DR SIMONA VAITKIENĖ (Orcid ID : 0000-0002-6550-1485)

Article type : Research Article

Antifungal activity of styrylpyridinium compounds against *Candida albicans*

Simona Vaitkienė[1*], Neringa Kuliešienė[1], Sandra Sakalauskaitė[1], Laura Bekere[2], Laura Krasnova[2], Brigita Vigante[2], Gunars Duburs[2] and Rimantas Daugelavičius[1]

[1]Department of Biochemistry, Vytautas Magnus University, Vileikos str. 8, Kaunas 44404, Lithuania;
[2]Latvian Institute of Organic Synthesis, Aizkraukles str. 21, Riga 1006, Latvia

[*]**Correspondence:** Simona Vaitkienė, Department of Biochemistry, Faculty of Natural Sciences, Vytautas Magnus University, Vileikos 8, LT-44404 Kaunas, Lithuania
E-mail: simona.vaitkiene@vdu.lt

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/CBDD.13777

Keywords:
Candida albicans
Styrylpyridinium compounds
Fungicidal activity
Cytotoxicity

Abstract

We synthesized a set of 13 new and earlier described styrylpyridinium compounds (*N*-alkyl styrylpyridinium salts with bromide or tosylate anions) in order to evaluate antifungal activity against *C. albicans* cells, to assay the possible synergism with fluconazole, and to estimate cytotoxicity to mammalian cells. All compounds were synthesized according to a well-known two-step procedure involving alkylation of γ-picoline with appropriate alkyl bromide and further condensation with substituted benzaldehyde.

Compounds with long *N*-alkyl chains (**$C_{18}H_{37}$–$C_{20}H_{41}$**) had no antifungal activity against the cells of all tested *C. albicans* strains. Other styrylpyridinium compounds were able to inhibit yeast growth at the concentrations of 0.06 to 16 μg/ml. At fungicidal concentrations, the compound with the CN- group was least toxic to mammalian cells, showed the most effective

synergism with fluconazole, and only slightly inhibited the respiration of *C. albicans*. The compound with the 4′-diethylamino group exhibited the strongest fungicidal properties and effectively blocked the respiration of *C. albicans* cells. However, toxicity to mammalian cells was also high.

Summarizing, the results of our study indicate that styrylpyridinium compounds are promising candidates in the development of new antifungal drugs.

1. Introduction

Yeasts of *Candida* genus, especially *Candida albicans* (*C. albicans*), are the most common causes of fungal infections. Recent studies have indicated that the frequency of invasive

candidiasis and candidemia is increasing [1]. The management of these infections depends on the immune status of a host, severity of a disease, and choice of antifungal drugs [2]. The leading cause of spread and progression of fungal infections is a weakened immune system, which is characteristic of elderly persons. The high-risk group also involves chemotherapy-treated cancer patients, persons after organ transplantations, or HIV-infected patients treated with immunosuppressant drugs. Moreover, the risk of being infected increases for diabetic patients, possibly due to the impact of glucose on *C. albicans* metabolism [3]. The developed severe fungal infection affects skin, mucosa and spreads to the organs and blood stream [4, 5].

The choice of medicines to treat fungal infections is limited due to emerging yeast resistance to antifungal compounds [6]. Most frequently, multidrug resistance (MDR) evolves due to transporters in the yeast plasma membrane that pump a broad spectrum of antimicrobial molecules out of cells [7]. *C. albicans* possesses two types of efflux pumps: ATP-binding cassette (ABC) family transporters and major facilitator superfamily (MFS) pumps that use ATP or electrochemical proton gradient, respectively, to drive active transport of their substrates across the plasma membrane. One of the possible ways to reduce *C. albicans* resistance to antimicrobials is to use inhibitors reducing efflux activity. Inhibitors can reduce the efflux of antimicrobial agents by inactivating pumps or competing with pump substrates [8]. Another way of fighting resistance—usage of drug combinations— appears as an effective approach to improve the antifungal efficiency of antimicrobials on multiple targets. Synergism of compounds leads to a shorter duration of treatment, lower doses, and increased efficacy of single drugs [9].

Reduced fungal susceptibility to polyenes, echinocandins, and azoles warrants the search for new antifungal agents, e.g., pyridine derivatives. N-containing heterocyclic compounds are widely found in nature, have diverse biological activities, and affect various cellular targets. Recently, pyridines have emerged as a substantial class of heterocycles that are endowed mainly with anticancer activities [10]. As a result, pyridines have become an appealing target for medicinal chemists around the world.

