1,4-Naphthoquinone derivatives potently suppress Candida albicans growth, inhibit formation of hyphae and show no toxicity toward zebrafish embryos

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Abstract

Purpose. In this study, we applied various assays to find new activities of 1,4-naphthoquinone derivatives for potential anti-Candida albicans applications.

Methodology. These assays determined (a) the antimicrobial effect on growth/cell multiplication in fungal cultures, (b) the effect on formation of hyphae and biofilm, (c) the influence on cell membrane integrity, (d) the effect on cell morphology using atomic force microscopy, and (e) toxicity against zebrafish embryos. We have demonstrated the activity of these compounds against different Candida species and clinical isolates of C. albicans.

Key findings. 1,4-Naphthoquinones significantly affected fungal strains at 8–250 mg l⁻¹ of MIC. Interestingly, at concentrations below MICs, the chemicals showed effectiveness in inhibition of hyphal formation and cell aggregation in Candida. Of note, atomic force microscopy (AFM) analysis revealed an influence of the compounds on cell morphological properties. However, at low concentrations (0.8–31.2 mg l⁻¹), it did not exert any evident toxic effects on zebrafish embryos.

Conclusions. Our research has evidenced the effectiveness of 1,4-naphthoquinones as potential anti-Candida agents.

INTRODUCTION

Candida species are major human fungal pathogens that cause both mucosal and deep tissue infections. The genus comprises a heterogeneous group of organisms, with more than 17 different Candida species known to be aetiological agents of human infections. However, more than 90% of invasive infections are caused by Candida albicans, Candida glabrata, Candida parapsilosis, Candida tropicalis and Candida krusei [1]. Candida pathogenicity is facilitated by a number of virulence factors, the most important of which are those of adherence to host tissues and medical devices, morphological transition between yeast and hyphal forms, biofilm formation and secretion of hydrolytic enzymes [2].

Factors predisposing for the development of systemic (disseminated) candidiasis include immunosuppressive therapy with antibiotics with a broad spectrum of activity, transplantations, total parenteral nutrition, chemotherapy, long-term hospitalization associated with the occurrence of other serious diseases, surgeries and invasive diagnostic procedures [3–6]. Candida infections also constitute the most common fungal diseases in AIDS patients. These patients predominantly develop oropharyngeal candidiasis, which can lead to malnutrition and interfere with drug absorption [7, 8].

Recently, there has been an increase in the level of infection caused by the spread of fungal resistance to commonly used chemotherapeutic agents. Drug resistance in Candida is an important factor in its contribution to human disease. In
some areas of the USA, the percentage of fluconazole-resistant Candida strains isolated from blood reached 15.5% [1]. Experiments carried out on Candida strains isolated from HIV patients have shown that almost 10% of the strains were resistant to this antibiotic [9]. Cells involved in biofilm formation are particularly resistant to most antibiotics used currently. Only two out of the four antifungal agent classes, namely polyenes and echinocandins (e.g. amphotericin B and caspofungin), have exhibited consistent in vitro activity against C. albicans biofilms. This unique biofilm resistance to antifungal compounds is related to the presence of the extracellular matrix, which limits the penetration of drugs, and surface-located cells are the only cells to come in contact with an effective dose of antibiotic [10]. The emergence of Candida strains with increasing drug resistance has led to increased development of new antimicrobials.

Naphthoquinones are natural aromatic compounds that can be found in several plant families, or isolated from fungi, algae and bacteria. These classes of organic compounds are highly reactive and have various properties and applications. They are traditionally used as natural or synthetic dyes whose colour ranges from yellow to red. Recently, a variety of biological activities of these natural and synthetic compounds has been reported. In most cases, these pharmacological activities are related to reactivity and acid–base properties, which can be modulated synthetically by changing the substituents attached to the 1,4-naphthoquinone ring [11]. To date, some promising groups of compounds have been shown to have antibacterial [12,13], antifungal [14–16], antiviral [17,18], anti-tumour [19–23] and antimarial [12,24,25] activities. Nowadays, a number of 1,4-naphthoquinones, such as phylloquinone (regulation of blood coagulation, bone metabolism and vascular biology), lawson (natural dye), naphthazarin (natural dye) and atovaquone (antineumococcal), are used both parenterally and externally. Simultaneously we determined that none of the compounds showed the strongest antibacterial properties and clinical activity. and the emergence of Candida strains with increasing drug resistance has led to increased development of new antimicrobials.

