A range of sulfone types (**2**, **4**, and **11**) was discovered that interfere with the ergosterol content and these sulfones exhibited antifungal action (Fig. 4A, B). **4** influence on ergosterol content depends on the *CNB1* double disruption, leading to the increased 4 sensitivity in *C. albicans*. Furthermore, resistant *C. albicans* showed no changes in ergosterol under the stress response generated by **3** and **12** (Fig. 4A).

The presence of exogenous ergosterol in the culture medium allows for the determination of the interaction with the applied compound through activity against *C. albicans*. The 96-h treatment of the *C. albicans* SC5314 cells with the

A

B

C

D

Fig. 5 Activity of sulfones **2**, **3**, **7**, and **11** against *C. albicans* SC5314 growing in medium with exogenous ergosterol (exErg). Activity of **2**, **3**, **7**, and **11** against *C. albicans* SC5314 growing in medium with exogenous ergosterol (exErg) at 400 μg/mL **A–C** and AmB **D**. The cell incubation was conducted for 48 h **A**, 72 h **B**, and 96 h **C**. **D** Activity of **2**, **3**, **7**, and **11** in combination with AmB and exEgr against *C. albicans* was assessed after 48 h. % cell growth inhibition (%IC) was calculated according to the following formula: $\%IC = 100\% \times [1 - (OD_{405}\ CTW - OD_{405}\ SCW) / (OD_{405}\ GCW - OD_{405}\ SCW)]$, where CTW is the absorbance of the tested group, SCW is a negative control, and GCW is the absorbance of the positive control group. The test was performed in 3 replications. The standard deviation was calculated and plotted on the graphs in the form of error bars

those compounds is not based on the binding to ergosterol, but there is a correlation that influences the activity against the *C. albicans* cells. The action mode of AmB is based on the formation of a complex with ergosterol, due to which pores in the membrane are formed, which ultimately leads to cell lysis [16]. The sulfones (**3**, **4**, **11**, and **12**) in combination with AmB in a medium with ergosterol had the strongest effect on the cell growth inhibition, reaching the inhibition degree equal to 100% for some concentrations (Fig. 5D). The sulfones (**3**, **4**, **11**, and **12**) at 0.5 to 16 μg/mL acted antagonistically with amphotericin B. Below this concentration the compounds showed a synergistic effect (Table S8).

By a consecutive testing regimen, preincubation in higher sulfone concentrations and subsequent incubation with AmB results in a clinically relevant antagonism and suggests various mechanisms of action of both antifungals. The absence of antagonism of sulfone to AmB was noted when yeast was incubated in lower concentrations of both compounds. The microscopic observations confirmed that sulfones are based on disturbance of the cell wall and plasma biogenesis/ content. The induction of oxidative stress under sulfones was detectable with the RH123 fluorescent probe. Under sulfones, the intensity of intracellular oxidative stress decreased. The cytometric and microscopic analyses confirmed the necrotic mechanism of cell death. On the contrary, **3** and AmB induce apoptotic cell death. Our study showed that sulfones are not a substrate for these pumps – they penetrate the cell interior without blocking the pump drug outflow.