Styrylpyridinium compounds—lipophilic cations linked to pyridine—were initially synthesized as fluorescent probes for studies on cell membranes in normal and pathological states [11-13]. Nowadays, the use of such compounds is wider [14-16], but still they mainly serve as probes for pathological studies. Studies on the activity of styrylpyridinium compounds against Gram-positive and Gram-negative bacteria are scarce [17].

Considering the information mentioned above, we aimed to evaluate the antifungal efficiency of newly synthesized and earlier described styrylpyridinium compounds on *C. albicans* cells, to estimate their cytotoxicity to mammalian cells, and to evaluate the synergistic antifungal effects in combination with fluconazole.

2. Material and methods

2.1. Synthesis of styrylpyridinium compounds

All 13 compounds (Table 1) were synthesized according to a two-step procedure involving alkylation of γ-picoline with appropriate alkyl bromide and further condensation with substituted benzaldehyde under conditions recently reported elsewhere [11]. The synthesis pathways of compounds I–XIII are depicted in Scheme 1. According to the reported procedure [18], the known 1-alkyl-4-methylpyridinium bromides were obtained by the treatment of γ-picoline with alkyl halides in acetonitrile solution at 80 °C temperature for 24 h. *N*-Alkyl styrylpyridinium bromides bearing long-chain alkyl groups (C_8, C_{10}, C_{12}, C_{18}, and C_{20}) at the nitrogen atom of the pyridine moiety and different substituents in the aryl group were obtained at moderate yields by the condensation of *N*-alkyl pyridinium bromides with appropriate substituted benzaldehyde in methanol solution at reflux for 5 h. For the synthesis of compound VII, *N*-hexyl pyridinium tosylate was used instead of the bromides mentioned above [13].

2.2. Chemistry

All reagents were purchased from Acros Organics, Sigma-Aldrich, or Alfa Aesar and used without further purification. One-dimensional ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz (^{1}H) and 100 MHz (^{13}C) operating frequency with a Varian 400 Mercury spectrometer. Chemical shifts of the hydrogen and carbon atoms are presented in parts per million (ppm) and referred to the residual signal of non-deuterated $CDCl_3$ (δ:7.26) or partially deuterated D_6-DMSO (δ: 2.50).

Low-resolution mass spectra (MS) were determined on an Acquity UPLC system (Waters) connected to a Waters SQ Detector 2 operating in the ESI positive ion mode on a Waters Acquity UPLC BEH C18 column. Elemental analyses were performed on an Elemental Combustion System ECS 4010 (Costech Instruments) at the Laboratory of Chromatography, Latvian Institute of Organic Synthesis. Logarithms of the partition coefficients logP were calculated by means of

Molinspiration; logP', topological polar surface area PSA, pKa and distribution coefficient logD were calculated by means of Chem3D Ultra 18.2, PerkinElmer Informatics.

The identities of *N*-alkyl 4-methyl pyridinium bromides and **Comps I–IV** and **VI–VIII**, which have been previously reported [11-13, 18-21, 23, 24], were confirmed by ^{1}H NMR. **Comps V**, **IX-X**, and **XI–XIII** were novel and were fully characterized by ^{1}H NMR, ^{13}C NMR, MS, and elemental analysis.

General procedure for the synthesis of 4-(substituted styryl)-1-alkylpyridinium bromides

The mixture of appropriate 1-alkyl-4-methylpyridinium bromide (1 mmol, 1 eq), substituted benzaldehyde (1 mmol, 1 eq), and piperidine (5 drops) in methanol (10 mL) was refluxed for 5 h. The solvent was removed in vacuo and the residue was triturated with dry acetone. After cooling, the precipitate was filtered and recrystallized from acetonitrile. All target compounds had >95% purity. The purities of all compounds were determined by HPLC on a Waters Alliance 2695 system and Waters 2489 UV-vis detector equipped with an Altima C18 column, 5 μM, 4.6 × 150 mm, using gradient elution with acetonitrile/H_3PO_4 (0.1%) in water at a flow rate of 1 mL/min**.**

(*E,Z*)-4-(3,4-Dimethoxystyryl)-1-dodecylpyridin-1-ium bromide (Comp V)

