Title: Blocking and dislocation of Candida albicans Cdr1p transporter bystyrylquinolines

Author: Joanna Szczepaniak, Wioleta Cieślik, Anna Romanowicz, Robert Musioł, Anna Krasowska

Citation style: Szczepaniak Joanna, Cieślik Wioleta, Romanowicz Anna, Musioł Robert, Krasowska Anna. (2017). Blocking and dislocation of Candida albicans Cdr1p transporter bystyrylquinolines. "International Journal of Antimicrobial Agents" (Vol. 50, iss. 2 (2017), s. 171-176), doi 10.1016/j.ijantimicag.2017.01.044

International Journal of Antimicrobial Agents 50 (2017) 171–176

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

journal homepage: www.elsevier.com/locate/ijantimicag

Blocking and dislocation of *Candida albicans* Cdr1p transporter by styrylquinolines

Joanna Szczepaniak [a,*], Wioleta Cieślik [b], Anna Romanowicz [a], Robert Musioł [b], Anna Krasowska [a]

[a] *Faculty of Biotechnology, University of Wroclaw, Wroclaw, Poland*
[b] *Institute of Chemistry, University of Silesia, Katowice, Poland*

ARTICLE INFO

Article history:
Received 22 September 2016
Accepted 30 January 2017

Keywords:
Candida albicans
Cdr1p
Styrylquinolines
ABC transporters
Drug design

ABSTRACT

Styrylquinolines are a novel group of quinoline drugs that are known to have p53-independent antiproliferative activity and antiviral properties. This study evaluated the antifungal activity of these drugs more deeply, particularly their activity modulation towards Cdr1p, the main multidrug transporter of *Candida albicans*. Styrylquinolines were found to have antifungal activity and to work synergistically with fluconazole. Additionally, they decreased the extracellular concentration of rhodamine 6G in ABC-transporter-expressing cells. The cellular localization of GFP-tagged Cdr1p was assessed by epifluorescent microscopy. Styrylquinolines induce expression of Cdr1p, as confirmed by Western blotting. Three of four drugs tested caused the partial delocalization of transport protein to the cytoplasm. These results show the first evidence that styrylquinolines decrease the activity of ABC multidrug transporters in *C. albicans*.

1. Introduction

Candida albicans is one of the leading causes of opportunistic mycoses, including hospital-borne infections. Most commonly, it causes superficial but often persistent oral or vaginal candidiasis. However, in patients with immunodeficiency infection, life-threatening systemic candidiasis can occur [1]. *C. albicans* can gain different mechanisms of resistance to commonly used antifungals such as azoles. The main reason for *C. albicans* drug resistance is the presence of ABC and MFS transporters in the plasma membrane. Three efflux pumps are responsible for decreasing the intracellular concentration of azoles. These pumps are encoded by genes for candida drug resistance (*CDR1* and *CDR2*) and multidrug resistance (*MDR1*). These proteins differ in the source of energy used for their activity and in their specificity to drug molecules [2]. The use of efflux pump inhibitors [3], or the synergistic action of two or more drugs with different mechanisms of action [4] could resolve the problem of candida drug resistance.

One of the most popular structural scaffolds is 8-hydroxyquinoline, which is found in many biologically active natural products and therapeutic agents [5]. Its derivatives have numerous applications, including antimicrobial agents for both Gram-negative and Gram-positive bacteria [6], antiviral [7] and anticancer [8] therapy, anti-epileptic and anti-obesity treatments [9], and pain management drugs [10]. It generally has comparably low cytotoxicity and high clinical applicability. Additionally, it is widely used as fungistatics in agriculture [11]. For this reason, the quinoline moiety has been considered among other mono- and diazanaphthalene scaffolds as a privileged structure that is believed to produce active molecules more often [12].

Styrylquinolines consist of quinoline and phenyl rings connected with an unsaturated ethylene linker, which results in a flat and rigid conformation that is structurally related to amphotericin B (AmB) and naftifine. Styrylquinolines are known to inhibit HIV integrase [13] and have p53-independent antiproliferative activity [14]. Due to their structure and lipophilic character, they can interact with the cell membrane during passive transport. As with other quinoline derivatives, styrylquinolines are known to have potent antifungal activity. However, their mechanism of action is largely unexplored. While 8-hydroxyquinoline may act as a metal chelator and disrupt cell wall biosynthesis, it has been suggested recently that the mechanism of action may consist of more complex interactions [15]. Moreover, in more complex quinoline derivatives, it has been shown that metal chelation only plays a minor role in their antifungal activity [11,16]. Other 8-hydroxyquinoline derivatives are postulated to act as inhibitors of (1,3)-β-D-glucan synthase, chitin synthase [17] or P450 14-α-demethylase [18], or by changing the pH in the phagocytic vacuole [19]. This study examined styrylquinolines as possible inhibitors of *C. albicans* ABC transporters. The results demonstrate that these compounds work

* Corresponding author. Faculty of Biotechnology, University of Wroclaw, ul, Fryderyka Joliot-Curie 14a, 50-383 Wroclaw, Poland.
E-mail address: joanna.szczepaniak@uwr.edu.pl (J. Szczepaniak).

http://dx.doi.org/10.1016/j.ijantimicag.2017.01.044

synergistically with fluconazole, and also inhibit the efflux of rhodamine 6G (R6G), a known substrate for ABC pumps.