We recently reported a new family of naphthalene-1,4-dione derivatives and their antimicrobial activity against selected bacterial species, e.g. Proteus, Escherichia, Klebsiella, Staphylococcus, Enterobacter, Pseudomonas, Salmonella and Enterococcus. A majority of the synthesized compounds showed the strongest antibacterial properties towards Staphylococcus aureus, with a high level of selectivity. Simultaneously we determined that none of the naphthalene-1,4-dione derivatives tested exhibited haemolytic activity against human erythrocytes [26]. In the present study, we expanded this research by indicating the strong antifungal potential of three of these synthesized compounds, namely 8, 9 and 14. The influence of these compounds on both reference Candida spp. and clinical isolates of C. albicans was tested. Next, the impact of naphthoquinones on C. albicans colony morphology and biofilm formation was verified. Using several tests, including AFM, the maintenance of the cell wall was explored. Additionally, the embryo- and genotoxic properties of the chemicals were tested.

### METHODS

#### 1,4-naphthoquinones

2-(2,4-dimethoxyphenyl)naphthalene-1,4-dione (8), 2-(2,4,6-trimethoxyphenyl)naphthalene-1,4-dione (9), and N-[4-(1,4-dioxo-1,4-dihydropyridine-2-yl)-3-methoxyphenyl]acetamide (14) were prepared by direct introduction of corresponding substituents into the naphthoquinone core, under oxidative conditions described previously [26]. In the first stage of synthesis, the naphthoquinone was activated with a strong mineral acid catalyst that enhanced its electrophilicity. Next, it underwent electrophilic aromatic substitution (SEAr) with an electron-rich nucleophilic aromatic compound with a rate, efficiency and selectivity corresponding to the values of local nucleophilicity of the chosen area. The dihydroxynaphthyl product formed in this stage of the reaction was oxidized in situ by either atmospheric oxygen or excess of naphthoquinone. The desired product was isolated chromatographically once the solvent had evaporated. In all cases, a solution of naphthoquinone and aromatic compounds used at a mol ratio of 2/1 in an appropriate solvent with an overall concentration of 1.2 (compounds 8 and 9) or 2.0 (compound 14) mol/ml, and in the presence of 1 mol% of the H$_4$PW$_{12}$O$_{40}$ catalyst, was kept in gentle reflux under the condenser for 24 h. The products were isolated by flash column chromatography on silica gel, using a gradient hexane/acetone mixture as an eluent. The DFT calculation of the reactivity of the aromatic compounds performed based on reactivity indices indicated that 3-methoxyphenylacetamide is much less reactive than 1,3-dimethoxy- and 1,3,5-trimethoxybenzene and, in consequence, the synthesis of 14 required a higher temperature and a medium of higher polarity. Thus, the synthesizes of compounds 8 and 9 was performed in acetone with 75 and 85% yields, respectively, while compound 14 was obtained in glacial acetic acid with a 15% yield. The spectral data of compounds 8, 9 and 14 and their melting point values were consistent with those mentioned in the literature. All compounds were obtained with purity above the 98% appropriate for biochemical studies.

#### Microbial strains

Naphthoquinones were screened for their in vitro antifungal activity against standard strains: Candida albicans ATCC 10231, Candida parapsilosis ATCC 22099, Candida tropicalis ATCC13803, Candida krusei ATCC14243, Candida glabrata ATCC15126, Candida kefyr ATCC 204093 and Candida lusitaniae ATTC 34449. One hundred clinical isolates of C. albicans derived from gynaecological patients (vaginal strains) and from sputum were kindly gifted by The John of God Independent Public Provincial Hospital in Lublin, Poland. The isolates were identified using VITEK 2 YST ID cards (Biomérieux).

