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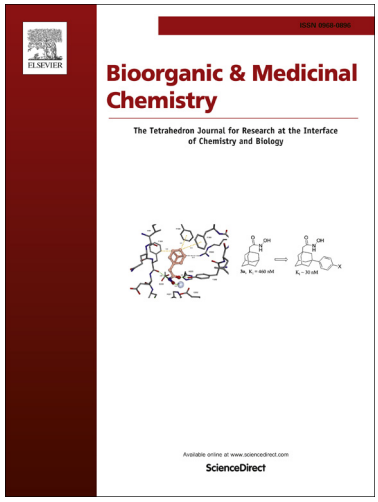

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New Synthetic Sulfone Derivatives Inhibit Growth, Adhesion and the Leucine Arylamidase *APE2* Gene Expression of *Candida Albicans in Vitro*

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Abstract

The successful preventing and effective treatment of invasive *Candida albicans* infections required research focused on synthesis of new classes of agents and antifungal activity studies. Bromodichloromethyl-4-chloro-3-nitrophenyl sulfone (named Compound 6); dichloromethyl-4-chloro-3-nitrophenyl sulfone (named 7); and chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone (named 11) on inhibition of planktonic cells' growth, leucine arylamidase *APE2* gene expression, and adhesion to epithelial cells were investigated. *In vitro* anti-*Candida* activities were determined against wild-types, and the morphogenesis mutants: Δ*efg1* and Δ*cph1*. MICs of Compounds 6, 7 and 11 (concentrated at 0.25-16 µg/ml) were determined using the Clinical and Laboratory Standards Institute Broth Microdilution Method (M27-A3 Document). *APE2* expression was analyzed using RT-PCR; relative quantification was normalized against *ACT1* in cells growth in YEPD and on Caco-2 cell line. Adherence assay of *C. albicans* to Caco-2 was performed in 24-well-plate.

The structure activity relationship suggested that sulfone containing hydrazine function at C-1 (Compound 11) showed higher antifungal activity (cell inhibition %=100 at 1-16 µg/ml) than the remaining sulfones with chlorine at C-1. *Δcph1/Δefg1* was highly sensitive to Compound 11, while the sensitivity was reduced in *Δcph1/Δefg1*::*EFG1* (%=100 at 16-fold higher concentration). Compound 11 significantly affected adherence to epithelium ($P \leq 0.05$) and hyphae formation. The *APE2* up-regulation plays role in sulfones' resistance on MAP kinase pathway. Either *CPH1* or *EFG1* play a role in the resistance mechanism in sulfones. The strain-dependent phenomenon is a factor in the sulfone resistance mechanism. Sulfones' mode of action was attributed to reduced virulence arsenal in terms of adhesiveness and pathogenic potential related to the *APE2* expression and morphogenesis.

Keywords: *Candida albicans,* sulfone derivatives, anti-virulence agents, *APE2*, adhesion, morphogenesis

1. Introduction

Candida albicans is the most common etiological agent of invasive fungal infections in immunocompromised hosts with cancer or AIDS and in organ-transplant patients.[1] The capacity of *C. albicans* to rapidly acquire resistance to antifungal drugs, such as amphotericin B, flucytosine, and azoles,[1] means that further studies are needed to examine the effect of new compounds on virulence factors of *C. albicans*.

The virulence factors, such as adhesion of the fungus to host cells and hyphae formation[2] have been suggested as attractive antifungal targets. Moreover, leucine arylamidase Ape2 is also believed to contribute to the invasion and to play a role in *C. albicans* virulence by removing the N-terminal L-leucine from peptide substrates.[3] Thus Ape2 facilitates the penetration of *C. albicans* into the host tissues.

Adherence to host tissues and morphological versatility are thought to be important in *C. albicans* virulence.[4, 5] Efg1 and Cph1 play a major role in promoting filamentous growth and regulates the expression of several genes with a crucial function in the invasion of host cells or in biofilm formation.[6, 7] As adhesion and morphogenesis are crucial for biofilm formation (difficult to eradicate with conventional antifungal therapy), it is fundamental to develop new approaches to managing the factors (*EFG1, CPH1, APE2*) associated with morphogenesis, adhesion, and tissue invasion. Thus the inhibition of morphogenesis factors represents a promising strategy for the design of anti-virulence drugs, especially in view of the alarming rise in life-threatening systemic fungal infections.[8] Moreover, potent degradative enzymes' inhibitors have been discussed as a novel antimycotic agent for the treatment of candidiasis.[8]

Sulfone derivatives provide an example of an important class of bioactive compounds with a wide spectrum of activities, as the sulfone group is an important core found in numerous biologically active compounds with a wide range of biological activity including antifungal properties.[9] There is evidence that the key feature of these compounds is a 6-

member heterocyclic ring attached to a sulfone, and additional modification of the benzene ring has been considered. Among these derivatives, phenyl trihalomethyl sulfone with different halogens: fluorine, chlorine and bromine prepared by Borys *et al.* [10] exhibited good inhibitory activity against plant pathogenic fungi. Moreover, Jha *et al.* [11] reported that 2-(5-sulfanyl-1,3,4-oxadiazol-2-yl)phenylacetate and 5-(pyridin-3-yl)-1,3,4-oxadiazole-2-thiol exhibit good antibacterial activities against *Escherichia coli* (MTCC 443). As part of our ongoing search for novel sulfone compounds displaying activity against the pathogenic *C. albicans* strains, new derivatives of bromodichloromethyl-4-chloro-3-nitrophenyl sulfone (named Compound 6) [12] and dichloromethyl-4-chloro-3-nitrophenyl sulfone (Compound 7),[12] and chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone (Compound 11)[10] were tested on some series of *C. albicans* strains. Moreover, studying *Candida* mutants lacking morphological transitionality (*EFG1* and/ or *CPH1*) may provide a deeper insight into the new compounds antifungal mechanisms under the epithelial growth model.

The goal of the present study was to investigate the *in vitro* susceptibility of planktonic cells of *C. albicans* wild-type strains and morphogenesis mutants to synthetic sulphones (named Compound 6, 7, 11 respectively). Furhtermore, we tested the effect of their activity on *C. albicans* adhesion to a monolayer cell culture of colorectal carcinoma of Caco-2 (ATCC). The next goal was to assess whether the expression of *APE2* is inhibited by the new Compound 11 (as the most effective from all the tested ones) during the development phase (adhesion) of the biofilm formation in *in vitro* mucosal infections. We evaluated the role that *APE2* as well as *EFG1* and *CPH1* play in the processes by inhibiting the enzymatic activity prior to the adhesion assay.