The title compound was synthesized according to the general procedure from 1-dodecyl-4-methylpyridinium-1 bromide (342 mg, 1 mmol) and 3,4-dimethoxybenzaldehyde (166 mg, 1 mmol) to afford V (210 mg, 51%) as yellow powder. ^{1}H NMR (400 MHz, $CDCl_3$): 9.01 (d, J = 6.9 Hz, 2H), 8.03 (d, J = 6.8 Hz, 2H), 7.67 (d, J = 16.1 Hz, 1H), 7.23–7.20 (m, 2H), 7.09 (d, J = 16.1 Hz, 1H), 6.90 (d, J = 8.9 Hz, 1H), 4.72 (t, J = 7.4 Hz, 2H), 3.98 (s, 3H), 3.93 (s, 3H), 2.02–1.92 (m, 2H), 1.30 (d, J = 14.1 Hz, 6H), 1.22 (s, 12H), 0.91–0.80 (m, 3H). ^{13}C ($CDCl_3$): 153.78, 151.87, 149.48, 143.93, 142.14, 127.73, 123.80, 123.67, 120.08, 111.16, 109.92, 60.73, 56.36, 56.07, 31.71, 31.65, 29.02, 29.00,26.11, 22.56, 14.04.MS (ES^+) *m/z*:411 ($[M+H-Br]^+$, 100). Anal.Calcd. for $C_{27}H_{40}BrNO_2$: C, 66.11; H, 8.22; N, 2.86; found: C, 65.85; H, 7.98; N, 2.76.

(*E,Z*)-4-(4-(Diethylamino)styryl)-1-dodecylpyridin-1-ium bromide (Comp IX)

The title compound was synthesized according to the general procedure from 4-methyl-1-dodecyl-4-methylpyridinium-1 bromide (342 mg, 1 mmol) and 4-diethylaminobenzaldehyde (177 mg, 1 mmol) to afford IX (346 mg, 69%) as red powder. ^{1}H NMR (400 MHz, DMSO-d_6): 8.75 (d, J =

7.6 Hz, 2H), 8.08–7.98 (m, 2H), 7.90 (d, *J* = 16.1 Hz, 1H), 7.60–7.51 (m, 2H), 7.60–7.51 (m, 2H), 7.17 (d, *J* = 16.1 Hz, 1H), 4.39 (t, *J* = 7.3 Hz, 2H), 3.42 (q, *J* = 7.1 Hz, 4H), 1.85 (t, *J* = 7.1 Hz, 2H), 1.23 (s, 18H), 1.12 (t, *J* = 7.1 Hz, 6H), 0.92–0.71(m, 3H). ^{13}C NMR (DMSO-d_6): 154.25, 149.96, 146.36, 143.87, 142.73, 137.96, 128.49, 125.97, 122.70, 111.87, 59.54, 44.34, 32.77, 30.94, 30.46, 29.37, 29.26, 29.19, 28.86, 25.89, 22.57, 21.25, 14.43, 12.97. MS (ES$^+$) *m/z*:422 ([M+H-Br]$^+$, 100). Anal. Calcd. for $C_{29}H_{45}BrN_2$: C, 69.44; H, 9.04; N, 5.61; found: C, 69.15; H, 9.10; N, 5.55.

(E,Z)-1-Octadecyl-4-(2-(2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1-i,j]quinolin-9-yl)vinyl)pyridine-1-ium bromide **(Comp X)**

The title compound was synthesized according to the general procedure from 4-methyl-1-octadecylpyridinium-1 bromide (426 mg, 1 mmol) and 2,3,6,7-tetrahydro-1*H*,5*H*-pyrido[3,2,1-*i,j*]quinoline-9-carbaldehyde (201 mg, 1 mmol) to afford X (320 mg, 50%) as red powder. ^{1}H NMR (400 MHz, $CDCl_3$): 8.81 (d, *J* = 7.0 Hz, 2H), 7.74 (d, *J* = 7.0 Hz, 2H), 7.49 (d, *J* = 15.8 Hz, 1H), 7.08 (s, 2H), 6.74 (d, *J* = 15.8 Hz, 1H), 4.62 (t, *J* = 7.4 Hz, 2H), 3.29 (dd, *J* =6.6, 4.9 Hz, 3H), 2.75 (t, *J* = 6.3 Hz, 3H), 2.00–1.88 (m, 6H), 1.23 (d, *J* = 8.1 Hz, 32H), 0.93–0.81 (m, 3H). ^{13}C ($CDCl_3$): 135.40, 147.76, 145.08, 143.68, 133.95, 129.58, 125.57, 125.04, 124.54, 123.23, 60.67, 56.00, 46.16, 31.87, 31.36, 29.61, 29.43, 29.37, 29.10 28.84, 28.70, 27.70, 26.15, 25.86,22.67, 21.30, 14.07. MS (ES$^+$) *m/z*:532 ([M+H-Br]$^+$, 100). Anal. Calcd. for $C_{37}H_{57}$ BrN_2: C, 72.88; H, 9.42; N, 4.59; found: C, 72.51; H, 9.21; N, 4.35.