The strains were stored in Microbank Mix kits (Biocorp, Poland), recommended for the storage of fungal strains at $-80^\circ$C, according to manufacturer’s instructions.
**MIC assay**

A previously described method was used to determine the susceptibility of *Candida* sp. to naphthoquinones [27, 28]. The yeast strains were inoculated in Sabouraud dextrose liquid medium (Biocorp, Poland) and incubated at 30°C with vigorous shaking (200 r.p.m.) for 24 h before performing the minimal inhibitory concentration (MIC) test. MIC was determined using the microbroth dilution method. Microbial cell suspensions at initial inocula of 3×10^5 colony-forming units per ml in Sabouraud dextrose broth were exposed to the tested compounds at adequate concentrations (range: 0.001–2.0 mg ml^-1) for 48 h at 30°C. MIC was defined as the lowest concentration of the compound that inhibited visible growth of the micro-organism. The experiments were performed in triplicate.

**Hyphal growth of Candida**

The effect of 1,4-naphthoquinones on hyphal growth of the *Candida* reference strain was evaluated using Spider medium as previously described [29]. *Candida* cells were grown overnight in Sabouraud broth medium (Biocorp, Poland) in a shaker at 180 r.p.m. and 37°C. At the late exponential growth phase, the yeast cells were harvested using a micro-centrifuge (Polygen 1-15PK, Poland) at 2300 g for 15 min. The yeast cells were washed twice with phosphate-buffered saline, pH 7.2, and resuspended in PBS to reach an optical density (OD_{600}) of 0.38 (10^6 cells ml^-1). One hundred microlitres of the suspension containing 10^6 cells ml^-1 were used for the assays. *Candida* cells were grown on Spider medium plates containing 10% fetal bovine serum (FBS) supplemented with or without the tested compounds at a concentration of 0.25–8 mg l^-1. The plates were incubated at 37°C for 36 h. The morphology of *Candida* colonies was inspected under a light microscope and imaged using a digital camera.

**In vitro biofilm formation assay**

Biofilm assays of the effect of the active chemicals on biofilm development and formation were carried out using a method previously reported [30]. The compounds were tested at concentrations corresponding to the values of MIC/2, MIC/4, MIC/8 and MIC/16, and 1% farnesol was used as a positive control [31]. The biofilm assays were performed using a micro-titre plate-based method. Sabouraud dextrose broth medium (Biocorp, Poland) was used to prepare the bacterial inoculum. To evaluate the effects of naphthoquinones on initial biofilm formation, the strain *C. albicans* was grown in Saburoud medium in a shaker (250 rev/min) at 37°C for 24 h. Then, the yeast culture was diluted (1:100) in the same medium containing sub-inhibitory concentrations (1/2, 1/4, 1/8 and 1/16 MIC) of the compounds. A volume of 200 μl of this mixture was inoculated into each well of a 96-well inert polystyrene micro-titre plate. After incubation of the microplate at 37°C for 24 h, the supernatants were removed and the biofilm was washed once with distilled water. It was dried and fixed at 65°C for 1 h. Finally, the wells were stained with crystal violet and washed, and the absorbance at 570 nm was determined using a microplate reader (BIOTEK SYNERGY HT). To examine the potential effects of the compound solvent in biofilm formation, 1% DMSO was used in place of the compounds tested in the experiment.

To analyse the effect of the active compounds on the mature biofilm, the growth of *C. albicans* biofilm was induced as described above but in the absence of the compounds, and was incubated for 24 h. Then, the supernatants were gently removed and the concentrations of 1/2, 1/4, 1/8 and 1/16 MIC for the compounds prepared in Sabouraud broth were added to each well of the micro-titre plate. After 24 h of incubation, the assay was read as described above. All experiments were performed at least three times with four replicates each, using 1% farnesol as positive control.

**C. albicans cell staining with propidium iodide (PI)**

The effect on the cytoplasmic membrane was tested using the method described by Lee and Kim [32]. *C. albicans* cells at the exponential phase (5×10^6 cells ml^-1) were incubated with each of the tested 1,4-naphthoquinones at a final concentration equivalent to their MICs, with shaking at 200 r.p.m. at 37°C for 4 h. The suspension was washed with phosphate-buffered saline (PBS, pH 7.4) and PI was added to create a final concentration of 10 µM. The cells were stained for 10 min in the dark at room temperature, and the effects of the tested naphthoquinones on the cytoplasmic membrane were evaluated with fluorescence microscopy (NIKON NIU).