2. Materials and Methods

2.1. The sulfone synthesis, purification, and analytical data

Bromodichloromethyl-4-chloro-3-nitrophenyl sulfone 6 was synthesized according to the described procedure,[12] starting from chlorobenzene 1, which was chlorosulfonated by chlorosulfonic acid and obtained 4-chlorobenzenesulfochloride 2 was transformed into natrium salt of 4-chlorobenzene sulfinic acid 3 by alkaline reduction with natrium sulfite. Obtained sodium 4-chlorobenzenesulfinate 3 was converted into dichloromethyl-4-chlorophenyl sulfone 4 by reaction with chloroform in alkaline solution. Sulfone 4 was then brominated by bromine chloride, fresh prepared with bromine and chlorine as 50% solution in carbon tetrachloride. The resulting bromodichloromethyl-4-chlorophenyl sulfone 5 was converted into nitrocompound 6 in the next step applying the mixture of the concentrated sulfuric acid and nitric acid (Fig. 1). Dichloromethyl-4-chloro-3-nitrophenyl sulfone 7 was obtained by nitration of dichloromethyl-4-chlorophenyl sulfone 4 under similar conditions to the previous synthesis of sulfone 6 (Fig. 1). Chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone 11 was synthesized starting from natrium salt of 4-chlorobenzene sulfinic acid 3, which was subjected to the reaction with dichloroacetic acid in alkaline solution. Obtained chloromethyl-4-chlorophenyl sulfone 8 was then brominated by sodium hypobromite according to the described procedure.[10] Chlorodibromomethyl-4-chlorophenyl sulfone 9 was nitrated to yield nitrosulfone 10, followed by transformation with the reaction of nucleophilic substitution with hydrazine to obtain the final product (Fig. 1).

2.2. Strains and Media

Candida albicans strains used in the current study are listed in Table 1.[6, 13, 14] All the strains used in the present study were stored on ceramic beads in Microbank tube (Prolab Diagnostics, Richmond Hill, ON, Canada) at -70°C. Prior to the respective examinations, routine culturing of strains for growth was conducted at 30°C for 18 h in YEPD.[15]

2.3. Anticandidal activity against planktonic growth

Antifungal susceptibility of the compounds was determined for each strain using the method M27-A3 (CLSI).[16] The final inoculum: from $0.5x10^2$ to $2.5x10^3$ cells/ml saline was prepared in synthetic RPMI medium (Sigma, USA). The compound test wells (CTW) were prepared with stock solution of the compound (1600 µg/ml) dissolved in water with 9% (v/v) addition of DMSO. Subsequently, serial two-fold dilutions were made, using RPMI as a solvent. Then, the fungal blastoconidial suspension and the compound (final dilution 1:100) were dispensed into 96-well microplates. The compound was tested at seven concentrations that ranged from 0.25 to 16 µg/ml. The DMSO concentration was maintained at 0.09% (v/v) in all the experiments, including the control ones. At this concentration, DMSO was not able to inhibit the growth of *C. albicans*. Growth control wells (GCW) (containing medium, inoculum, the same amount of DMSO used in CTW, but compound-free) and sterility control wells (SCW) (sample, medium, and sterile water replacing inoculum) were included for each strain tested. The microplates were read with the Infinite M200 PRO NANOQuant (Tecan Group Ltd., Austria). MIC was read spectrophotometrically (optical density OD_{405}) and the end point was calculated as a 100% reduction in OD_{405} as compared to the growth in the control well. Growth reduction for each compound concentration was calculated as follows: % of inhibition: 100 - (OD_{405} CTW - OD_{405} SCW)/(OD_{405} GCW - OD_{405} SCW). Amphotericin B (Sigma-Aldrich, USA) was diluted in DMSO (1600 µg/ml) to be subsequently used in the assay as a reference antifungal at the concentration of 1 µg/ml (100% cell inhibition).

2.4. Cultivation and Infection of Caco-2 Cell Line (ATCC HTB27, LGC, Poland)

Following the supplier's guidelines, monolayers of the colon adenocarcinoma derived cell line were maintained in a humidified incubator at 37°C in 5% CO_2. For the experiment 1.2 x 10^5 of Caco-2 cells per milliliter were seeded into 24-well-plates (Corning, USA) and cultured in the EMEM medium (10% FCS, 1mM pyruvic acid, without antibiotics or antifungal agents) up to 18 h. Next, after 18-h post seeding the Caco-2 monolayers were inoculated with

10^5 log phase yeast cells of *C. albicans* wild types and mutants. After 18 h of incubation the Caco-2 was lysed by adding sterile water in the result of which the *C. albicans* cells were recovered.

2.5. Assay of Adherence to Human Line Caco-2 Epithelial Cells

Adherence of *C. albicans* to the Caco-2 cell line (ATCC HTB-37™) was performed as described previously.[5] Briefly, the Caco-2 cell line was cultivated as described above 2.4. Subsequently, the blastoconidia were grown overnight in the YEPD medium at 30°C. Then, final density 10^4/ml was added to each well of the epithelial cells to be afterwards incubated for 90 min (adhesion phase). Next, the non-adherent cells were removed by rinsing with PBS. Then, Caco-2 was lysed by adding sterile water resulting with *C. albicans* cells recovery. After 18-h growth on Sabouraud dextrose agar plates at 30°C, the number of adherent cells was determined by colony counting. Adherence was expressed as a percentage of the total number of cells added (control cells).

2.6. Inhibition of *Candida Albicans* Adhesion: Macrowell- Based Assay

The cells grown for 18 h in YEPD at 30 °C were then pre-treated with the Compounds 6, 7 and 11. Briefly, 200 µl of the blastoconidial suspension (at the final density of 10^4/ml) were preincubated with 1800 µl of the RPMI medium (containing selected concentration of the sulfones) for 2 h on a shaker at 35°C. Then, the cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 2000 µl of fresh RPMI medium. The wells of the plate containing adherent endothelial Caco-2 cells were washed twice with PBS and then incubated with the blastoconidial suspension (pre-treated with the sulfones) for 2 h at 37°C at 5% (v/v) CO_2. The *C. albicans* cells recovery and the assassing of adherent cells were conducted as described above 2.5.