2. Materials and methods

2.1. Compounds

All four styrylquinolines were obtained as part of the search for novel active antifungal agents [16,20]. They were synthesized according to known methods from aromatic aldehydes and appropriate quinaldines [14,21]. Stepwise partial hydrolysis was afforded in acetylated (WK14B, WK15B) or fully hydrolysed products (WK14, WK15). After column chromatography purification, the compounds were used for biological tests. Styrylquinolines, fluconazole (Sigma Aldrich, Poznan, Poland) and AmB (Sigma Aldrich) were dissolved in dimethyl sulphoxide (DMSO, Bioshop, Rzeszów, Poland), and fluphenazine (Sigma Aldrich) was dissolved in sterile water.

2.2. Strains and growth media

The *C. albicans* strains used in this study were a generous gift from Prof. Dominique Sanglard (Table 1). All strains were kept as frozen stocks in glycerol at –80 °C and grown routinely at 28 °C on YPD medium with 2% dextrose, 1% Bacto peptone (Difco, Warsaw, Poland) and 1% yeast extract (Difco). For all experiments, with the exception of a rhodamine 6G assay, strains were subcultured in yeast nitrogen base (YNB) liquid medium [0.67% YNB (Difco) and 2% dextrose] at 28 °C and diluted to desirable optical density at 600 nm (OD_{600}). For growth on solid media, 2% agar (Difco) was added.

2.3. Susceptibility testing

Drug susceptibility testing was performed in microtitre plates with two-fold serial dilutions of tested compounds (Fig. 1) according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST E.DEF 7.3) specifications with modifications. Cultures were grown for 18 h in YNB and diluted to A_{600} of 0.1 in a total volume of 200 μL, with the tested compound in concentration so that the DMSO concentration was 1%. Microtitre plates were incubated at 28 °C for 24 h, shaken, and optical densities were read with a microtitre plate reader at a wavelength of 600 nm. The results are presented as the percentage of growth relative to control samples, and MIC_{50} values are given as the lowest concentration inhibiting 50% of growth according to EUCAST standards. The experiment was performed in three biological replicates with three technical replicates for each of the four styrylquinolines.

Table 1
Collection of *Candida albicans* strains used in this study.

Strain	Genotype	Reference
CAF 2-1	*ura3Δ::imm434/URA3*	[22]
DSY 448	*cdr1Δ::hisG-URA3-hisG/cdr1Δ::hisG*	[23]
DSY 653	*cdr2Δ::hisG-URA3-hisG/cdr2Δ::hisG*	[24]
DSY 654	*cdr1Δ::hisG/cdr1Δ::hisG cdr2Δ::hisG-URA3-hisG/cdr2Δ::hisG*	[24]
ASCa1	*ura3Δ::imm434/URA3 CDR1-GFP*	[25]

Strain CAF 2-1 was treated as the wild-type strain.

2.4. Fluconazole–styrylquinoline combination assays

Interactions between fluconazole and tested drugs (Fig. 1) were measured by obtaining the fractional inhibitory concentration (FIC) index values [26]. Cultures were grown for 18 h in YNB and diluted to A_{600} of 0.1 in a total volume of 200 μL with a mixture of fluconazole and styrylquinoline, each at a starting concentration of MIC_{50}. Microtitre plates were incubated at 28 °C for 24 h, shaken, and optical densities were read with a microtitre plate reader at a wavelength of 600 nm. The sum of the FICs (ΣFIC) was calculated for each well with the equation ΣFIC = FICA + FICB = (MICA + B/MICA) + (MICB + A/MICB), where MICA and MICB are the MICs of drugs A and B alone, respectively, and MICA + B and MICB + A are the concentrations of the drugs in combination, respectively, in all the wells corresponding to an MIC_{50}. The experiment was performed in three biological replicates with three technical replicates.

2.5. Permeability assays

Yeast cultures were subcultured overnight in YNB and diluted to OD_{600} of 0.4 in fresh medium. The styrylquinoline compounds, fluphenazine (Sigma Aldrich) or AmB (Sigma Aldrich) were added and incubated at 28 °C with shaking at 180 rpm. Aliquots of cultures were taken after 4 h and stained with propidium iodine [27,28] for 5 min at room temperature to assess membrane permeability. Observations were made using fluorescent microscopy (Zeiss AxioVision, Poznan, Poland). The experiment was performed in three biological replicates.