**Effect of 1,4-naphthoquinone derivatives on DPH binding to C. albicans cells**

A method previously described was used to evaluate the effect of 1,4-naphthoquinones on the binding of 1,6-diphenyl-1,3,5-hexatriene (DPH) to the *C. albicans* membrane [32]. *C. albicans* cells (5×10^6 cells ml^-1) were incubated with 2× or 4×MIC 1,4-naphthoquinone derivatives at 37°C on a shaking incubator at 200 r.p.m. for 120 and 150 min, respectively. The control cells were incubated with the same volume of DMSO as in the case of the tested compounds. The cells were separated from the growth medium and the plant extract by centrifugation at 4000 g for 5 min, washed, and resuspended in PBS (pH 7.4). Each suspension was adjusted to an optical density of 595 nm for 1×10^8 c.f.u. ml^-1. To evaluate the effect of the tested naphthoquinones on 1,6-diphenyl-1,3,5 hexatriene (DPH) binding to the *C. albicans* membrane, the yeast cells were incubated with the fluorescence probe DPH at a final concentration of 2µM at room temperature for 30 min in the dark. The samples were washed with PBS (pH 7.4), and fluorescence was measured in a black, 96-well microplate using a spectrofluorometer (BIOTEK) at 360 nm excitation and 460 nm emission wavelength. The results represent an average of quadriplicate measurements from three independent assays.

**Effect of naphthoquinones on membrane permeability**

Alteration of membrane permeability was investigated using a crystal violet assay proposed by Lee et al. [32]. *C. albicans*...
cells at the exponential phase were harvested by centrifugation at 4500 g at 4 °C for 5 min. The cells were washed twice and resuspended in 0.85 % NaCl. The tested 1,4-naphthoquinones corresponding to the concentrations of 1, 2 or 4 × MIC were added to the suspension and incubated at 37 °C, 200 r.p.m. for 8 h. Solvent (DMSO) controls were included for each compound. Cells were harvested and washed in 0.85 % NaCl, and the cell density was adjusted in each experimental group to equate to 1 × 10⁸ cells ml⁻¹. Next, the cells were resuspended in 0.85 % NaCl solution containing 10 µg ml⁻¹ of crystal violet. The cell suspensions were incubated at 37 °C, 200 r.p.m. for 10 min. The cells were precipitated by centrifugation at 12 000 g at 4 °C for 15 min, and the amount of crystal violet remaining in the supernatant was measured at 590 nm using a spectrophotometer. The OD values of the initial crystal violet solution used in the assay were regarded as 100 %. The percentage of crystal violet uptake of all cells was calculated as follows: uptake of crystal violet (%) = (A_{590} of the sample)/(A_{590} of crystal violet solution) × 100.

**Loss of 260 nm-absorbing materials and proteins**

In order to investigate the antifungal effect of 1,4-naphthoquinone derivatives on the integrity of the *C. albicans* cell membrane, the release of 260 nm-absorbing materials and proteins was determined spectrophotometrically using the method of Kahn et al. [33] with slight modifications. Briefly, *C. albicans* cells (1 × 10⁹ cells ml⁻¹) at the exponential phase were washed twice and dissolved in 0.85 % NaCl. The suspension was treated with 4 × MIC compounds or the same volume of DMSO (control) as in the case of the tested compounds. At each time point (0, 1, 2 and 3 h), 0.5 ml of the cell suspension was taken and filtered through a 0.22 µm filter. To measure the leakage of cellular materials absorbing at 260 nm, the absorbance of the filtrate was read at 260 nm using a UV spectrophotometer, from which the absorbance of the DMSO control was subtracted. To evaluate protein leakage, the filtrate was mixed with Bradford reagent according to the method provided by the manufacturer (SIGMA Aldrich) and absorbance at 595 nm was read using a spectrophotometer. The data represent the absorbance at 595 nm of the sample at each time point in reference to the control (DMSO). The data are representative of three independent experiments carried out in duplicate.

**Sorbitol protection assay**

Duplicate plates containing 1,4-naphthoquinones or amphotericin B were prepared. One plate contained twofold dilutions of the tested compounds and the other contained the tested compounds and 0.8 M sorbitol as an osmotic protectant. All the wells inoculated with the *C. albicans* cell suspension were incubated at 37 °C, and MICs were determined using the method described above at 24 and 72 h.