2.7. RT-PCR Analyses to Assess the Effect of the Chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone 11 on the APE2 Gene Expression

Total RNA from cells was extracted as described Amberg *et al.* [17] RNA was isolated from cells after 18-h growth in YEPD at 30°C. Simultaneously, the cells after having been grown in the YEPD medium were washed with water and then 200 µl of the suspension was added to 1800 µl of the RPMI medium (the final density of 10^4 cfu/ml) and inoculated onto the Caco-2 monolayer. Incubation was conducted for 18 h at 37°C until the RNA extraction.

For the cells pre-treated with Compound 11, blastoconidia grown in YEPD (10^4 cfu/ml) after having been washed with water were suspended in the YEPD medium containing 16 µg/ml of Compound 11. Then, after 2-h incubation with Compound 11 the cells were washed with water and resuspended in 2000 µl YEPD for 18 h at 30°C. Simultaneously, the blastoconidial cells pre-icubated with 16 µg/ml of Compound 11 in RPMI after washing were resuspended in 2000 µl of RPMI and inoculated onto the Caco-2 monolayer for 18 h at 37°C. Prior to further examinations *C. albicans* total RNA was stored at -20°C.

First-strand cDNA synthesis was performed using the Enhanced Avian HS RT-PCR kit (Sigma-Aldrich, USA) according to the manufacturer's instructions. Appropriate Taq-Man primer set was designed for *APE2* by using Primer3 (Primer-BLAST, NCBI, Table 2). The primer set of *ACT1* (Table 2) was used as described previously by Naglik *et al.* [18] cDNA was quantified using the FastStart Essential DNA Green Master (Roche, Germany) according to the manufacturer's instructions. Each reaction mixture (15 µl) contained FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, 2 µl (250 nM) of each forward and reverse primer, water PCR grade, and 5 µl of template 60 ng cDNA.[18]. For reliable normalization of the *APE2* gene expression data in *C. albicans* cells grown for 18 h in both media we used the housekeeping gene *ACT1* as a reference gene. The real time PCR reactions were performed as described previously by Naglik *et al.*:[18] at 95°C for 15 min, followed by 45 cycles of 15 s at 94°C and 1 min at 60°C with the LightCycler 96 Instrument (Roche Diagnostics GmbH, Germany). A dissociation curve was generated at the end of each PCR

cycle to very that a single product was amplified. The C_T values were provided from RT-PCR instrumentation and were imported into a spreadsheet Microsoft Excel 2010. The relative quantification was calculated using Eq.,[19] where ΔC_T = Avg. *APE2* C_T – Avg. *ACT1* C_T and $\Delta\Delta C_T = \Delta CT - \Delta CT_{\text{parental strain}}$. Finally, $2^{-\triangle\triangle Ct}$ was calculated.

2.8. Phase-contrast Microscopy (Docuval, Carl Zeiss, Germany)

The cells of 90028 pre-treated with Compound 11 at 16 µg/ml for 18 h according to the CLSI document M27-A3[16] were examined under the phase-contrast microscope. Washed once with disitilled water, the cells were then suspended in 250 µl of water. Fifty µl of the suspension were pipetted onto microscope glass slides with coverslips on top to be later examined under the microscope. The control morphologies of 90028 (untreated with Compound 11) were generated in GCW wells (as described above 2.3.)

2.9. Statistical Analysis

Each experiment was performed in triplicate on three separate occasions. The percentage of cell growth inhibition as well as inhibition of adhesion and the *APE2* expression were formulated as a mean ± standard deviation. Statistical differences were evaluated through comparison with the non-parametric Wilcoxon test, $P \leq 0.05$ was considered significant.

3. Results

3.1. Antifungal Activity

At each concentration tested the % of inhibition displayed by the compounds was determined and compared with MIC of amphotericin B, with values summarised in Tables 3, 4, and 5.

Compounds 6 and 7, having bromodichloromethylsulfonyl and dichloromethylsulfonyl group respectively, at the para position of the phenyl rings attached to C-1, C-2 and C-4 chlorine, nitro and sulfone moiety respectively showed moderate activity against all the strains tested even at the maximum concentration of 16 µg/ml. Thus, MIC_{90}=16 µg/ml was assessed as a supra-MIC concentration (the highest concentration with incomplete killing). Interestingly,

the introduction of the bromodichloromethylsulfonyl group at the C-4 position of the benzene ring (Compound 6) exerted moderate activity against the null Δ*efg1* mutant and the rescued Δ*cph1::CPH1* strain ($P \leq 0.05$, Table 3). On the contrary, dichloromethylsulfonyl substituted compound at the C-4 position of the benzene ring (Compound 7) was more active against the following strains: ⊿*cph1* and⊿*cph1*/⊿*efg1* ($P \leq 0.05$, Table 4).

Compound 11 with hydrazino function at the C-1 position of the phenyl ring attached to C-2 and C-4 of the nitro and sulfone moiety respectively along with chlorodibromomethyl substituent at the C-4 position of the phenyl ring exerted a greater anti-*Candidal* potential.

The Table 5 shows, Compound 11 which was more effective against ⊿*cph1*, exhibited 100% of cell inhibition at 2 µg/ml ($P \leq 0.05$). In the case of ⊿*efg1*, the same level of cell inhibition was observed at a two-fold higher concentration ($P \leq 0.05$). Moreover, 100% of cell inhibition was noted for ⊿*cph1*/⊿*efg1* at the range of 1-16 µg/ml. Compound 11 out of all the compounds screened was the most effective (Table 5), with a clear end point (100% cell reduction) at 8 µg/ml against 90028. On the other hand, Compound 11 did not show MIC_{50} against 90028 at the lowest concentration tested of 0.25 µg/ml. Contrariwise, compounds 6 and 7 were found comparably effective, yet neither Compound 6 nor 7 displayed 100% of cell inhibition.

3.2. Impact of Sulfone Derivatives on Adhesive Properties of the *C. albicans* Species

Pre-treating the cells with sulfone 6 (8-16 µg/ml) did not significantly affect adhesion of all the *Candida* strains tested compared to their non-treated counterparts ($P \geq 0.05$, Table 6).