2.6. Western blotting

The assay was performed in accordance with a previous method [29] with modifications. Crude protein extract was prepared from

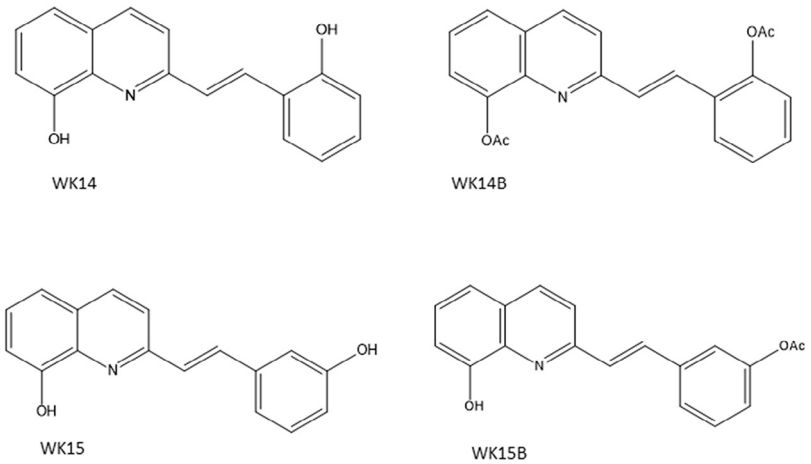

Fig. 1. Styrylquinoline compounds used in this study.

cell suspensions after 4 h of induction with the tested compounds. Aliquots of cell suspensions were pelleted by centrifugation at 2260 × *g* for 5 min and resuspended in 1 mL of deionized water. Cells were lysed by adding 150 µL of 1.85 M NaOH–7.5% β-mercaptoethanol and incubated on ice for 10 min. Proteins were precipitated by adding 150 µL of 50% trichloroacetic acid and incubated on ice for 10 min. Samples were centrifuged at 10,000 × *g* for 5 min at 4 °C, washed in 1 mL of 1 M Tris–HCl pH 8.0, and resuspended in 50 µL of sample buffer (40 mM Tris–HCl, 8 M urea, 5% SDS, 0.1 mM EDTA 1% β-mercaptoethanol, 0.1 mg/mL bromophenol blue). Following incubation at 37 °C for 30 min, samples were loaded on 6% sodium dodecyl sulphate–polyacrylamide gel and developed in a Mini-PROTEAN II electrophoresis cell (Bio-Rad, Poznan, Poland). Samples were then transferred onto nitrocellulose membrane using a Mini-PROTEAN Tetra System electrophoresis cell (Bio-Rad). Membranes were stained with Poncau S to check for equal loading of gels. Immunodetection of Cdr1p was performed using polyclonal mouse anti-GFP antiserum and horseradish-peroxidase-conjugated antimouse antiserum as a secondary antibody. Signals were detected using an ECL kit (PerkinElmer, Krakow, Poland) according to the manufacturer's instructions. The experiment was performed in four biological replicates.

2.7. Rhodamine 6G assay

R6G assay was performed according to Nakamura et al [30] with modifications. The cell culture was pelleted in the log phase and washed twice in double-distilled water and once in HEPES buffer (50 mM, pH 7.0). Cells were diluted in fresh HEPES buffer to OD_{600} of 1.0 and incubated for 60 min at 30 °C and 200 rpm with 5 mM 2-deoxy-D-glucose (Sigma Aldrich). Next, the tested compounds were added in half MIC_{50} concentrations, and cells were incubated for 5 min in the same conditions. Next, 10 µM R6G was added and the cell suspension was incubated for an additional 90 min. Cells were pelleted, washed twice in HEPES buffer, and suspended in fresh HEPES at OD_{600} of 10.0. The cell suspension was incubated for 5 min at 30 °C with shaking, R6G efflux was initiated by adding 10 mM glucose, and suspensions were incubated for 30 min with aliquots removed at 15-min intervals. Aliquots of 400 µL were pelleted, and three duplicates of 100 µL of supernatant were added to black microtitre plates. Fluorescence was measured in a Cary Eclipse spectrofluorimeter (Agilent Technologies, Santa Clara, CA, USA) at an excitation wavelength of 529 nm (slit 5) and emission of 553 nm (slit 10). The experiment was performed in three biological replicates with three technical replicates.

2.8. Cytotoxicity assay

Normal human fibroblasts were grown in MEM alpha medium with foetal bovine serum supplementation at 5%. Cells were cultured in 25-cm^2 tissue culture flasks at 37 °C in 5% CO_2/95% humidified air, and passaged twice per week, using 0.25% trypsin solution with 0.05% EDTA added. Cells were seeded in 96-well plates. They were grown for 24 h and styrylquinoline cytotoxicity was tested by MTT assay [31]. Styrylquinolines were added to the cells and incubated for 48 h, after which 5 µL of MTT stock solution (5 mg/mL) was added. Cells were incubated for 4 h at 37 °C. Following removal of the medium, 50 µL of DMSO was added to the cells and plates were stirred for 10 min. The absorbance of formazan, a metabolite of MTT, was measured at a wavelength of 570 nm using an ASYS UVM340 microplate reader (Biogenet, Jozefow, Poland). The experiment was performed in two biological replicates with three technical replicates.