**Ergosterol binding assay**

Duplicate plates containing 1,4-naphthoquinones or amphotericin B (positive control) were prepared. One plate contained twofold dilutions of the tested compounds and the other contained the tested compounds and 200 µg ml⁻¹ of ergosterol. Each well, inoculated with 100 µl of the cell suspension (1–5 × 10⁶ cells ml⁻¹), was incubated at 37 °C. MIC end-points were determined after 2 and 7 days.

**Genotoxicity of naphthoquinones**

*C. albicans* genomic DNA was isolated (using a kit from A and A Biotechnology, Poland) from logarithmic cells cultured in the presence/absence of naphthoquinones at a concentration of MIC/2. For the *in vitro* assay, prior to agarose electrophoresis, 100 ng of DNA was incubated with chemicals at the MIC concentration at 37 °C for 24 h. The condition of DNA was inspected using agarose electrophoresis.

**Influence of naphthoquinones on zebrafish embryo development**

The collected embryos were transferred to a Petri dish with E3 medium (5 mM NaCl, 0.33 mM MgCl₂, 0.33 mM CaCl₂, 0.17 mM KCl; pH 7.2) and then placed in 6-well plates, 10 embryos per well. Stock solutions of naphthoquinones 8, 9 and 14 were prepared in DMSO. In these experiments, three series of solutions with differing naphthoquinone concentrations were employed, which were freshly prepared by dissolving stock solutions in the E3 solution each time directly before addition to the wells. The solutions were changed once daily and the embryos were maintained in the incubator at 28.5 °C. Zebrafish embryos were evaluated for developmental abnormalities and viability at 24 h intervals for up to 5 days using a stereomicroscope (Zeiss Axio Vert, ZEISS, Germany). Morphological deformities of the heart, dorsal string and tail development were measured and compared to the control embryos. Image analysis was performed to determine the percentage of dead and malformed embryos over time using Zeiss software. The same embryos (n=10) were followed throughout the study.

**Atomic force microscopy (AFM) analysis of *C. albicans* cells treated with naphthoquinones**

Five millilitres of *Candida albicans* cell suspension cultured under the pressure of amphotericin-B and the tested naphthoquinone 8 at concentrations of MIC/10 (0.2 mg l⁻¹) and MIC/4 (7.5 mg l⁻¹), respectively (DMSO was used as a control) in liquid medium were centrifuged for 8 min at 2000 g. The supernatant was removed from the precipitate and 5 ml of distilled water was added to the tube and centrifuged for 8 min at 2000 g. After centrifugation, the supernatant was removed and 3 ml of distilled water were added. The slurry was applied to degreased glass slides (10 µl) and allowed to dry at room temperature.

Analysis of the topography and sample properties was performed using atomic force microscopy (AFM) Ntega Spectra C from NT MDT. Analysis of the data was carried out using NOVA 1.1.0.1824 software. Topography measurements, including error signal and phase contrast, were performed in semi-contact mode (tapping mode) with a 135 µm NT-MDT NSG03 Cantilever with typical resonant frequency 90 kHz and force constant 1.74 N/m. Scanning
RESULTS AND DISCUSSION

1,4-naphthoquinones show fungistatic activity against Candida albicans

The synthesis and antibacterial activities of the newly synthesized family of 1,4-naphthoquinones have been reported [26]. Consecutive studies revealed that three of the nineteen compounds, namely 8, 9 and 14 (Fig. 1a), showed strong antifungal potential against C. albicans. Initially, the chemicals were tested against a panel of seven reference Candida species with the use of microdilution assay. Although five of the tested species were susceptible to the action of the examined compounds, only C. albicans was potently affected, with MIC values of 8.0–31.2 mg l⁻¹ (Fig. 1b). The activity level of compounds 8, 9 and 14 was the same as that of ketoconazole and up to 17–100-fold lower in reference to caspofungin (Fig. 1a). The compounds did not show a fungicidal effect up to the concentration of 4 × MIC (data not shown). The fungistatic effects of 8, 9 and 14 were also confirmed by the growth curves of C. albicans cultured at different concentrations of the compounds (Fig. 1c). Next, the fungistatic activity of the chemicals was tested against 83 clinical vaginal and sputum isolates of C. albicans. The majority (83–87%) of the clinical isolates was refractory to the action of 8, 9 and 14 in the range of concentrations tested (0–250 mg l⁻¹). The percentage of susceptible isolates was comparable for all three compounds, i.e. 12, 16 and 16%, respectively (Fig. 1d).