Conclusion

Our in vitro studies enhanced our understanding of the new sulfone potential against *Candida*. Sulfones focused on *Candida* virulence targets may influence clinical drug development. The presence of chlorine halogens in halogenomethylsulfonyl group increases the antifungal action by inhibiting metabolic activity. The antifungal action of bromodichloromethylsulfonyl or dichloromethylsulfonyl benzoic acid was exaggerated via mutation in calcineurin.
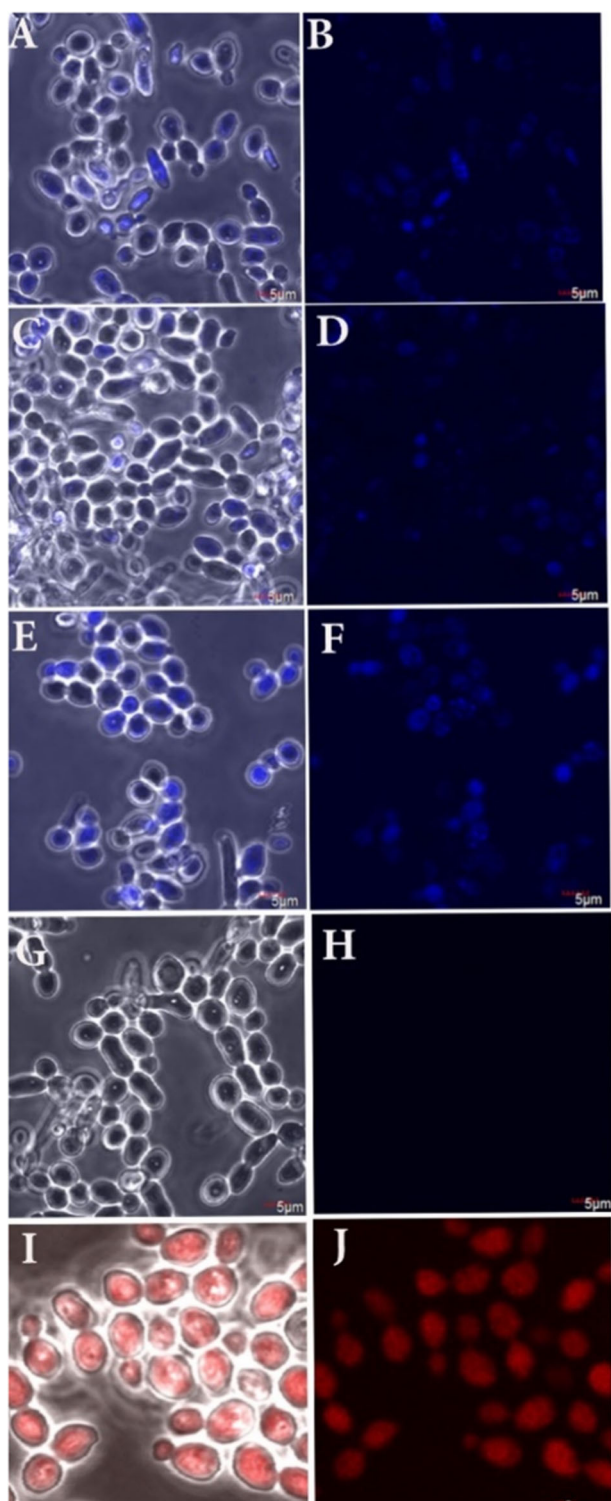
sulfonic derivatives: **2**, **3**, **7**, and **11** in the presence of exogenous ergosterol resulted in inhibition of the cell growth by up to 100% (Fig. 5A–D and Table S5). Based on these results, it can be assumed that the action mode of

Fig. 6 A–J Sulfones activity against unfixed *C. albicans* sessile cells. Sessile cells incubated with Rhodamine 123 (RH-123) at 15 µM and glucose at 5 mM for 5 h followed by treatment with sulfones marked with ZnO quantum dots at 128 µg/mL for 18 h at 37 °C. **A–F** Blue fluorescence is visible inside cells after the treatment with sulfones marked with ZnO dots: **A, B** L1, **C, D** L2, **E, F** L3. The sulfone treatment enhanced the RH-123 efflux. **G, H** Cells treated with ZnO quantum dots (negative control) at 128 µg/mL after 18 h at 37 °C. Lack of blue fluorescence inside cells. **I, J** Control cells were treated with RH-123 (15 µM) and glucose (5 mM) for 5 h at 37 °C. Control cells display red fluorescence generated by RH-123. Merged brightfield (differential interference contrast DIC) and fluorescence **A, C, E, G, I** and DIC images of the unfixed sessile cells **B, D, F, H, J**

starting compound in the synthesis of sulfones was commercially available sodium p-toluene sulfinate, which was subjected to the reaction with chloroform and potassium hydroxide solution. Obtained dichloromethyl-p-toluene sulfone **1** was then oxidized to the 4-dichloromethylbenzoic acid by potassium dichromate **2**. Compound **3** was then brominated with sodium hypobromite to give bromodichloromethylsulfonyl benzoic acid **4**.

Sulfones **7** and **8** were prepared according to Fig. 7B and Supplementary data. The substrate for this synthesis was commercial 3-phenylpropanoic acid, which in reaction with chlorosulfonic acid gave 3-(4-chlorsulfonylphenyl) propanoic acid **5**, which after reduction with sodium sulfite gave sodium 4- (2-carboxyethyl phenyl) methanesulfinate **6**. This salt was used for the reaction with chloroform in the presence of potassium hydroxide to give 3- [4-(dichloromethylsulfonyl) phenyl] propanoic acid **7**. This compound was converted into 3-[4-(bromodichloromethylsulfonyl) phenyl] propanoic acid **8** by reaction with an aqueous solution of sodium hypobromite.