3. Results

3.1. Antifungal activity of styrylquinolines

The MICs of styrylquinolines were determined for the wild-type strain and for single and double *cdrΔ* mutants. All four tested drugs were diluted in DMSO and then diluted in YNB to final concentrations of 0.4, 0.2, 0.1 and 0.05 mM, followed by incubation for 24 h as described above. The two compounds with a hydroxyl group in the phenyl ring, WK14 and WK15, had the lowest MIC_{50} values among the drugs tested (Table 2). Control compounds 8-hydroxyquinoline and 8-hydroxyquinaldine had a similar effect on the strains. Substitution of an acetoxy group in the same positions (WK14B, WK15B) decreased drug activity, particularly for *cdr2Δ* and wild-type strains. In general, the cells with *CDR1* deletion showed a significant increase in sensitivity to styrylquinolines in contrast to 8-hydroxyquinoline and 8-hydroxyquinaldine, suggesting that styrylquinolines are Cdr1p substrates. This indicates a possible mode of action as competitive inhibitors of this transporter. As Cdr1p has a higher impact on fluconazole resistance than Cdr2p, the strain without Cdr1p transporters is characterized by a lower fluconazole MIC than strains that express Cdr1p transporters (0.25 µg/mL vs. 2 µg/mL for *cdr2Δ* and wild-type strains). However, the simultaneous presence of fluconazole and styrylquinolines abolishes the Cdr1p-induced resistance, as evidenced by the FIC index values in Table 3. No synergistic effect was observed in the case of the *cdr1Δ* strain. This presumably occurs because styrylquinolines only have a minor effect on Cdr2p, as the MIC profile of the *cdr1Δ* strain is comparable with that of the wild-type strain (Table 2). Interestingly, the compound with the highest synergistic activity, WK14B, has acetoxy groups in both the phenyl and quinolone rings. For all subsequent studies, the styrylquinoline concentration was half MIC_{50} if not noted otherwise.

3.2. Styrylquinolines inhibit R6G efflux

One of the hypotheses explaining the preference for some structures in synergistic activity is the competitive export of compounds with varying intensity outside the cells. An R6G efflux assay was used to determine if styrylquinolines can influence ABC transporters in *C. albicans*. Tested compounds are structurally related to AmB, which may result in membrane permeabilization, so this drug was used as a control. As expected, high levels of R6G were absorbed

Table 2
Minimum inhibitory concentration (mM) at which 50% of isolates were inhibited (MIC_{50}) for tested styrylquinoline compounds ($n = 3$).

	Wild-type	*cdr1Δ*	*cdr2Δ*	*cdr1Δ cdr2Δ*
WK14B	>0.4	0.1	>0.4	0.05
WK15B	>0.4	0.4	>0.4	0.1
WK15	0.1	0.01	0.2	0.01
WK14	0.1	0.1	0.1	0.05
8HQ	0.12	0.25	0.12	0.12
8HQD	0.12	0.25	0.12	0.12
AmB	0.001	0.001	0.001	0.001

Table 3
Synergistic effect of tested compounds and fluconazole on *Candida albicans* strains. The results are presented as fractional inhibitory concentration values for tested styrylquinoline compounds ($n = 3$).

	Wild-type	*cdr1Δ*	*cdr2Δ*
WK14B	0.26	1	0.03
WK15B	0.26	1.2	0.02
WK15	2	1	2
WK14	1	2	1

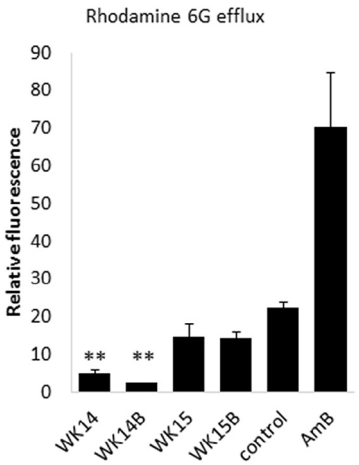

Fig. 2. Influence of styrylquinolines (half of minimum inhibitory concentration) on energy-dependent rhodamine 6G efflux, 30 minutes after efflux was induced by addition of glucose (**$P < 0.005$; $n = 3$).

in the sample treated with AmB (Fig. 2). However, as in the fluconazole synergy experiment, WK14B and (surprisingly) WK14 showed inhibition of R6G efflux. WK15 and WK15B had no effect on R6G export from cells. It seems that the change of substituents in the ortho position on the phenol ring reduced the affinity of these compounds for pumps and efflux from the cell. However, in combination with fluconazole, WK15B was more active than WK15, indicating that the –OAc group is significant for synergistic action of the tested drugs.

To confirm that the cell membrane of *C. albicans* is not disrupted, cells treated with styrylquinolines were stained with non-permeable dye propidium iodine and observed under a fluorescent microscope. As shown in Fig. 3a, none of the tested compounds showed red staining characteristic of membrane disruption, as shown in cells after treatment with AmB. However, when the concentration of styrylquinolines was increased, cells were stained with propidium iodine (Fig. 3b).

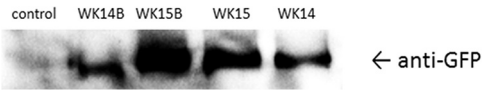

Fig. 4. Cdr1p-GFP protein levels in crude protein extracts from cells after treatment with styrylquinolines (half of minimum inhibitory concentration) as evidenced by anti-GFP Western blot ($n = 4$). DMSO-treated *Candida albicans* cells were used as a control.

3.3. Styrylquinolines induce Cdr1p production and affect its localization in cells

The treatment of *C. albicans* cells with ABC transporter substrates increases their expression [32]. Fluorescent microscopy (Fig. 3) shows a stronger signal from GFP-tagged Cdr1p for cells incubated with styrylquinolines than control samples. The Cdr1p-GFP protein level in crude extract was also higher in treated cells (Fig. 4). WK14B, WK14 and WK15 at half MIC_{50} showed partial delocalization of the signal to the inside of the cell (Fig. 3). At concentrations higher than MIC_{50} of WK14 and WK15, the signal came from the whole cells (Fig. 3b). The AmB-positive control for delocalization showed complete intracellular delocalization of the signal, but as this drug is not a substrate for ABC transporters, there was no increase in Cdr1p levels (Fig. 3).