Attachment of *Candida* cells to the Caco-2 monolayer was inhibited ($P \geq 0.05$) at the highest concentration tested (16 µg/ml). Interestingly, in half of the strains tested cell adhesion increased after 90-min pre-treatment with two-fold lower concentration (8 µg/ml). On the contrary, the remaining strains displayed decreased adhesion at 8 µg/ml. At the concentration of 8 µg/ml, 90028 exhibited increase in attachment to Caco-2 compared to the non-treated

counterparts. In the case of the resued *⊿cph1::CPH1* the same phenomenon was noted, yet the increase in adhesion did not go beyond the adhesion level of the non-treated cells (observed in 90028). The adhesion level was 5.8-fold higher at 8 µg/ml *versus* to 16 µg/ml. Moreover, this feature rose three- and five-fold at 4 µg/ml and 2 µg/ml respectively compared to 16 µg/ml. The similar trend was observed for *⊿efg1* and *⊿cph1/⊿efg1,* i.e., 2.4- and 2.0-fold at 4 and 2 µg/ml respectively compared to the highest concentration tested.

Sulfone 7 did not significantly ($P \geq 0.05$) affect *Candida* cells' adhesion to epithelium at 2-16 µg/ml. Generally, the concentration of 16 µg/ml inhibited *Candida* cells' attachment to Caco-2, except for the rescued *⊿cph1/⊿efg1::EFG1* strain (increased adhesion, $P \geq 0.05$). The latter strain and 90028 also displayed increased adhesion ability at 8 µg/ml. The mutants (*⊿cph1*, *⊿efg1* and *⊿cph1/⊿efg1*) were inhibited in the attachment to the Caco-2 monolayer from 1.5- to 3.4-fold in comparison to the non-treated cells. Moreover, there was no considerable increase in adhesion at lower concentrations compared to 16 µg/ml.

Compound 11 altered adhesion of the strains significantly ($P \leq 0.05$) at all the concentrations tested. In 62% (5 strains) of all the strains tested the concentration of 16 µg/ml inhibited attachment of cells to Caco-2. The exceptions are as follows: 90028, *⊿cph1/⊿efg1*, and the latter one with one copy of *EFG1* reintroduced, in which adhesion increased under the 16 µg/ml treatment. In the case of 90028 the increase observed at 8 µg/ml was 1.7-fold higher compared to non-treated counterparts and cells treated with 16 µg/ml. Additionally, slightly increased adhesion at the concentration 8 µg/ml was noted for *⊿cph1* and its counterpart reverted with one copy *CPH1.* In the remaining strains, the level of attachment was reduced at the latter concentration compared to the control cells. Moreover as for the concentration of 4 µg/ml, an increased adhesion was seen for *⊿cph1/⊿efg1.* This feature was higher at 2 µg/ml in the following strains: *⊿cph1* reverted with *CPH1, ⊿efg1,* and *⊿cph1/⊿efg1* (2.3-, 1.6- and 1.3-fold compared to 16 µg/ml respectively).

3.3. Compound 11 Modulates the *APE2* mRNA Expression

The *APE2* expression significantly differed ($P \leq 0.05$) between the cells treated with Compound 11 and untreated and subsequently growing for 18 h both in the in the YEPD medium (Table 7) as well as on the Caco-2 monolayer (Table 8).

In the case of SC5314, the expression of *APE2* in the cells grown either in YEPD or on Caco-2 (pre-treated with Compound 11) was down-regulated ($P \leq 0.05$). *APE2* up-regulation was observed in the cells of *∆cph1* treated with Compound 3 grown both in YEPD and on Caco-2. In the cells of *∆efg1* up-regulation of *APE2* was noted under both tested conditions. The *∆efg1* strain reverted with one copy of *EFG1,* showed differences in the *APE2* expression between the cells preincubated with Compound 11 grown in YEPD and those grown on the epithelial monolayer. In the cells of *∆cph1/∆efg1* treated with Compound 11 an up-regulation of *APE2* in YEPD and a significant down-regulation (10-fold compared to the untreated cells) on Caco-2 ($P \leq 0.05$) was noted.

3.4. Microscopy Study. Compound 11 Perturbs *C. albicans* Morphogenesis

To visualize the disturbed hyphae formation under Compound 11, we performed a phase-contrast microscopy on *C. albicans* 90028 cells exposed to 16 µg/ml for 18 h (Fig. 2). Images revealed long true-hyphal morphologies of the untreated cells (Fig. 2(A)), and spherical blastoconidia with polarly located budding without filament formation under Compound 11 (Fig. 2(B)).

4. Discussion

The inhibition of degradative enzymes,[8] adhesion and morphogenesis factors[8, 18, 20] represents a promising strategy for the design of new compounds with effective antifungal abilities targeting *Candida* spp. In our study, Compounds 6 , 7, and 11 first tested for their activity against the pathogenic *C. albicans* planktonic cells differed in the inhibition abilities of yeast growth depending on their derivatives (Table 3, 4, and 5). By using the genetic alternations in

EFG1 and *CPH1* we tested whether these morphogenesis factors could have an impact on *C. albicans* resistance to the tested sulfones.

The lack of both *CPH1* and/or *EFG1* linked to the dysfunction of the cell wall integrity and filamentous growth of *C. albicans* significantly increased the fungicidal potential of Compound 11. As described Zavrel *et al.* [21] Efg1 and Cph1, strongly impact cell wall thickness as well as polysaccharide composition even if only one copy of the *EFG1* or *CPH1* genes is removed. In our study (Table 5), *Δcph1/Δefg1* was highly sensitive to the sulfone containing hydrazine function at benzene ring (Compound 11), while the sensitivity was reduced in the rescued *Δcph1/Δefg1*::*EFG1* strain (%=100 at a 16-fold higher concentration). Although deletion of the *CPH1* gene reduces hyphal growth on solid medium[6] we showed (unpublished data) that it still forms hyphae during adhesion to a polarized monolayer of Caco-2 epithelial cells. In stark contrast, the Δ*efg1* mutant was slightly attenuated in the developing filaments.[22] In our study, deletion of either *CPH1* or *EFG1* provoked excellent adhesion to the epithelial cells relative to 90028 and SC5314 (Table 6). Interestingly, a 90-min pre-treatment of the planktonic cells of the strains tested with Compound 11 significantly affected adherence to epithelial cells ($P \leq 0.05$) compared with the non-treated counterparts. Conversely, for the strains: 90028, *Δcph1/Δefg1,* and *Δcph1/Δefg1::EFG1,* the number of the adherent cells on the Caco-2 surface was not reduced significantly with Compound 11 at 16 µg/ml (short 90-min pre-treatment induced adherence) relative to the control cells. The development of Compound 11 tolerance was also likely to have impelled the remodelling of the cell wall components in *Δcph1/Δefg1*.[21] Moreover, under Compound 11 the compensation of adhesins recognizing ligands (*ALS3*, *HWP1*) can occur in the initial attachment to Caco-2.[23] Thus suggesting their contribution to the resistance to sulfone derivatives. Furthermore, over-expression of the efflux pump *MDR* gene[24, 25] induced by the sulfones tested ought to be considered. Our results demonstrated that the rescued Δ*cph1::CPH1* strain exposed to