The syntheses of compounds **11** and **12** are shown in Fig. 7C and Supplementary data. The starting material was commercially available 4-methylbenzenethiol **9**, which was reacted with difluorochloromethane in a water-dioxane sodium hydroxide solution. The obtained difluoromethyl-4-toluene sulfide **10** was oxidized to difluoromethyl 4-methylphenyl sulfone **11** by hydrogen peroxide in acetic acid. Then the methyl group in sulfone **11** was oxidized to the carboxyl moiety using potassium dichromate in sulfuric acid to give sulfone **12**.

Sulfones **7, 12**, and **8** were attached to ZnO quantum dots that could act as fluorescent markers. Surrounding reactions ZnO quantum dots by sulfone ligands were carried out according to the unique procedure developed by [17], the so-called OSSOM method (one-pot self-supporting organometallic approach), shown in Fig. 7D and Supplementary data. Stable ZnO nanoparticles were not obtained as a result of the L-3 and Et$_2$Zn reaction. In this case, an alternative method of conjugate synthesis was used. In the first step, non-functionalized ZnO nanoparticles were obtained as a

Materials and methods

Chemistry

Sulfones **2, 3**, and **4** were synthesized according to the scheme shown in Fig. 7A and Supplementary data. The
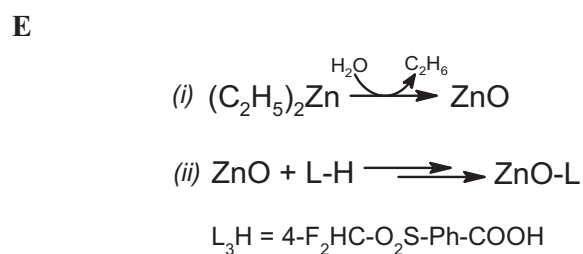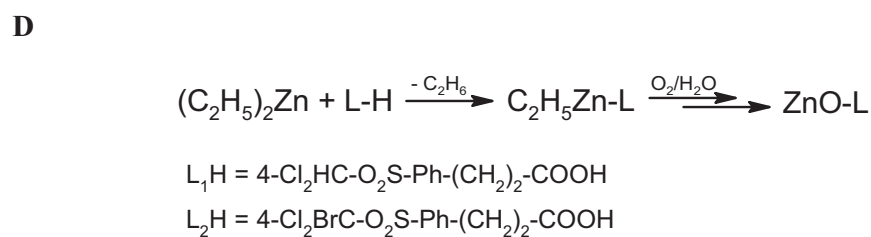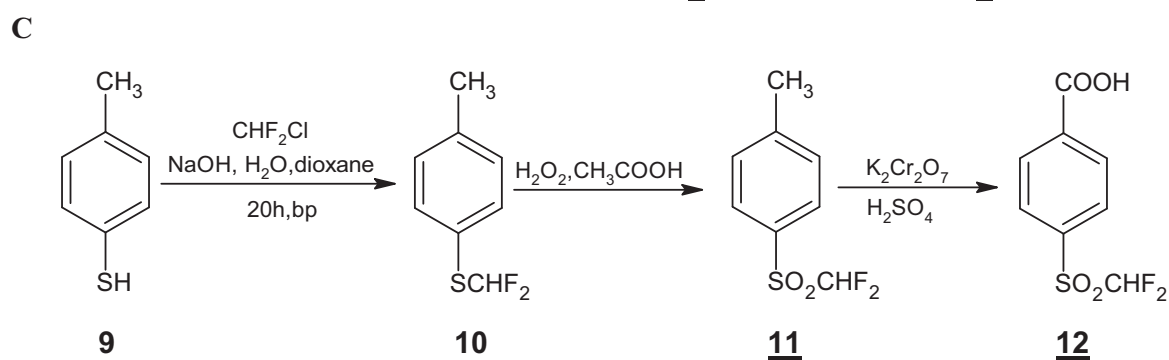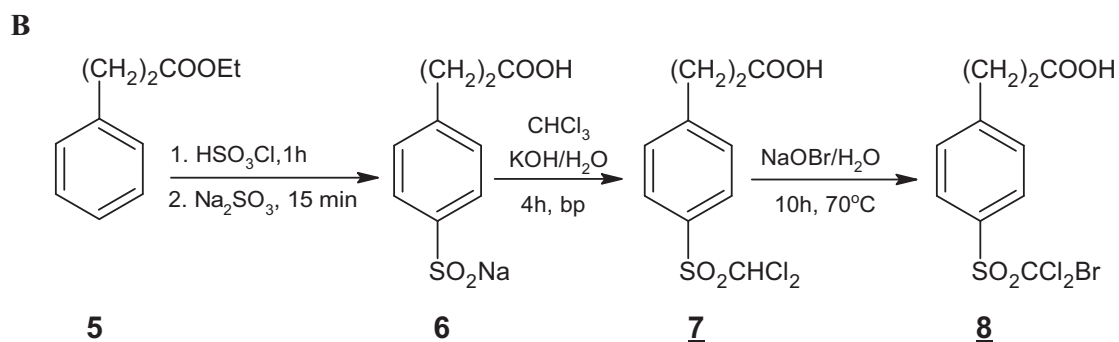
A