(*E,Z*)-4-(4-Cyanostyryl)-1-dodecylpyridin-1-ium bromide (Comp XI)

The title compound was synthesized according to the general procedure from 4-methyl-1-dodecyl-4-methylpyridinium-1 bromide (342 mg, 1 mmol) and 4-cyanobenzaldehyde (131 mg, 1 mmol) to afford XI (270 mg, 59%) as red powder. ^{1}H NMR (400 MHz, DMSO-d_6): 9.07–8.99 (m, 2H), 8.32–8.24 (m, 2H), 8.08 (d, *J* = 16.4 Hz, 1H), 7.97 (d, *J* = 8.5 Hz, 2H), 7.92 (d, *J* = 8.5 Hz, 2H), 7.72 (d, *J* = 16.4 Hz, 1H), 4.52 (t, *J* = 7.4 Hz, 2H), 1.96–1.85 (m, 2H), 1.23 (s, 18H), 0.89–0.78 (m, 3H). ^{13}C NMR ($CDCl_3$): 153.80, 151.77, 149.43, 143.90, 142.14, 127.70, 123.82, 123.60, 120.10, 111.16, 110.50, 109.80, 60.72, 56.37, 56.07, 31.71, 31.64, 29.02, 29.00, 26.11, 22.55, 14.04. MS (ES$^+$) *m/z*:376 ([M+H-Br]$^+$, 100). Anal. Calcd. for $C_{26}H_{35}BrN_2$: C, 68.56; H, 7.75; N, 6.15; found: C, 68.12; H, 7.65; N, 6.05.

(*E,Z*)-4-(2-([1,1`-Biphenyl]-4-yl)vinyl)-1-octadecylpyridin-1-ium bromide (Comp XII)

The title compound was synthesized according to the general procedure from 4-methyl-1-octadecylpyridinium-1 bromide (426 mg, 1 mmol) and [1,1′-biphenyl]-4-carbaldehyde (182 mg, 1 mmol) to afford XII (300 mg, 51%) as yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6): 8.98 (d, J = 6.9 Hz, 2H), 8.30-8.24 (m, 2H), 8.09 (d, J = 16.4 Hz, 1H), 7.88–7.80 (m, 4H), 7.78–7.73 (m, 2H), 7.60 (d, J = 16.4 Hz, 1H), 7.50 (dd, J = 8.3, 6.8 Hz, 2H), 7.44–7.38 (m, 1H), 4.50 (t, J = 7.3 Hz, 2H), 2.91 (t, J = 7.1 Hz, 2H), 1.22 (s, 30H), 0.90–0.76 (m, 3H).^{13}C NMR (DMSO-d_6): 160.38, 144.78, 144.12, 140.86, 139.80, 139.07, 137.46, 130.54, 129.54, 129.45, 129.36, 129.32, 129.07, 127.95, 127.75, 127.70, 127.18, 126.86, 126.84, 124.35, 60.31, 46.16, 31.76, 31.00, 30.90, 29.51, 29.47, 29.37, 29.17, 28.84, 28.70, 25.86, 22.56, 14.42. MS (ES$^+$) *m/z*:511 ([M+H-Br]$^+$, 100). Anal. Calcd. for $C_{37}H_{52}BrN$: C, 75.23; H, 8.87; N, 2.37; found: C, 74.84; H, 8.88; N, 2.27.

(*E,Z*)-4-(2-([1,1′-Biphenyl]-4-yl)vinyl)-1-icosylpyridin-1-ium bromide (Comp XIII)

The title compound was synthesized according to the general procedure from 1-icosyl-4-methylpyridinium-1 bromide (440 mg, 1 mmol) and [1,1′-biphenyl]-4-carbaldehyde (182 mg, 1 mmol) to afford XIII (350 mg, 56%) as yellow powder. ^{1}H NMR (400 MHz, DMSO-d_6): 8.98 (d, J = 6.5 Hz, 2H), 8.30–8.23 (m, 2H), 8.09 (d, J = 16.3 Hz, 1H), 7.88–7.9 (m, 4H), 7.80–7.71 (m, 2H), 7.59 (d, J = 16.3 Hz, 1H), 7.50 (dd, J = 8.4, 6.9 Hz, 2H), 7.45–7.38 (m, 1H), 4.50 (t, J = 7.4 Hz, 2H), 1.96–1.84 (m, 2H), 1.22 (s, 34H), 0.89–0.77 (m, 3H). ^{13}C NMR ($CDCl_3$): 153.39, 144.21, 143.53, 141.67, 139.71, 133.52, 129.08, 128.99, 128.80, 128.10, 127.67, 126.99, 126.84, 124.30, 122.08, 60.98, 31.92, 31.72, 29.72, 29.67, 29.63, 29.61, 29.54, 29.41, 29.36, 29.11, 26.14, 22.69, 14.13. MS (ES$^+$) *m/z*:539 ([M+H-Br]$^+$, 100). Anal. Calcd. for $C_{39}H_{56}$ BrN: C, 75.70; H, 9.12; N, 2.26; found: C, 73.15; H, 9.18; N, 2.11.