The anti-Candida activity of 1,4-naphthoquinone derivatives, both from natural sources and chemical synthesis, was previously reported. The representatives of natural compounds, i.e. 2-methoxy-1,4-naphthoquinone from Impatiens balsamina, plumbagin isolated from Plumbago scandens and shikonin isolated from Lithospermum erythrorhizon, showed potency towards Candida sp. with MIC values of 0.6–25 mg l⁻¹ [34–36].

1,4-naphthoquinones inhibit cell adhesion of Candida albicans

Since the biofilm appears to be a determinant of poor prognosis in candidiasis, the biofilm formation process is a common therapeutic target [8]. The macroscopic observations on C. albicans colonies grown in the presence of the tested compounds revealed that, starting from the concentration of MIC/16, the naphthoquinones effectively interfered with the hyphal growth process (Fig. 2a, b). The strongest effect was observed in the case of compounds 8 and 14, which completely abolished hyphal formation at all concentrations tested. Since the yeast–hyphae transition is crucial in biofilm maturation and maintenance [37], the influence of the compounds on mature C. albicans biofilm was investigated. As expected, naphthoquinones were able to disrupt the biofilm almost completely at the concentration of MIC/2, although anti-biofilm activity was observed at the concentrations from MIC/16 (0.5–1.95 mg l⁻¹). Surprisingly, the activity of 14 was found comparable to a known anti-biofilm agent, farnesol (Fig. 2c).

Considering compounds 8, 9 and 14 as effective anti-biofilm drug candidates, the influence of the chemicals on the first, hyphae-independent, phase of biofilm formation, namely adhesion, was tested. The observed effect was significantly lower, although compound 8 at the concentration of MIC/2 inhibited the adhesion phase by ~50% (Fig. 2d).

The tested compounds showed significant differences in activity against the mature biofilm and adhesion phase in comparison to ketoconazole [38]. Compound 14 at the concentration of MIC/8 (1 mg l⁻¹) reduced mature biofilm by 85%, while ketoconazole at the concentration of 0.4 mg l⁻¹ reduced the process by 65%. On the other hand, 14 did not show any effect on biofilm synthesis while ketoconazole reduced it by 75% at the concentration of 0.4 mg l⁻¹. Such differences probably indicate different mechanisms of action of these compounds.

These results suggest that the morphological switching of C. albicans cells can be considered as a potential target for 1,4-naphthoquinones.

Cell membrane of C. albicans is affected by 1,4-naphthoquinones

Binding of DPH and its derivatives to intra-membranes is coupled with a strong increment in their fluorescence [39]. Therefore, the influence of 1,4-naphthoquinones on the C. albicans plasma membrane was examined using the DPH fluorescence probe. DPH fluorescence intensity indicated a reduction in numbers of 1,4-naphthoquinone-treated C. albicans cells. The treatment with compound 9 for 150 min resulted in a fluorescence reduction to 66.7 and 50% at the concentrations of 2 and 4 × MIC, respectively (Fig. 3a).

It has been suggested that altered membrane potential is correlated with enhancement of membrane permeability [40]. To check whether naphthoquinones also affected the permeability of the C. albicans cell membrane, crystal violet and propidium iodide assays were performed. As shown in Fig. 3(b), there was a concentration-dependent increase in crystal violet uptake by 41.3, 27.9 and 37.7% for compounds 8, 9 and 14, respectively. A similar effect was observed when PI was added to the cell suspension (Fig. 3c).

Disruption of the physical integrity of the fungal cell membrane has been associated with antifungal agents that exhibit rapid fungicidal activity. To check whether the tested
compounds would show such activity, leakage of the intracellular content was studied. The 1,4-naphthoquinones induced steady leakage of the cellular components in a time-dependent manner (Fig. 3d, e). The treatment of the fungi with compounds 8 and 14 resulted in a nearly linear release of the cellular components absorbing light in the 260 and 595 nm wavelength regions.