1   2   3   4

B

5   6   7   8

C

9   10   11   12

D

$$(C_2H_5)_2Zn + L\text{-}H \xrightarrow{\text{- } C_2H_6} C_2H_5Zn\text{-}L \xrightarrow{O_2/H_2O} ZnO\text{-}L$$

$L_1H = 4\text{-}Cl_2HC\text{-}O_2S\text{-}Ph\text{-}(CH_2)_2\text{-}COOH$

$L_2H = 4\text{-}Cl_2BrC\text{-}O_2S\text{-}Ph\text{-}(CH_2)_2\text{-}COOH$

E

$$(i) \ (C_2H_5)_2Zn \xrightarrow{\substack{H_2O \quad C_2H_6}} ZnO$$

$$(ii) \ ZnO + L\text{-}H \longrightarrow ZnO\text{-}L$$

$L_3H = 4\text{-}F_2HC\text{-}O_2S\text{-}Ph\text{-}COOH$
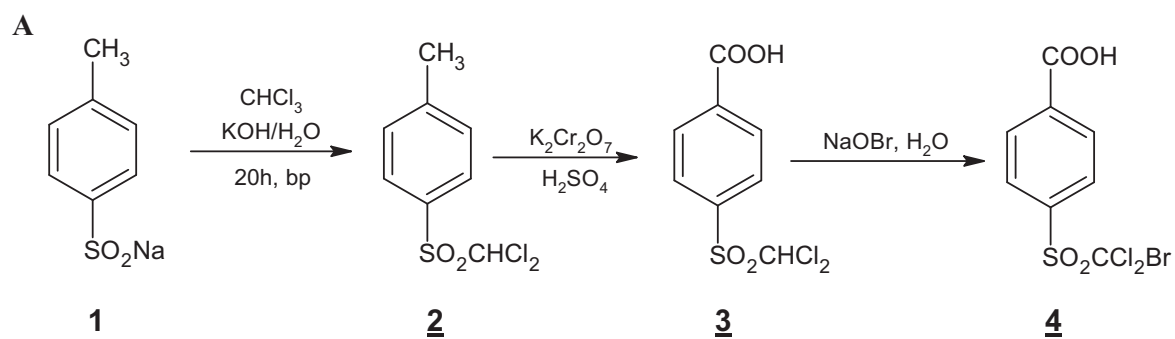
Fig. 7 Synthesis of sulfone derivatives **A 1, 2** and **3, B** dichloro- and (bromodichloromethylsulfonyl)phenylpropanoic acids **7** and **8, C 11** and **12** and **D, E**. attachment of carboxylic acids to ZnO quantum dots. Conjugate synthesis L-1, L-2, L-3

Table 3 Candida albicans strains

Strain	Parental	Genotype	Source of information
ATCC SC5314	–	Wild type	[24]
CAI4	SC5314	ura3Δ::imm434/ura3Δ::imm434	[24]
CAF2-1	SC5314	ura3Δ::imm434/URA3	[24]
cnb1Δ/CNB1	CAI4	cnb1Δ::URA3dpl200/CNB1	[25]
cnb1Δ/cnb1Δ	cnb1Δ::URA3dpl200/CNB1	cnb1Δ::URA3dpl200/cnb1Δ	[25]

result of a direct reaction of diethyl zinc with water. In the second step, an equilibrium amount of ligand was added to the ZnO nanoparticles (Fig. 7E).