2.3. Strains, cell lines, and media

The susceptibility of *C. albicans* wild-type (wt) strain ATCC10231, clinical isolate strain SV1, isolated from patients' ascitic fluid, and DSY448 (*cdr1*Δ), DSY653 (*cdr2*Δ), DSY465 (*mdr1*Δ), and DSY654 (*cdr1*Δ*cdr2*Δ) mutant strains (Table 2) was tested against 13 styrylpyridinium compounds. The mutant cells were selected to check the relationship between the compounds and the activity of efflux pumps responsible for MDR.

C. albicans mutant strains used in this study were a generous gift by Prof. Dominique Sanglard (Institute of Microbiology, University of Lausanne and University Hospital Center, Lausanne, Switzerland). The clinical isolate strain SV1 was gifted from the Laboratory of Microbiology, Republican Panevėžys Hospital, Lithuania.

2.4. Gene expression analysis

Expression of *CDR1*, *CDR2*, and *MDR1* genes was evaluated to estimate the characteristics of clinical isolate strain SV1. Yeast cultures were grown for 18 h in an YPD medium, and pellets were collected by centrifugation at 4 °C for 10 min at 3000*g* (Heraeus Megafuge 16R, ThermoFisher Scientific, Germany). Total RNA from SV1 and ATCC10231 cells was extracted using a Trizol Plus RNA kit (Invitrogen, USA) with ZR BashingBead Lysis Tubes (Zymo Research, Irvine, CA, USA). cDNA synthesis was performed using a high capacity cDNA reverse transcription kit (Applied Biosystems, USA), according to the manufacturer's instructions. RT-PCR was performed using StepOne™ and StepOnePlus™ Systems (Applied Biosystems, USA) in 20-µl reaction volume consisting of Power SYBR Green RT-PCR Mix (ThermoFisher Scientific), cDNA template, and forward and reverse primers (ThermoFisher Scientific). RT-PCR conditions for *CDR1*, *CDR2*, and *MDR1* were as follows: 95 °C for 5 min, followed by 40 cycles at 95 °C for 15 s, 60 °C for 30 s. To determine the level of *CDR1, CDR2*, and *MDR1* expression, the differences (Δ) between the threshold cycles (Ct) were measured. Data are presented as the fold change in gene expression normalized to the *ACT1* gene as a control.

The following primers were used in the study:

CDR1: 5′-GTACTATCCATCAACCATCAGCACTT-*3′* (forward)

5′ GCCGTTCTTCCACCTTTTTGTA-*3′* (reverse);

CDR2: *5′*-TGCTGAACCGACAGACTCAGTT-*3′* (forward)

5′- AAGAGATTGCCAATTGTCCCATA -*3′* (reverse);

MDR1: *5′*-TCAGTCCGATGTCAGAAAATGC-*3′* (forward)

5′- GCAGTGGGAATTTGTAGTATGACAA-*3′* (reverse);

ACT1 (house-keeping gene and for confirmation of the PCR process):

5′-GCTTTTGGTGTTTGACGAGTTTCT-3′ (forward)

5′-GTGAGCCGGGAAATCTGTATAGTC-3′ (reverse).