3.4. Cytotoxic activity of styrylquinolines

An MTT assay on normal human fibroblasts was performed to examine the toxicity of styrylquinolines to human cells. The results showed (Table 4) that three compounds (WK14, WK15 and WK15B) had the same cytotoxicity value (0.1 mM), with WK14 and WK15 having comparable MIC_{50} and cell viability values. However, the cytotoxicity level of WK14B was >0.1 mM.

4. Discussion

Styrylquinolines consist of two aromatic parts connected by an unsaturated ethylene linker that results in a flat rigid structure with relatively high lipophilicity. Such molecules are generally

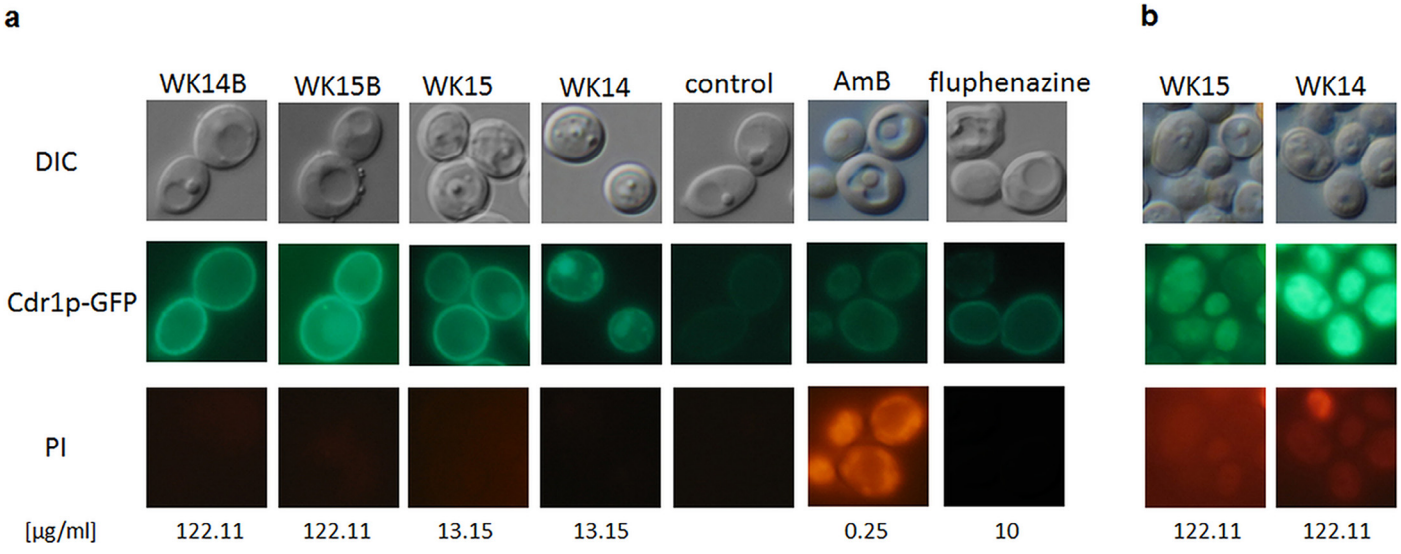

Fig. 3. Microscopic imagining of *Candida albicans* cells. (A) After treatment with styrylquinolines (half of minimum inhibitory concentration), amphotericin B (0.25 µg/mL) or fluphenazine (10 µg/mL). (B) After treatment with WK14 and WK15 (0.4 mM). Top panels, differential interference contrast; middle panels, Cdr1p-GFP; bottom panels, propidium iodine staining ($n = 3$).

Table 4
Effect of styrylquinolines on cell viability (IC_{50}) as measured by MTT test.

	Normal human fibroblast IC_{50} (mM)
WK14B	>0.1
WK15B	0.1
WK15	0.1
WK14	0.1

expected to penetrate cell membranes effectively; moreover, they may interact with lipid structures such as ergosterol. The polyenes, such as AmB, are a class of these drugs [33]. Other compounds with large, flat aromatic structures may act similarly, including doxorubicin, tetracycline and thioridazine [34]. Lipophilic drugs are known for their unspecific interactions, disrupting membrane integrity [35].

As a result of screening for antifungal activity of a large group of styrylquinolines, four compounds with interesting structure–activity relationships were selected for inclusion in this study.

WK14 and WK15 have a hydroxyl group on both the phenyl and quinolone rings, and have a high antifungal activity, regardless of the position of the –OH group. The use of both compounds at MIC_{50} causes membrane permeabilization in *C. albicans* (Fig. 3b). Replacement of the –OH group with the –OAc group strongly reduces the activity of these compounds (Table 2), which is consistent with other reports [16]. The antifungal and anticandida properties of phenolic compounds have been widely described, and they are known for activity such as inactivation of enzyme production [36], antibiofilm effects and anti-adhesive effects [37,38]. Some phenolic compounds can damage the cell membrane of *C. albicans* [39,40], cause considerable reduction in ergosterol biosynthesis [41], or bind to chitin in the cell wall [42]. It appears that the –OH group plays an important role in the destruction of fungal cells.