Biology

Strains and media

Candida albicans strains used in the study are listed in Table 3. The quality control wild-type strain C. albicans SC5314 was obtained from the American Type Culture Collection (ATCC). All the strains were stored on ceramic beads in a Microblank tube (Prolab Diagnostics, Canada) at −70 °C. Before the respective examinations, routine cultures were performed in yeast extract-peptone-dextrose medium (YEPD) at 30 °C for 18 h [4].

Antifungal activity assays against the C. albicans planktonic growth

A susceptibility of SC5314 and CNB1 mutant planktonic cells to novel sulfone derivatives was determined using the M27-A3 method [5]. The final inoculum of 2.5×10^2 cells/mL was prepared in the synthetic RPMI 1640 medium (ThermoFisher). Compound concentrations ranging from 0.5 to 256 µg/mL were prepared with a stock solution (12,800 µg/mL) dissolved in DMSO. The cell growth was measured using a microtiter plate reader Synergy H4 Hybrid Reader (BioTek Instruments, USA) after incubation at 35 °C without agitation for 23, 28, and 48 h. Minimal inhibitory concentration (MIC) was defined as the lowest concentration of the compounds yielding cell growth (visual assessment) [18]. The percentage of cell growth reduction was calculated according to the following formula: % inhibition = 100% x [1 - (OD$_{405}$ CTW − OD$_{405}$ SCW)/(OD$_{405}$ GCW − OD$_{405}$ SCW)]. CTW was prepared with compound, medium, and C. albicans inoculum; SCW sterility control wells contained compound, RPMI 1640 medium, and sterile water replacing inoculum; GCW growth control wells were prepared with inoculum, RPMI 1640 medium, and the same amount of DMSO used in CTW. The minimal fungicidal concentration (MFC) was determined as described previously [6]. Briefly, 100 µL of aliquots from the selected wells of plates were removed after incubation at 35 °C for 48 h. Next, 100 µL of aliquots (1–10,000 diluted) were placed onto YEPD and incubated at 35 °C for 48 h. The MFC results for each sulfone derivative concentration tested were defined as the reduction viability record (R) and calculated using the formula: R = lg A − lg B, where A means CFU/mL of growth control wells (GCW), B means CFU/mL of sulfone-treated wells (CTW), and A or B were calculated according to the following quotation: CFU/mL= (mean number of colonies from 3 plates) x (inverse dilution coefficient plated) x 10 (counted per 1 mL). The lowest sulfone concentration that killed ≥ 99.9% of viable cells, compared with the GCW (lgR ≥ 3) was defined as MFC [18] and the concentration of a compound is considered to be fungistatic when 0 ≤ lgR < 3.

Metabolic activity assay

Antifungal activity of sulfone derivatives was assessed using the MTS test (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, MTS, Promega, USA) [5, 6]. Briefly, 10 µL of MTS was added to each well after incubation of cells with the compounds for 48 h. The optical density was measured at 490 nm and 660 nm (reference wavelength) using a microtiter plate reader after 3h-incubation of MTS with the treated cell suspension. Specific absorbance (SA) was calculated using the following formula: SA = A$_{490}$ − A$_{660}$. The reduction of viability was calculated using the following formula: % inhibition = 100% x [1 - (SA CTW − SA SCW)/(SA GCW − SA SCW)] [5, 6].

Antifungal activity of sulfone derivatives against the C. albicans biofilm

Biofilms of C. albicans were formed using a 96-well microtiter plate as described previously [6, 19]. The antifungal effect of sulfone derivatives against the C. albicans mutants' and wild-type strains' biofilm was monitored using the metabolic (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS, Promega, USA) assay. After 48-h incubation at 35 °C, the microplates were read as described above. The antifungal effect was measured by comparing the reduction in the mean absorbance of the compound-treated well to that of the untreated growth (control). The lowest concentration

showing a distinct reduction in the metabolic activity compared with the growth control was determined and the results were plotted as % of metabolic activity. Three replicates of biofilm were included for each experiment [6, 19].