2.5. *Susceptibility testing*

The effects of 13 synthesized styrylpyridinium compounds on the growth of *C. albicans* cells were assayed according to the specifications of the European Committee on Antimicrobial Susceptibility Testing (EUCAST E.DEF 7.3). Yeast cultures were grown for 18 h in an YPD medium, and suspensions with a final cell concentration of 1.0×10^3 CFU/ml were prepared in a modified RPMI-1640 medium, supplemented with 2% glucose and L-glutamine, without sodium bicarbonate, and buffered to pH 7.0 with 0.165 M 4-morpholinepropanesulfonic acid (MOPS, Sigma–Aldrich, GmbH, Steinheim, Germany). Yeast suspensions with the serially diluted compounds at concentrations varying from 0.25 to 16 μg/ml and serially diluted fluconazole at concentrations varying from 0.0625 to 4 μg/ml (total volume of 100 μl), were incubated at 37 °C for 24 h without agitation. To assess the growth, ODs of yeast suspensions were measured spectrophotometrically at 612 nm using the TECAN GENios Pro™ plate reader. The growth of yeast cultures in the absence of compounds was used as a control. The results are presented as the percentage of growth relative to control samples, and MIC_{50} and MIC_{90} values are given as the lowest concentration inhibiting 50% and 90% of growth.

2.6. *Susceptibility testing of styrylpyridinium compounds in combination with fluconazole*

Interactions between fluconazole and the tested compounds were evaluated by determining the fractional inhibitory concentration (ΣFIC) index values [27]. *C. albicans* cells were grown the same as for susceptibility testing (see 2.5). Diluted yeast suspensions, 0.25 μg/ml of styrylpyridinium compounds, and serially diluted fluconazole (0.0625–4 μg/ml) as well as 0.0625 μg/ml of fluconazole in combination with serially diluted styrylpyridinium compounds (0.25 to 16 μg/ml) were used for synergy evaluations (total volume of 100 μl, incubated without agitation at 37 °C for 24 h). After incubation, OD_{612} was read spectrophotometrically using the TECAN GENios Pro™ plate reader. The sum of FICs (ΣFIC) was calculated using the equation ΣFIC = FIC of agent A + FIC of agent B, where:

$$\text{FIC (A)} = \frac{\text{MIC}^{90} \text{ of compound A in combination}}{\text{MIC}^{90} \text{ of compound A alone}}$$

$$\text{FIC (B)} = \frac{\text{MIC}^{90} \text{ of Fluconazole in combination}}{\text{MIC}^{90} \text{ of Fluconazole alone}}$$

MIC_{90} = concentration (μg/ml) required to inhibit the growth of cells by 90%. Synergy of fluconazole and the compound was considered effective when the ΣFIC value was ≤0.5.

2.7. Electrochemical measurements

To study the interaction of the studied compounds with yeast, *C. albicans* cells were grown with aeration in 10 ml of YPD medium (1% yeast extract, 2% peptone, 2% glucose) at 37 °C for 18 h. The overnight culture was diluted 1:50 with a fresh YPD medium and grown to A_{600} of 0.6 at the same conditions. The cells were collected by centrifugation at 4 °C for 10 min at 3000*g* (Heraeus Megafuge 16R, ThermoFisher Scientific, Germany). Pelleted cells were resuspended in a modified RPMI-1640 medium, as described previously, to obtain 1/100 of the original cell culture volume, kept on ice and used within 4 h.

Measurements of dissolved oxygen concentration in a medium were performed by adding 1.0×10^8 CFU yeast suspension to a thermostated (37 °C) and magnetically stirred reaction vessel, containing 5 ml of RPMI-1640 medium (pH 7.0) and 32 μg/ml of the tested compound. At the end of the experiment, solid $Na_2S_2O_4$ was added to the vessel to approximately 20 mM to exhaust dissolved oxygen. Oxygen concentration in the suspension after $Na_2S_2O_4$ addition was set as 0%, while the concentration in an RPMI-1640 medium before the addition of cells was set as 100%.

2.8. Cytotoxicity assay

Chinese hamster ovary (CHO-K1) cells were used for the evaluation of cytotoxicity of the compounds. The cells were grown in a Dulbecco's modified Eagle's medium (DMEM) (GIBCO, Life Technologies), supplemented with fetal bovine serum (10% v/v, GIBCO, Life Technologies), streptomycin/penicillin (2% v/v, GIBCO, Life Technologies), and amphotericin B (1% v/v; GIBCO; Life Technologies) in 75-cm^2 Falcon culture flasks under standard conditions of 5% CO_2 in air at 37 °C, with culture medium renewal every 2–3 days. After reaching sufficient confluence, the cells were passaged by trypsinization. The effects of the compounds on CHO-K1 cells were examined using a 2,3-bis [2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide

(XTT) viability assay. This assay is based on the ability of metabolically active (live) cells to reduce XTT tetrazolium salt, leading to the formation of orange-colored formazan compounds.