Compound 11 showed increasing adhesion following decreased sulfone concentrations (concentration-dependent manner), but still lower than the non-treated cells. In the cells pre-treated with Compound 11, the loss of the morphogenesis factors either *CPH1* or *EFG1* decreased the adhesive properties. Conversely, the rescued Δ*cph1::CPH1* strain showing a higher adhesion level compared with Δ*cph1* under Compound 11, the differences were not signifiact ($P \geq 0.05$). Our studies not only provided a comprehensive evaluation of morphogenesis mutants as the determinants of the sulfone resistance, but also showed that each strain is likely to possess an altered cell wall composition influencing their susceptibility to these compounds. Thus the strain-dependent phenomenon ought to be considered as a factor in the sulfone resistance mechanisms. Furthermore, we demonstrated that it was particularly *C. albicans* heterozygousity that significantly influences the latter phenomenon (e.g., in the case of the rescued strains tested).

The reintroduction of an ectopic copy of the wild-type allele back into each mutant reversed their respective resistance to Compound 11. These results harmonize with the ones[8] showing that heterozygous cells are more resistant to antifungals. Furthermore, we found susceptibility in the MAP kinase pathway mutant against Compound 11, which confirms sulfones also targetting cell wall biosynthesis in *C. albicans.* Thus the cell wall playing a critical role in the sulfone resistance mechanism requires further investigation.

The exposure to antifungals induced up-regulation of drug targets and genes as contributing to resistance.[25, 26] In our study, the cells exposed to Compound 11 resulted in the *APE2* increased expression, which can imply resistance to this compound. The disruption of *CPH1* in *C. albicans* cells generated an *APE2* down-regulation. Conversely, under Compound 11 in the *Δcph1::CPH1* rescued strain *APE2* was downstream of *CPH1* on the MAP kinase pathway. This is in agreement with earlier results[27] that growth inhibition resulting from treatment with antifungals required both a functional histidine kinase and an inact HOG pathway. In our

study, Efg1-compromised cells displayed up-regulation of *APE2*, suggesting an Ras1-cAMP-independent mechanism of *APE2*. The activity of transcriptional factors is regulated in many different ways.[27] Cph1 and Efg1 were shown to be transferred to the nucleus and bound to its up-stream promoter regions of target genes in the absence of inducers.[28] Nobile *et al.*[29] showed that Efg1 is both an activator and repressor of its target genes. In compliance with the latter, *APE2* was up- or down-regulated depending on the regulator gene mutant background combination. It seems likely that these regulators are necessary as activators (Cph1) or negative regulators (Efg1) for *APE2* during the attachment to the Caco-2 monolayer (in stress: exposure to Compound 11). Conversely, both Cph1 and Efg1 negatively regulated the *APE2* expression during the epithelium colonization *in vitro* (without exposure to Compound 11). Furthermore, as found Pierce and Kumamoto[7] the expression of *C. albicans'* virulence factors was Efg1-dependant or not with respect to growth conditions. Our studies revealed that the *C. albicans* sulfone resistance events are associated with differences in the target gene transcription. Consistently with the previous data,[7] while comparing planktonic with sessile growth we revealed that Cph1 and Efg1 do not regulate adhesion. In order to interpret our whole data, it should be borne in mind that binding of the regulators is associated with differential transcription in the biofilm *vs* planktonic cultures.[29] Other possible explanation for the results obtained here is that Compound 11 can actually be a substrate of Ape2. As Ape2 is implicated in several facets of biological processes of fungal cells and in the interaction with the host,[3] it offers a promising target for the action of sulfones.

In our study genetic changes in both *EFG1* and/or *CPH1* enabled Compound 11 to gain access to intracellular targets, facilitating its membrane transience and increasing its contact with intracellular targets (transcription of *APE2*). Under Compound 11, adhesion of the wild-type cells was not reduced but the cells were defective in hyphae formation (Fig. 2B). Since hyphal development is an important step in a normal biofilm development *in vivo* (causing the

majority of infections in humans), it is worth indicating that we found Compound 11 inhibiting the morphogenesis process. Using our data, we identified the *APE2* gene that can be expected to play an important role in sulfone resistance (Compound 11). The overall results indicate that the performed antifungal screens are a valid approach to understanding how *C. albicans* cells respond to sulfone derivatives.

Performing genetic screens of *APE2* regulators is our future challenge allowing to answer whether *APE2* is controlled by more than one regulator under the sulfone influence. Moreover, sulfones' interaction with the cell wall biosynthesis pathway will provide an effective strategy to blocking *C. albicans'* invasion of epithelial cells.

5. Conclusions

To our knowledge, ours is the first study of sulfone derivatives' influence on virulence factors of *C. albicans*. Our data provide evidence that the sulfones' mode of action is associated with a reduced virulence arsenal in terms of pathogenic potential related to the expression of the *APE2* gene and the morphogenesis factors. The use of sulfone derivatives can successfully inhibit degradative enzyme production and the induction of hyphal forms constitutively expressed in *C. albicans* biofilms difficult to prevent by the current antifungal drugs. The results obtained may be of great value for the design of new efficient inhibitors of the cell wall components.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

6. Acknowledgements

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studies were funded by the National Science Centre (No. DEC-2011/03/D/NZ7/06198). The sulfones synthesis was supported by Warsaw University of Technology, Faculty of Chemistry.

Appendix A. Supplementary data

Supplementary material related to this article can be found at http://dx.doi.org/...../j.bmc....