In vitro cytotoxicity

The sulfone cytotoxicity against the kidney epithelial cell line Vero (ATCC CCL-81, LGC Poland) was assessed by MTS (3-(4.5-dimethylthiazol-2-yl)-5-(3-carboxymethox-yphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay) (MTS, Promega, USA) [6]. Vero cells were maintained in EMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco) at 37 °C and 5% CO_2. Vero cells (1×10^6 cells/mL of EMEM) were plated in 96-well microtiter plates for 24 h, following which the cell monolayer was treated with sulfone derivatives at different concentrations and incubated under the same conditions for 20 h. Subsequently, 10 μL of MTS was added to each well, and incubation for another 3 h in darkness proceeded. The concentration reducing cell viability by 50% compared with the untreated control was recorded as the cytotoxic concentration (CC_{50}) [7]. Additionally, the cytotoxicity of **4** and **12** was evaluated against the lung fibroblast cell line MRC-5 (ATCC CCL-171) maintained and collected as described above, using MTS as an assessment of cytotoxicity.

Rhodamine 123 (RH-123) dye efflux property

The final inoculum of 1.2×10^8 SC5314 cells/mL was prepared in phosphate-buffered saline (PBS) pH 7.4 with the addition of glucose and RH-123 at 5 mM or 15 μM [20, 21]. After 30 min of incubation at 35 °C, the cells were pelleted at 6000 x g for 2 min and resuspended in PBS containing 1 mM glucose and sulfones (**4, 7, 8, 12**) at 16 μg/mL or 128 μg/mL. Incubation at 35 °C was carried out for 15, 30, 45 min, 1 h, and 18 h, after which the cells were centrifuged (6000 × g, 2 min), and RH-123 fluorescence in the supernatants was assessed spectrophotometrically (Synergy H4 Hybrid Reader BioTek Instruments, USA) using excitation at 521 nm and emission at 627 nm. Additionally, *CNB1/cnb1Δ* and *cnb1Δ/cnb1Δ* treated with **4** or **12** were analyzed. The fluorescence of RH-123 released from cells treated with sulfones compared with the untreated control was recorded as % efflux. The experiment was conducted in three independent replications. The antifungal sulfone concentrations were selected based on the MIC/MFC studies [20, 21].

Flow cytometry analyses

Flow cytometry was applied to evaluate the mechanism of the *C. albicans* cell death caused by the tested sulfone derivatives at concentrations of 16 μg/mL and 128 μg/mL [5]. The final inoculum of 6.9×10^{11} cells/mL was prepared in sterile Mili-Q water, harvested (1500 × g, 4 °C, 10 min), and cells were incubated in 2.5 mL of buffer I (1 M sorbitol; 1 mM EDTA (pH 8.0); 50 mM 2-mercaptoethanol) at 28 °C for 30 min (180 rpm). The cell pellet collected by centrifugation under the same condition as before was washed twice in 3 mL 1 M sorbitol. Then, the cells were harvested in buffer II (1 M sorbitol; 1 mM EDTA, pH 5.8; 10 mM sodium citrate) and digested with lyticase (12.5 μg/mL) for 1 h at 37 °C (200 rpm). The pellet was centrifuged (3000 rpm, 4 °C, 10 min) and washed twice with 2.5 mL 1 M sorbitol. The protoplasts were resuspended in 1 mL of sterile Mili-Q water. The suspensions of cells or protoplasts were prepared in an MMD medium with the addition of tested compounds and incubated at 28 °C for 24 h (180 rpm). After centrifugation, the pellet was resuspended in Mili-Q water, and cells and protoplasts were stained with annexin V (Invitrogen), and incubated for 15 min at room temperature. The cell/protoplast suspensions were prepared in an annexin binding buffer (BD Pharmingen) and propidium iodide (PI) (Invitrogen) was added. 50 μL of the suspension was mixed with 450 μL PBS before the cytometric analysis was undertaken [5].

Ergosterol assay (HPLC)

The effect of sulfone derivatives on ergosterol synthesis in *Candida* SC5314 wild-type cells was determined using the previously described method [22]. Briefly, yeast cells at a density of 7×10^6 cells/mL in MMD with compounds (**2, 3, 11**, and **12**) at two concentrations of 0.5 μg/mL or 16 μg/mL were incubated for 24 h with shaking (100 rpm) at 30 °C. The cells were harvested by centrifugation at 1500 × g for 5 min, washed, and weighed. Each pellet was then treated with 3 mL of freshly prepared alcoholic potassium hydroxide solution (25%) and vortexed for 1 min. Cell suspensions were then incubated for 1 h at 80 °C in a water bath and cooled. Sterols were extracted by adding 3 mL of petroleum ether followed by shaking for 3 min. The organic phase was then transferred to a clean glass tube to evaporate petroleum ether on a rotary evaporator (Bunchi) at 60 °C under a vacuum. The extracted sterols were redissolved in 1 mL of methanol (Chempur) before HPLC-UV analysis. The sample containing ergosterol was analyzed at 281 nm with a C_{18} reverse-phase column. The mobile phase was a solution of acetonitrile/ methanol (97/3 v/v).