To check whether 1,4-naphthoquinones would bind membrane ergosterol, an essential lipid for many aspects of the physiology of *C. albicans*, the MICs of 1,4-naphthoquinones were evaluated in the absence and presence of ergosterol in the broth. As shown in Fig. 3(f), ergosterol did not modify the activity of 8, 9 or 14, which is the result of the absence of interactions between the lipid and naphthoquinones. The data confirm that, in contrast to AMP-B, 1,4-naphthoquinones do not bind ergosterol directly in the *C. albicans* membrane.

The damaging effects of an antimicrobial compound on the cell wall can be reversed in the presence of osmoprotectants such as sorbitol. No such effect was observed in the case of naphthoquinones (Fig. 3).

This result suggests that 1,4-naphthoquinones are involved in a manner that alters the membrane composition of *C. albicans* cells, leading to a change in membrane permeability and disruption of their physical integrity. In this respect, the compounds show similarities to the action of known antifungal polyenes (e.g. amphotericin-B, nystatin) which bind to ergosterol and disrupt cell membrane permeability causing leakage of the cellular content [41].

**AFM imaging of *C. albicans* cells treated with naphthoquinone 8**

Atomic force microscopy analysis demonstrated particular differences in the topography of *C. albicans* cells treated with naphthoquinone 8 in comparison to the controls (Fig. 4). The control cells (a1) had a smooth surface and regular shape, while the surface of cells treated with the antifungal compounds, namely amphotericin-B (b1) and naphthoquinone 8 (c1), was rough.
Phase contrast imaging allows visualization of areas on the surface of cells of varying softness. Cells treated with AMP-B (b2) and chemical 8 (c2) were softer than the control cells (the scale shows force values ranging from 70 to 170 degrees). Additionally, cells grown under the pressure of compound 8 (c2) were softer than those treated with AMP-B (b2). The adhesion force measured for the control cells (a3) was 31.75 nN (min. 29.62 nN–max. 40.46 nN, SD 3.13). Comparable values were obtained for cells treated with compound 8 (c3): 30.50 nN (min. 27.23 nN–max. 38.52 nN, SD 3.26). The highest values of adhesion force 48.32 nN (min. 45.78 nN–max. 58.07 nN SD 3.54, $P<0.001$) were found for cells treated with AMP-B (b3), which may suggest changes to their surface.

In AFM analyses, the magnitude of cantilever vibration is dependent on sample stiffness (the z-scale shows values ranging from 0 to 12 nA). The surface of cells treated with AMP-B exhibited areas with loss of stiffness (b4).

The height of \textit{C. albicans} cells is shown in Figs 4 and 5. The grey line in Fig. 4 represents the location of the height measurement. The difference in height between the control cells and those treated with AMP-B and naphthoquinone 8 is illustrated in Fig. 4(d, e). The average height of \textit{C. albicans} control cells (C) is 1394.05 nm (min. 1026.0 nm–max. 1743.66 nm), 1539.76 nm for AMP-B (min. 1289.14 nm–max. 1794.93 nm) and 1695.41 nm for compound 8 (min. 1409.59 nm–max. 1943.80 nm). The height of the control cells (1394.05 nm, SD 159.76) is significantly lower compared to cells treated with AMP-B (1539.76 nm, SD 154.00, $P<0.005$) and cells treated with compound 8 (1695.41 nm, SD 122.95, $P<0.0000001$). The differences in the sizes of cells treated with AMP-B or naphthoquinone are also statistically significant ($P<0.001$).

As in the case of amphotericin-B, AFM revealed changes in the physical properties of \textit{Candida} cells treated with 8. The effect of this compound on cell morphology suggests that targeting of certain molecular events is responsible for maintaining the proper cellular structure.

### Toxic effect of 1,4-naphthoquinones on genomic DNA and zebrafish embryos

In order to verify their application as potential antimicrobial therapeutics, the tested compounds were examined in terms of their toxic activity. Previously, we reported that compounds 8 and 14 are not harmful to human erythrocytes up to a concentration of $8 \times$ MIC [26]. Here, the absence of any genotoxic effect of 1,4-naphthoquinones on \textit{C. albicans} genomic DNA is demonstrated (Fig. 5c).