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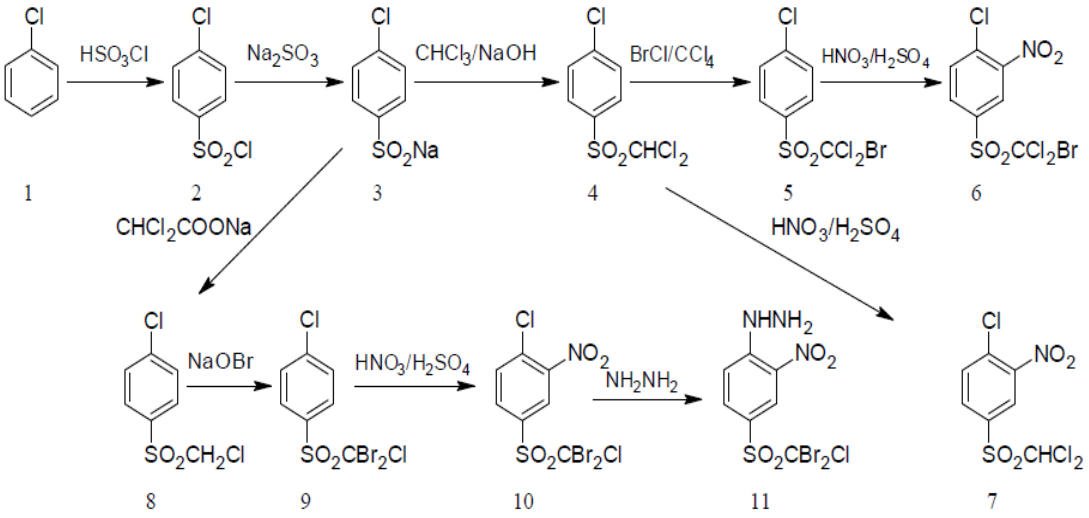
Figure Captions

Fig. 1. The sulphone derivatives synthesis. Detail procedures and characteristic data of synthesised compounds are given in Supplementary data

Fig. 2. Phase-contrast microscopy of *Candida albicans* untreated cells (A), and cells treated with 16 µg/ml of chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone (B). (A) After 18 h, note true hyphal forms grown in RPMI medium contained 0.09% DMSO. Oval blastoconidial mother cell (arrowhead) with polarly filamented true hyphal forms (open arrow) were discerned. (B) Note clumps of budding blastoconidial cells (arrowhead) upon exposure to Compound 11



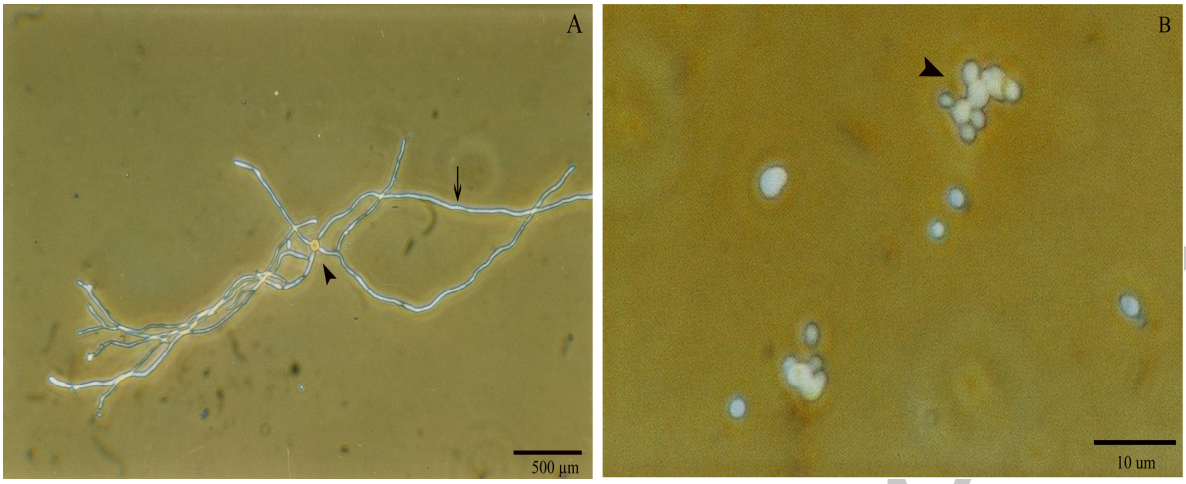


Table 1
Candida albicans strains used in the study

Strain	Parental strain	Relevant characteristics or genotype	Reference
SC5314		Wild type, reference strain	[13]
90028		Wild type, reference strain	[14]
Δ*cph1*	CAI4	*ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG-URA3-hisG*	[6]
Δ*cph1* (*CPH1*)	CAI4	*ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG (CPH1)*	[6]
Δ*efg1*	CAI4	*ura3::1imm434/ura3::1imm434 efg1::hisG/efg1::hisG-URA3-hisG*	[6]
Δ*cph1*/Δ*efg1*	CAI4	*ura3::1imm434/ura3::1imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG-URA3-hisG*	[6]
Δ*efg1* (*EFG1*)	CAI4	*ura3::1 imm434/ura3::1 imm434 efg1::hisG/efg1::hisG (EFG1)*	[6]
Δ*cph1*/Δ*efg1* (*EFG1*)	CAI4	*ura3::1 imm434/ura3::1 imm434 cph1::hisG/cph1::hisG efg1::hisG/efg1::hisG (EFG1)*	[6]

Table 2
List of primers used in this study

Primers	Sequence (5'-3')
APE2-1	GCACTGAATTCCCAACCAGT
APE2-2	TGGTTTAGTCGCTGATGCTG
ACT1-1	GACAATTTCTCTTTCAGCACTAGTAGTGA
ACT1-2	GCTGGTAGAGACTTGACCAACCA

Table 3
Antifungal activity (cells inhibition %) of bromodichloromethyl-4-chloro-3-nitrophenyl sulfone (Compound 6) against *Candida albicans* strains after 48 h

Candida albicans	**16**[b]		**8**		**4**		**2**		**1**		**0.5**		**0.25**		**1**[a]
90028	99.60±	0.32	99.51±	1.44	99.40±	0.25	98.90±	0.23	98.75±	0.35	98.72±	0.15	98.63±	0.60	100
Δ*cph1*	99.63±	0.1	99.32 ±	1.6	98.74±	0.1	98.90±	0.61	97.75±	0.14	97.42±	0.1	97.40±	0.25	100
Δ*cph1* (*CPH1*)	**99.80±**	**2.40**	**99.80±**	**0.34**	**99.72±**	**1.12**	**99.20±**	**6.84**	**98.72±**	**5.8**	**97.10±**	**2.5**	**96.70±**	**2.53**	100
Δ*efg1*	**99.61±**	**0.40**	**99.55±**	**0.8**	**99.00±**	**0.07**	**98.91±**	**0.07**	**98.53±**	**0.41**	**98.73±**	**0.1**	**98.40±**	**0.30**	100
Δ*efg1 (EFG1)*	99.45±	5.60	99.40±	5.14	99.30±	2.60	99.3±	3.83	99.2±	1.4	99.00±	1.22	99.0±	0.30	100
Δ*cph1*/Δ*efg1*	99.52±	7.7	99.41±	8.4	99.00±	0.32	98.75±	0.3	98.81±	0.06	98.62±	0.4	98.31±	0.6	100
Δ*cph1*/Δ*efg1 (EFG1)*	99.80±	1.55	99.80±	1.25	99.52±	3.4	99.30±	1.05	98.90±	2.95	98.80±	4.64	96.50±	2.34	100