For further explanation of the effects of styrylquinolines on ABC transporters in *C. albicans* cells, strains with deletions in genes encoding Cdr1p and Cdr2p pumps were tested. The cells with *CDR1* deletion showed a significant increase in sensitivity to styrylquinolines, suggesting that these compounds are Cdr1p substrates. This indicates a possible mode of action as competitive inhibitors of this transporter. In the case of WK14, a significant reduction in resistance was only observed in the case of double cdrΔ mutant. Both observations imply that Cdr2p removes the tested styrylquinolines from cells less effectively than Cdr1p.

Another strategy in combating fungal infections is to study the synergism of a new class of compounds and existing antifungal agents in order to maximize the antifungal effect. Promising synergism was found between WK14B, WK15B and fluconazole against strains of *C. albicans*. All styrylquinolines were tested in double-diluted concentrations starting with MIC_{50}. In this system, WK14 and WK15 with fluconazole were less active than WK14B and WK15B (Table 3). Fluconazole is a substrate for the ABC and MFS transporters of *C. albicans*. No synergistic effect was observed in the case of the *cdr1Δ* strain. This presumably occurs because styrylquinolines have only a minor effect on Cdr2p, as the MIC profile of the *cdr2Δ* strain is comparable with that of the wild-type strain (Table 2). Interestingly, the compound that had the highest synergistic activity, WK14B, has acetoxy groups in both the phenyl and quinolone rings, indicating that while the –OH group is important for compound antifungal activity, the –OAc group increases synergistic action.

Very similar results to wild-type strain MICs were obtained in cytotoxicity testing using normal human fibroblast cells. This suggests excluding the use of higher concentrations of styrylquinolines as antifungal drugs. However, in combination, styrylquinolines increased sensitivity to fluconazole in concentrations as little as 64 times lower than MIC_{50}.

One of the hypotheses explaining the preference for some structures of compounds in synergistic activity is the competitive pumping of compounds with varying intensity outside the cells. The experiment with R6G showed that WK14 and WK14B significantly block the efflux of R6G, unlike WK15 and WK15B (Fig. 2). It seems that the substitution of the –OH or –OAc groups in the ortho position on the quinolone ring reduced the affinity of these compounds for the rhodamine-binding site in ABC transporters [43]. However, in combination with fluconazole, WK15B was more active than WK15, indicating an additional but yet unknown mechanism of action.

The delocalization of Cdr1p from the membrane into the cell is observed under certain conditions and in the presence of some compounds [25,44]. Kumar et al [45] found that higher membrane fluidity can lead to incorrect localization of the Cdr1p pump and a lack of pump activity. In some cases, decreases in cellular ergosterol level and mis-sorting of Cdr1p into vacuoles were observed during the dysfunction of mitochondria [46]. The structures of styrylquinolines and their lipophilic properties predispose them to accumulate in cellular membranes. Their intrinsic mechanism of action may consist of non-specific interactions or alteration of the arrangement of ergosterol. Except for WK14B, the tested compounds caused intracellular delocalization of Cdr1p (Fig. 4). One of the mechanisms of action could be reducing the activity of Cdr1p and its relocation by disrupting the construction of the membrane. Another study has shown that some phenolic compounds significantly decrease the expression levels of *CDR1* and *MDR1* genes in fluconazole-resistant *C. albicans* [47]. However, the present results indicate an increase in Cdr1p concentration in candida cells under the action of styrylquinolines (Fig. 4), which suggests that the excess quantity of transporter cannot anchor in the plasma membrane where Cdr1p localization is supposed to occur.

5. Conclusions

Four promising styrylquinolines with high activity against pathogenic fungi were tested for their anti-*C. albicans* activity. In combination with fluconazole, the tested compounds showed synergistic interactions that may result from mutual efflux by Cdr proteins. The styrylquinolines may also interact with the cell membrane, leading to delocalization of pumps. These findings are promising for designing novel antifungal agents. Highly active compounds that disrupt natural fungal defence against xenobiotic substances may be particularly desirable for effective therapy, which is even more important in highly resistant strains of candida.

Acknowledgements

The authors wish to thank Prof. Dominique Sanglard for the kind gift of *C. albicans* strains, and Mohamed Mahmud for help with the cytotoxicity assay.

Funding: This study was supported by the Polish National Centre for Science NCN Grant 2013/09/B/NZ7/00423 and by Wroclaw Centre of Biotechnology, Programme: the Leading National Research Centre (KNOW) for 2014–2018.

Competing interests: None declared.

Ethical approval: Not required.

References

[1] Pfaller MA, Diekema DJ. Epidemiology of invasive candidiasis: a persistent public health problem. Clin Microbiol Rev 2007;20:133–63.
[2] Cannon RD, Lamping E, Holmes AR, Niimi K, Baret PV, Keniya MV, et al. Efflux-mediated antifungal drug resistance. Clin Microbiol Rev 2009;22:291–321.
[3] Szczepaniak J, Łukaszewicz M, Krasowska A. Detection of inhibitors of *Candida albicans* Cdr transporters using a diS-C3(3) fluorescence. Front Microbiol 2015;6:1–6.