Antifungal activity of sulfone derivatives against exogenous ergosterol

The tested sulfone derivatives' interaction with exogenous ergosterol (Sigma) was determined using the method M27-

A3 [18] with a few modifications. The compounds (**2, 3, 7,** and **11**) were tested at the conc. range from 0.5 to 16 µg/mL. Briefly, the final inoculum of yeast suspension (10^3 CFU/mL) was prepared in MMD supplemented with ergosterol and distributed to the wells, giving ergosterol a final concentration of 400 µg/mL in the tested wells [22]. The ergosterol was prepared directly before the experiment. It was dissolved in 10% DMSO (v/v), and 1% Triton X-100 (v/v), heated to augment the solubility, and then diluted with the MMD medium. The tested compounds were added to the microtiter plate wells. Yeast growth control and sterility control were also analyzed. The plates were incubated at 30 °C for 4 days. Absorbance measurements were taken at 405 nm with an automatic plate reader every 24 h [22].

Antifungal activity of sulfone derivatives and amphotericin B (AmB) against exogenous ergosterol – assessment of the fractional inhibitory concentration (FIC)

The activity of sulfones (**2, 3, 11**, and **12**) at final conc.: 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 4 µg/mL, 8 µg/mL and 16 µg/mL against *C. albicans* SC5314 wild type strain (1×10^3 CFU/mL) in the presence of ergosterol (400 µg/mL), was tested with addition of AmB at final cons.: 0.04 µg/mL, 0.09 µg/mL, 0.16 µg/mL, 0.31 µg/m, 0.63 µg/mL and 1.25 µg/mL, respectively. The antifungal effect was measured by comparing the mean absorbance reduction of the compound-treated well to that of untreated growth control at 405 nm after 3 days using an automatic plate reader Synergy H4 Hybrid Reader (BioTek Instruments, USA). To characterize the interaction of each combination tested, the fractional inhibitory concentrations (FICs) of each agent tested and their sums are used to calculate the FIC index for the cell metabolic reduction, according to the following formula: index $FIC = A/MIC_A + B/MIC_B$, where A/B denotes the concentrations of tested compound (A) or amphotericin B (B), MIC_A and MIC_B mean the concentrations of a tested compound or AmB, respectively, which were defined as MIC of compound against *C. albicans*. The FIC index of <1 is the expression of the agents' synergism, whereas the FIC index of >1 represents an antagonism [23].

In addition, the minimal fungicidal concentration (MFC) was determined as described previously. Briefly, to verify if 0.5 µg/mL of **2, 3, 11**, or **12** used with 0.09 µg/mL of AmB could be considered as a fungicidal cons., the cell suspension treated for 48 h with the compounds and exogenous ergosterol (400 µg/mL) was plated on agar medium. The fungicidal conc. induced reduction of cells in 99.9%. The decimal logarithm of R was calculated.

Confocal laser scanning microscopy

The suspension of SC5314 (1×10^5 CFU/mL) in YEPD was inoculated on sterile glass disks in the 24-well culture plates to obtain biofilms by incubation at 37 °C for 20 h. The biofilm was washed with PBS and fresh YEPD medium with 15 µM rhodamine 123 and 5 mM glucose. After 5-h incubation, the cells were rinsed and treated with fluorescently labeled sulfones (**L1, L2**, and **L3**) at 128 µg/mL (selected based on previous studies), and incubated for 18 h at 37 °C. Subsequently, the observations were made using a confocal scanning microscope (CLSM, Fluoroview FV1000, Olympus) at excitation and emission wavelengths: $\lambda_{ex} = 553$ nm and $\lambda_{am} = 627$ nm (rhodamine 123), $\lambda_{ex} = 400$ nm and $\lambda_{am} = 655$ nm (fluorescently labeled compounds) [23].

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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