[a], Amphotericin B in concentration of 1 μg ml^{-1} was used as a control (% inhibition=100). [b], MIC_{90} - the highest concentration with incomplete killing for the reference strain 90028. Values in bold indicate significant inhibtion ($P \leq 0.05$) of cells growth compared to the reference strain 90028

Table 4
Antifungal activity (cells inhibition %) of dichloromethyl-4-chloro-3-nitrophenyl sulfone (Compound 7) against *Candida albicans* strains after 48 h

Candida albicans	**16**[b]		**8**		**4**		**2**		**1**		**0.5**		**0.25**		**1**[a]
90028	99.42±	0.05	99.30±	0.003	99.20±	0.003	99.50±	0.007	99.24±	0.008	99.14±	0.003	99.11±	0.0006	100
Δ*cph1*	**99.60±**	**0.003**	**99.24 ±**	**0.004**	**99.12±**	**0.005**	**99.00±**	**0.003**	**99.35±**	**0.002**	**99.12±**	**0.008**	**97.7±**	**0.005**	100
Δ*cph1* (*CPH1*)	99.38±	0.006	99.36±	0.004	98.71±	0.045	98.63±	0.15	98.85±	0.01	96.74±	0.01	91.83±	0.01	100
Δ*efg1*	99.51±	0.006	99.42±	0.002	99.49±	0.003	99.40±	0.01	99.42±	0.02	99.36±	0.01	99.10±	0.01	100
Δ*efg1 (EFG1)*	99.20±	0.001	98.90±	0.004	98.53±	0.03	98.10±	0.01	96.05±	0.01	95.41±	0.01	95.2±	0.01	100
Δ*cph1*/Δ*efg1*	**99.72±**	**0.002**	**99.40±**	**0.001**	**99.25±**	**0.004**	**99.10±**	**0.01**	**99.10±**	**0.02**	**99.10±**	**0.01**	**99.05±**	0.01	100
Δ*cph1*/Δ*efg1 (EFG1)*	99.40±	0.002	99.32±	0.01	99.30±	0.002	99.22±	0.02	99.01±	0.01	99.01±	0.01	98.8±	0.02	100

[a], Amphotericin B in concentration of 1 μg ml^{-1} was used as a control (% inhibition=100). [b], MIC_{90} - the highest concentration with incomplete killing for the reference strain 90028. Values in bold indicate significant inhibition ($P \leq 0.05$) of cells growth compared to the reference strain 90028

Table 5
Antifungal activity (cells inhibition %) of chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone (Compound 11) against *Candida albicans* strains after 48h

Candida albicans	**16**		**8**		**4**[b]		**2**		**1**		**0.5**		**0.25**		**1**[a]
90028	100±	0.5	100±	15.1	99.73±	0.2	99.6±	0.2	97.40±	0.1	81.75±	1.44	17.0±	0.7	100
Δ*cph1*	**100±**	**0.15**	**100 ±**	**0.7**	**100±**	**0.3**	**100±**	**0.8**	**99.85±**	**0.4**	**96.6±**	**1.9**	**20.0±**	**0.5**	100
Δ*cph1* (*CPH1*)	99.81±	1.7	99.7±	2.0	99.1±	1.3	99.3±	0.95	98.8±	1.0	98.75±	0.5	98.6±	0.15	100
Δ*efg1*	**100±**	**0.62**	**100±**	**1.5**	**100±**	**3.0**	**99.74±**	**3.95**	**99.5±**	**1.40**	**97.9±**	**2.23**	**70.80±**	**1.4**	100
Δ*efg1 (EFG1)*	99.81±	1.7	99.80±	2.0	99.31±	0.95	99.1±	1.3	98.80±	1.0	98.75±	0.5	98.6±	0.15	100
Δ*cph1*/Δ*efg1*	**100±**	**0.11**	**100±**	**0.3**	**100±**	**2.44**	**100±**	**0.23**	**100±**	**0.35**	**97.0±**	**0.64**	**70.0±**	**0.40**	100
Δ*cph1*/Δ*efg1 (EFG1)*	100±	0.85	99.44±	0.9	99.1±	0.9	99.3±	1.05	98.8±	0.81	98.8±	1.0	72.0±	2.10	100

[a], Amphotericin B in concentration of 1 μg ml^{-1} was used as a control (% inhibition=100); [b], MIC_{90} - the highest concentration with incomplete killing for the reference strain 90028. Values in bold indicate significant inhibtion ($P \leq 0.05$) of cells growth compared to the reference strain 90028

Table 6

The percentage of adhesion of *C. albicans* cells to Caco-2 cell line after pre-treatment with the bromodichloromethyl-4-chloro-3-nitrophenyl sulfone (named Compound 6), dichloromethyl-4-chloro-3-nitrophenyl sulfone named (Compound 7), and chlorodichloromethyl-4-hydrazino-3-nitrophenyl sulfone (Compound 11). Adhesion data calculated for cells grown on Sabouraud agar of 24-well-plate. Adherence was expressed as a percentage of the total number of cells added (control cells non-treated). Data are expressed as the mean±SD of three independent experiments. Values in bold indicate significant reduction of cells adhesive properties compared to non-treated counterparts ($P \leq 0.05$)