Since the zebrafish is a promising model for whole-organism toxicology screening due to its rapid development, morphological changes in zebrafish embryos induced by
naphthoquinones were further evaluated. Lethality of 100% induced by naphthoquinones 8 and 14, and a 50% rate for 9, were observed after 1 day at a concentration of 10×MIC, while the dose of 1×MIC resulted in 100% lethality only in the case of compound 8, and in 20% after 24 h and 40% after 48 h in the case of 14. The experiment revealed no morphological changes in zebrafish embryos or toxic effects of compounds 8, 9 and 14 used at doses of 0.1×MIC during 5 days of observation. Significant morphological changes were observed after the application of 0.4×MIC of compound 8 on the second day, and 0.4×MIC of compound 9 on the fourth day of observation, while the dose 0.4×MIC of compound 14 did not induce any changes. Morphological disorders were noted after the application of 1×MIC of compound 9 on the third day of observation, and of 1×MIC of 14 on the fourth day (Figs 5a and S1, available in the online version of this article).

Fig. 3. (a) Influence of the tested compounds on the ability of DPH to bind to the intra-membrane space of C. albicans cells. Rate of crystal violet (b) and propidium iodide (c) uptake by Candida cells incubated in the presence of compounds 8, 9 and 14. Release rate of cellular components absorbing light in the 260 nm (d) and 595 nm (e) wavelength regions from Candida cells incubated in the presence of compounds 8, 9 and 14. Influence of ergosterol (f) and sorbitol (g) on the activity of the tested naphthoquinones.
Compounds 8 and 9 at a concentration of $0.4 \times \text{MIC}$ were found to cause pericardial oedema (PE), scoliosis (S) and tail autolysis (TA) (Figs S2 and S3). In turn, compound 14 was safe at the above-mentioned concentration ($0.4 \times \text{MIC}=3.2 \text{ mg l}^{-1}$) and was found to induce pericardial oedema (PE) and scoliosis (S) from a concentration of $1 \times \text{MIC}$ (Figs Sb and S4). This result shows the lower toxicity of 14 in comparison to difenoconazole, which was investigated by Mu et al. [42, 43]. This known antifungal agent at a concentration of $0.5–2 \text{ mg l}^{-1}$ caused a large suite of symptoms including hatching regression, heart rate decrease, growth inhibition and teratogenic effects. Currently, little is known about the effect of antifungal agents on the embryonic development of *Danio rerio* and the results presented results may represent a significant contribution in this field.

**Conclusions**

The panel of assays presented revealed that 1,4-naphthoquinones may be promising lead compounds for further

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**Fig. 4.** Multiparametric imaging of *Candida albicans* cells by AFM. (a), (b) and (c) show AFM images of *C. albicans* control cells, positive control (AMP-B) and cells treated with naphthoquinone 8, respectively, as a representation of error signal (1), phase contrast (2), adhesion (3), stiffness (4) and height (5). (d), (e) height of the cells in the colony. The line of measurement is marked in white in (a)–(c).
development of agents against candidiasis. The compounds showed MIC values in the range 8–31 mg l\(^{-1}\) against the reference \(C.\ albicans\) strain. Despite lower activity in comparison to the reference strain, naphthoquinones showed fungistatic activity against clinical isolates of \(C.\ albicans\). The compounds effectively interfered with various aspects of living \(C.\ albicans\) cells. At concentrations from 0.25 mg l\(^{-1}\), they inhibited hyphal formation and influenced the adhesion phase during biofilm formation. Further tests revealed that the compounds affected the integrity of the

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**Fig. 5.** (a) Influence of the tested compounds on the embryonic development of \(Danio\ rerio\). (b) Morphological abnormalities of zebrafish embryos induced by naphthoquinone 14 (PE: pericardial oedema; S: scoliosis) observed on one particular day of the experiment (table). (c) Influence of naphthoquinones on \(C.\ albicans\) genomic DNA under \textit{in vitro} and \textit{in vivo} conditions.
fungal cell wall. The value of naphthoquinones as potential antifungals is increased by the acceptable toxicity level demonstrated.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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