[4] Musiol R, Mrozek-Wilczkiewicz A, Polanski J. Synergy against fungal pathogens: working together is better than working alone. Curr Med Chem 2014;21:870–93.
[5] Sridharan V, Suryavanshi PA, Menéndez JC. Advances in the chemistry of tetrahydroquinolines. Chem Rev 2011;111:7157–259.
[6] Zheng QZ, Cheng K, Zhang XM, Liu K, Jiao QC, Zhu HL. Synthesis of some N-alkyl substituted urea derivatives as antibacterial and antifungal agents. Eur J Med Chem 2010;45:3207–12.
[7] Su D-S, Lim JJ, Tinney E, Wan B-L, Young MB, Anderson KD, et al. Substituted tetrahydroquinolines as potent allosteric inhibitors of reverse transcriptase and its key mutants. Bioorg Med Chem Lett 2009;19:5119–23.
[8] Nagashima S, Akamatsu S, Kawaminami E, Kawazoe S, Ogami T, Matsumoto Y, et al. Novel malonamide derivatives as alpha v beta 3 antagonists. Syntheses and evaluation of 3-(3-indolin-1-yl-3-oxopropanoyl)aminopropanoic acids on vitronectin interaction with alpha v beta 3. Chem Pharm Bull (Tokyo) 2001;49:1420–32.
[9] Isaac M, Slassi A, Brien AO, Edwards L, Maclean N, Bueschkens D, et al. Pyrrolo[3,2,1-ij]quinoline derivatives, a 5-HT2c receptor agonist with selectivity over the 5-HT 2a receptor: potential therapeutic applications for epilepsy and obesity. Bioorg Med Chem Lett 2000;10:919–21.
[10] Doherty EM, Fotsch C, Bo Y, Chakrabarti PP, Chen N, Gavva N, et al. Discovery of potent, orally available vanilloid receptor-1 antagonists. Structure–activity relationship of N-aryl cinnamides. J Med Chem 2005;48:71–90.
[11] Musiol R, Serda M, Hensel-Bielowka S, Polanski J. Quinoline-based antifungals. Curr Med Chem 2010;17:1960–73.
[12] Polanski J, Kurczyk A, Bak A, Musiol R. Privileged structures—dream or reality: preferential organization of azanaphthalene scaffold. Curr Med Chem 2012;19:1921–45.
[13] Jiao Z-G, He H-Q, Zeng C-C, Tan J-J, Hu L-M, Wang C-X. Design, synthesis and anti-HIV integrase evaluation of N-(5- Chloro-8-Hydroxy-2-Styrylquinolin-7-yl)benzenesulfonamide derivatives. Molecules 2010;15:1903–17.
[14] Mrozek-Wilczkiewicz A, Spaczynska E, Malarz K, Cieslik W, Rams-Baron M, Kryštof V, et al. Design, synthesis and in vitro activity of anticancer styrylquinolines. The p53 independent mechanism of action. PLoS ONE 2015;10:e0142678.
[15] Melbourne R, St L. Fungitoxicity of oxine and copper oxinate: effects of pH, metals and chelating agents on activity. Sabouraudia 1999;103:1085–97.
[16] Cieslik W, Musiol R, Nycz JE, Jampilek J, Vejsova M, Wolff M, et al. Contribution to investigation of antimicrobial activity of styrylquinolines. Bioorg Med Chem 2012;20:6960–8.
[17] Vargas MLY, Castelli MV, Kouznetsov VV, Urbina GJM, López SN, Sortino M, et al. In vitro antifungal activity of new series of homoallylamines and related compounds with inhibitory properties of the synthesis of fungal cell wall polymers. Bioorg Med Chem 2003;11:1531–50.
[18] Gerami-Nejad M, Forche A, McClellan M, Berman J. Analysis of protein function in clinical *C. albicans* isolates. Yeast 2012;29:303–9.
[19] Levitz SM, Harrison TS, Tabuni A, Liu X. Chloroquine induces human mononuclear phagocytes to inhibit and kill *Cryptococcus neoformans* by a mechanism independent of iron deprivation. J Clin Invest 1997;100:1640–6.
[20] Musiol R, Jampilek J, Buchta V, Silva L, Niedbala H, Podeszwa B, et al. Antifungal properties of new series of quinoline derivatives. Bioorg Med Chem 2006;14:3592–8.
[21] Musiol R, Podeszwa B, Finster J, Niedbala H, Polanski J. An efficient microwave-assisted synthesis of structurally diverse styrylquinolines. Monatshefte Für Chemie—Chem Mon 2006;137:1211–17.
[22] Fonzi W, Irwin M. Isogenic strain construction and gene mapping in *Candida albicans*. Genetics 1993;134:717–28.
[23] Sanglard D, Ischer F. Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob Agents Chemother 1996;40:2300–5.
[24] Sanglard D, Ischer F, Monod M, Bille J. Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology 1997;143:405–16.
[25] Szczepaniak J, Łukaszewicz M, Krasowska A. Estimation of *Candida albicans* ABC transporter behavior in real-time via fluorescence. Front Microbiol 2015;6:1–6.
[26] Hall MJ, Middleton RF, Westmacott D. The fractional inhibitory concentration (FIC) index as a measure of synergy. J Antimicrob Chemother 1983;11:427–33.