Antifungal Compounds concentrations (µg/ml)		**90028**	**SC5314**	***Δcph1***	***Δcph1(CPH1)***	***Δefg1***	***Δefg1/Δcph1***	***Δefg1(EFG1)***	***Δcph1/Δefg1 (EFG1)***
Compound 6	**16**	**0.68±0.46**	**0.71±0.00**	**0.56±0.51**	**0.48±0.22**	**0.99±0.24**	**0.43±0.29**	**0.77±0.25**	**0.75±0.49**
	8	2.20±1.33	0.36±0.00	0.35±0.11	2.78±4.77	0.49±0.33	0.38±0.08	1.13±0.27	1.26±0.62
	4	nt	nt	0.55±0.46	1.39±0.06	1.14±1.16	1.02±0.37	nt	nt
	2	nt	nt	0.43±0.26	2.38±1.09	0.90±0.74	0.78±0.16	nt	nt
Compound 7	**16**	1.19±0.74	0.68±0.17	1.03±0.49	2.28±0.52	1.06±0.08	0.42±0.21	0.79±0.34	1.68±0.32
	8	2.05±0.87	nt	0.80±0.47	0.71±0.00	0.69±0.14	0.48±0.10	1.18±0.83	2.24±0.50
	4	nt	nt	0.99±0.54	1.28±0.24	0.97±0.84	0.36±0.09	nt	nt
	2	nt	nt	0.80±0.43	1.43±1.01	1.10±0.28	0.47±0.11	nt	nt
Compound 11	**16**	**1.60±1.59**	**0.80±0.40**	**0.37±0.12**	**0.78±0.75**	**0.81±0.36**	**2.25±1.12**	**0.89±0.42**	**1.50±0.74**
	8	**2.66±2.00**	**0.45±0.17**	**0.56±0.89**	**0.90±0.59**	**0.63±0.40**	**1.66±0,17**	nt	**0.75±0,50**
	4	**0,91±0.44**	nt	**0.22±0,08**	**0.76±0,05**	**0.69±0,17**	**2.74±1.03**	**0.91±0.44**	nt
	2	nt	nt	**0.51±0.23**	**1.86±0.35**	**1.32±0.36**	**2.94±0.59**	**0.51±0.23**	nt
Non treated cells		1.58±0.67	1.10±1.10	1.60±0.65	5.37±12.40	2.10±3.52	1.22±0.78	2.60±2.33	1.24±1.06

nt, not tested

Table 7
Analysis of the *APE2* gene relative expression compared to the *ACT1* reference gene in *C. albicans* cells untreated and after treatment with chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone (Compound 11). The cells were grown for 18 h in YEPD medium at 30°C.

C. albicans	Not treated cells				Cells treated with sulfone 3			
	C_t*APE2*	C_t*ACT1*	$\Delta C_{(t)}$	$2^{-\Delta\Delta C(t)}$	C_t*APE2*	C_t*ACT1*	$\Delta C_{(t)}$	$2^{-\Delta\Delta C(t)}$
SC5314	21.68±3,05	30.72±1.38	-9,04	3,81	26.55±3.05	29.39±0.50	-2,84	0,02
90028	23.04±1,75	28.27±0.73	-5,23	-[1]	26.79±1.35	29.61±0.45	-2,82	-[1]
Δcph1	24.85±2.92	31.15±11.99	-6,95	1,73	26.53±2.47	26.64±3.80	-0,11	-2,71
Δcph1 (CPH1)	20.46±0.03	31.56±2.24	-11,10	4,15	26.51±2.84	31.22±2.04	-4,71	4,60
Δefg1	22.34±2.52	28.57±3.29	-6,23	-4,87	26.87±2.85	30.56±2.74	-3,69	-1,02
Δcph1/Δefg1	23.79±1.82	30.94±2.03	-7,15	0,92	27.24±3.30	31.29±1.86	-4,05	0,36
Δefg1 (EFG1)	21.39±0.49	32.28±3.4	-10,89	3,74	26.38±2.33	29.82±0.01	-3,44	-0,61
Δcph1/Δefg1 (EFG1)	25.35±3.14	30.09±3.94	-4,74	-6,16	26.88±3.05	30.18±1.38	-3,30	-0,14

C_t- mean for three independent experiment ± SD; [1]strain 90028 – kalibrator in $2^{-\Delta\Delta C(t)}$

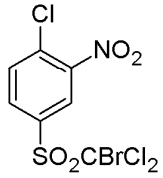
Tabela 8

Expression of the *APE2* gene compared to the *ACT1* reference gene in *C. albicans* cells incubated on Caco-2 cell line. The *C. albicans* cells were previously untreated and pre-treated with chlorodibromomethyl-4-hydrazino-3-nitrophenyl sulfone (Compound 11)

Candida albicans	**Time of incubation[a]**	**C_t*APE2***	**C_t*ACT1***	**ΔC_t**	**$2^{-\Delta\Delta Ct}$**	**Time of incubation[b]**	**C_t*APE2***	**C_t*ACT1***	**ΔC_t**	**$2^{-\Delta\Delta Ct}$**
SC5314	18	25.92±2.96	35.85±3.53	-9.93	3.49	6	25.65±2.91	32.03±3.42	-6.39	-1.05
						18	25.89	29.72	-3.83	-0.3
90028		25.91±3.35	32.35±2.64	-6.44	[1]	6	24.46±2.05	31.90±3.69	-7.44	[1]
						18	26.09	30.22	-4.13	[1]
Δcph1		21.85±1.35	32.45±2.54	-10.61	4.17	6	20.47±0.77	31.30±2.67	-10.83	3.39
						18	25.89	29.94	-4.05	-0.08
Δcph1 (CPH1)		23.31±1.58	33.01±3.23	-9.70	-0.90	6	21.21±1.56	31.46±3.80	-10.25	-0.59
						18	19.79	29.08±0.33	-9.29	5.24
Δefg1		22.05±1.36	31.04±2.27	-9.00	-0.70	6	20.96±1.26	31.23±2.86	-10.27	0.03
						18	19.79	28.86	-9.07	-0.22
Δcph1/Δefg1		22.78±0.71	32.79±3.40	-10.01	1.01	6	20.86±1.34	31.11±3.08	-10.26	-0.02
						18	26.53	25.15	1.38	-10.45
Δefg1 (EFG1)		23.71±0.89	31.06±2.46	-7.35	-2.66	6	20.75±1.34	31.45±3.05	-10.70	0.45
						18	20.35	29.07	-8.72	10.10
Δcph1/Δefg1 (EFG1)		24.25±0.81	30.85±1.65	-6.60	-0.75	6	21.50±1.65	30.92±2.90	-9.42	-1.28
						18	26.26	29.81	-3.55	-5.17

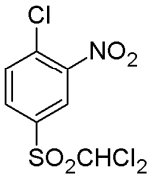
C_t - mean for three independent experiment ± SD; [1] - szczep 90028 - kalibrator in $2^{-\Delta\Delta Ct}$; [a], untreated cells; [b], pre-treated cells

Sulfones mode of action attributs to reduced virulence arsenal of *Candida albicans*



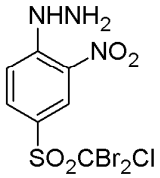
6

MIC_{90} = 16μg/mL



7

MIC_{90} = 16mμ/mL



11

100% cell inhibition at 1-16μg/mL