For the evaluation of cytotoxicity, CHO-K1 cells were grown in 96-well plates (starting 1×10^4 cells per well) for 24 h in a culture medium. After an incubation medium was carefully aspirated (avoiding damage of the attached cells), wells were two times gently washed with PBS. Solutions of the compounds (see 2.5) were added to each well and incubated for 24 h at 37 °C. Controls consisted of the untreated cells. After an incubation media was removed from all wells, 100 µl of fresh DMEM and 50 µl of XTT solution were added to each well. The plates were covered to avoid direct light and incubated for 2 h at 37 °C to induce the formation of a formazan dye. After incubation, the plates were gently shaken, and absorbance at 492 nm was spectrophotometrically read using the TECAN GENios Pro™ plate reader.

CHO-K1 cells grown under the same conditions without compounds served as a control. Viability was calculated according to the equation:

$$\text{Viability (\%)} = \frac{\text{absorbance of cells treated with compounds}}{\text{absorbance of control cells}} \times 100\ \%$$

2.9. Statistical analysis

Statistical analysis was performed by using the GraphPad Prism 8 (ver. 8.01, 2018; GraphPad Software Inc., San Diego, CA). All the experiments were performed in three biological replicates with three technical repetitions. Results are expressed as means ± standard error of the mean (SEM).

3. Results and discussion

The synthesis of styrylpyridinium compounds **Comps I-IV** and **VI-IX** was described, and fluorescence properties along with antimicrobial activities against bacteria were reported elsewhere [11-13, 18-20]. It has been demonstrated that styrylpyridinium compounds (sometimes described as pyridinium and quinolinium stilbenes) are more effective against Gram-positive than Gram-negative bacteria [16]. This difference in sensitivity can be explained by the presence of the outer membrane in Gram-negative bacteria [28].

Due to the lack of information about the antifungal activity of the compounds, we evaluated the activity of the known and newly synthesized styrylpyridinium derivatives against *C. albicans* cells.

3.1. Characterization of styrylpyridinium compounds

We synthesized a set of compounds by changing two structural elements: substituents at the phenyl ring of the styryl part and alkyl substituents at the pyridinium N-atom. In the first case, we regulated an electronic structure of the conjugated system of the styrylpyridinium moiety.

In the second case, the length of pyridinium *N*-alkyl chain changed the lipophilicity of the molecule and the processes of association with and incorporation into membranes. The alkyl-chain varied from *N*-hexyl (**Comp VII**) followed by *N*-octyl (**Comps I** and **VIII**), *N*-decyl (**Comp II**), *N*-dodecyl (**Comps III**, **V**, **IX**, and **XI**), *N*-octadecyl (**Comps IV**, **VI**, **X**, and **XII**) to *N*-icosyl (**Comp XIII**)). In parallel, the logP values increased in a row (see Table 1): 0.59 (**Comp VII**) → 1.02 (**Comp I**), 1.60 (**Comp VIII**) → 2.03 (**Comp II**) → 3.04 (**Comp III**), 3.16 (**Comp V**), 4.37 (**Comp IX**), 3.27 (**Comp XI**) → 6.60 (**Comp IV**), 6.19 (**Comp VI**), 7.62 (**Comp X**), 8.29 (**Comp XII**) → 8.86 (**Comp XIII**).

From the other point of view, in the first case, we used electron-donating or electron-withdrawing substituents: powerful electron-withdrawing substituent 4′-CN, providing – I and – M effects (**Comp XI**), medium electron-donating substituents 3′,4′-dimethoxy (**Comps V** and **VI**) and 4′-methoxy (**Comp IV**), more powerful electron-donating substituents (providing – I and + M effects) 4′-dimethylamino (**Comps VII** and **VIII**) and 4′-diethylamino (**Comp IX**), and fixed dipropylamino moiety included in the julolidine framework (**Comp X**). For **Comp IX**, the diethylamino group makes it less water soluble than the cyano group (**Comp XI**). Also, the dipropylamino moiety in the tricyclic system makes **Comp X** less water soluble than one or two methoxy groups (**Comps IV** and **VI**). Biphenyl substituents lead to low water solubility (**Comps XII** and **XIII**).

In case of **Comps I**, **II**, and **III,** p-OH groups can be deprotonated. Calculated pKa values of studied **Comps I**, **II** and **III** are at 8.87, near to published data at 8.7 – 8.5 [19]. In the cytosol, hydroxy- and quinonoid-type derivatives have to be in equilibrium. Accordingly, we calculated the coefficient of distribution for these compounds: logD at 6.30 – 7.41. It is quite close to logP data, that is, the partition coefficient is not far from the distribution coefficient.

All studied compounds have polar surface area less than 90 A^2. It means that molecules can permeate cell membranes, even blood-brain barrier.