[27] Zucker RM. Parameters and probes. In: Shapiro HM, editor. Practical flow cytometry. 4th ed. Hoboken: John Wiley & Sons, Inc.; 2003. p. 273–410.
[28] Delattin N, Bardiot D, Marchand A, Chaltin P, De Brucker K, Cammue BPA, et al. Identification of fungicidal 2,6-disubstituted quinolines with activity against candida biofilms. Molecules 2012;17:12243–51.
[29] Hiller D, Sanglard D, Morschhäuser J. Overexpression of the MDR1 gene is sufficient to confer increased resistance to toxic compounds in *Candida albicans*. Antimicrob Agents Chemother 2006;50:1365–71.
[30] Nakamura K, Niimi M, Niimi K, Holmes AR, Yates JE, Decottignies A, et al. Functional expression of Candida albicans drug efflux pump Cdr1p in a *Saccharomyces cerevisiae* strain deficient in membrane transporters. Antimicrob Agents Chemother 2001;45:3366.
[31] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[32] Henry KW, Cruz MC, Katiyar SK, Edlind TD. Antagonism of azole activity against Candida albicans following induction of multidrug resistance genes by selected antimicrobial agents. Antimicrob Agents Chemother 1999;43:1968–74.
[33] Gray KC, Palacios DS, Dailey I, Endo MM, Uno BE, Wilcock BC, et al. Amphotericin primarily kills yeast by simply binding ergosterol. Proc Natl Acad Sci 2012;109:2234–9.
[34] Smídková M, Spundová M, Marecek Z, Entlicher G. Effect of drugs on cholesterol crystallization in an artificial bile model and relation of this effect to drug binding to albumin. Fundam Clin Pharmacol 2003;17:331–9.
[35] Lúcio M, Lima JLFC, Reis S. Drug–membrane interactions: significance for medicinal chemistry. Curr Med Chem 2010;17:1795–809.
[36] Evensen NA, Braun PC. The effects of tea polyphenols on *Candida albicans*: inhibition of biofilm formation and proteasome inactivation. Can J Microbiol 2009;55:1033–9.
[37] Shahzad M, Sherry L, Rajendran R, Edwards CA, Combet E, Ramage G. Utilising polyphenols for the clinical management of *Candida albicans* biofilms. Int J Antimicrob Agents 2014;44:269–73.
[38] Rane HS, Bernardo SM, Howell AB, Lee SA. Cranberry-derived proanthocyanidins prevent formation of *Candida albicans* biofilms in artificial urine through biofilm- and adherence-specific mechanisms. J Antimicrob Chemother 2014;69:428–36.
[39] Yun J, Lee H, Ko HJ, Woo E-R, Lee DG. Fungicidal effect of isoquercitrin via inducing membrane disturbance. Biochim Biophys Acta 2014;1848:695–701.
[40] Lee W, Lee DG. An antifungal mechanism of curcumin lies in membrane-targeted action within *Candida albicans*. IUBMB Life 2014;66:780–5.
[41] Ahmad A, Khan A, Manzoor N, Khan LA. Evolution of ergosterol biosynthesis inhibitors as fungicidal against candida. Microb Pathog 2010;48:35–41.
[42] Mahata D, Mandal SM, Bharti R, Gupta VK, Mandal M, Nag A, et al. Self-assembled cardanol azo derivatives as antifungal agent with chitin-binding ability. Int J Biol Macromol 2014;69:5–11.
[43] Rawal MK, Khan MF, Kapoor K, Goyal N, Sen S, Saxena AK, et al. Insight into pleiotropic drug resistance ATP-binding cassette pump drug transport through mutagenesis of Cdr1p transmembrane domains. J Biol Chem 2013;288:24480–93.
[44] Mukhopadhyay K, Prasad T, Saini P, Pucadyil J, Chattopadhyay A, Prasad R, et al. Membrane sphingolipid-ergosterol interactions are important determinants of multidrug resistance in *Candida albicans*. Antimicrob Agents Chemother 2004;48:1778–87.
[45] Kumar A, Radhakrishnan VS, Singh R, Kumar M, Mishra NN, Prasad T. A clinical resistant isolate of opportunistic fungal pathogen, *Candida albicans* revealed more rigid membrane than its isogenic sensitive isolate. In: Méndez-Vilas A, editor. Multidisciplinary approaches for studying and combating microbial pathogens. Boca Raton (FL): Brown Walker Press; 2015. p. 1–7.
[46] Thomas E, Roman E, Claypool S, Manzoor N, Pla J, Panwar SL. Mitochondria influence CDR1 efflux pump activity, Hog1-mediated oxidative stress pathway, iron homeostasis, and ergosterol levels in *Candida albicans*. Antimicrob Agents Chemother 2013;57:5580–99.
[47] Ahmad A, Khan A, Manzoor N. Reversal of efflux mediated antifungal resistance underlies synergistic activity of two monoterpenes with fluconazole. Eur J Pharm Sci 2013;48:80–6.