3.2. Gene expression

In order to get more information about the clinical isolate SV1 from patients' ascitic fluid, we evaluated the gene expression of *CDR1*, *CDR2*, and *MDR1* efflux pumps. We found that the expression of *CDR1* gene in SV1 cells was 5.59 ± 1.8-fold higher compared with ATCC10231 cells (Fig. 1). At the same time, the expression of *CDR2* and *MDR1* was a 2.80 ± 0.99-fold and a 12.86 ± 1.88-fold greater, respectively, in SV1 cells than ATCC10231 cells (Fig. 1).

3.3. Antifungal activity of styrylpyridinium compounds

For susceptibility evaluation, stock solutions of all tested compounds were prepared in ethanol, serially diluted using an RPMI-1640 medium, and incubated with yeast suspensions in 96-well plates for 24 h as described above (chapter 2.5.).

In case of the compounds with a similar structure (**Comps I**, **II**, and **III**), fungicidal efficiency was dependent on the length of *N*-alkyl chain. MIC_{50} and MIC_{90} of **Comp II** ($C_{10}H_{21}$) and **Comp III** ($C_{12}H_{25}$) for ATCC10231 cells varied from 0.5 to 2 μg/ml, and for SV1 cells with increased expression of efflux pump *CDR1*, *CDR2*, and *MDR1* genes, higher concentrations ranging from 6 to 8 μg/ml were required. The pump mutant strains were more susceptible: concentrations between 0.25 and 1.5 μg/ml diminished cell growth up to 90%. At the same time, **Comp I** with the shortest *N*-alkyl chain (C_8H_{17}) and the lowest logP (1.02) had higher MIC_{50} and MIC_{90} values for the cells of almost all studied strains (Table 3).

Meanwhile, **Comp V** with a shorter *N*-alkyl chain ($C_{12}H_{25}$) compared to **Comp VI** with a similar structure but longer *N*-alkyl chain ($C_{18}H_{37}$) showed a higher antifungal activity considerably inhibiting yeast growth. MIC_{50} and MIC_{90} of **Comp V** for ATCC10231 cells were 0.5 and 1 μg/ml, respectively, and for mutant strains varied from 1 to 3 μg/ml. Concentrations up to 4 μg/ml were required to inhibit the growth of SV1 strain (Table 3). A similar effect of **Comp V** on the cells of mutant strains, ATCC10231, and SV1 could indicate that this compound is not the substrate of efflux pumps.

Comps VII and **VIII** with short *N*-alkyl chains (C_6H_{13} and C_8H_{17}) were also effective against the cells of all *C. albicans* strains tested. **Comp VII** at the concentrations of 1 to 4 μg/ml inhibited the growth of wt and efflux-defective strains by 50%, while concentrations at 16 μg/ml were required to inhibit the growth of SV1 cells by 90% (Table 3). In case of **Comp VIII** with an octyl chain, a stronger fungicidal effect was obtained along with the increase of logP. MIC_{50} for all strains varied between 0.25 and 2 μg/ml, while MIC_{90} varied from 0.75 to 2 μg/ml for ATCC10231 and efflux mutant strains, and higher concentrations were required for pumps overexpressing SV1 cells (Table 3).

Comps IX and **XI**, having the same *N*-alkyl chain ($C_{12}H_{25}$), but different substituents at position R2 (-NEt_2 or -CN), demonstrated different antifungal effects. **Comp IX** had the lowest MIC_{50} and MIC_{90} values among all compounds tested. ATCC10231 and SV1 strains demonstrated the same susceptibility to this compound, MIC_{50} for the cells of both strains was 0.6 μg/ml, while 1 μg/ml was needed to inhibit the growth of these strains by 90%. At the same time, the efflux pump mutants showed increased sensitivity to the tested compound, and 90% inhibition of the yeast growth was observed at concentrations between 0.25 and 0.5 μg/ml. MIC_{50} of **Comp XI** for wt and the mutant strains was in the range of 1 to 2.8 μg/ml, and MIC_{90} varied from 3 to 8 μg/ml. The SV1 strain demonstrated increased resistance to this compound: more than 16 μg/ml was required to inhibit the growth of these cells by 90% (Table 3).

Compounds having a medium long *N*-alkyl chains (C_6H_{13} – $C_{12}H_{25}$) are the most appropriate antifungal agents, we can use just this subtype of compounds to compare efficacy. **Comp XI** having a strong electron withdrawing substituent CN has medium antifungal activity. **Comp V** possessing methoxy group has quite high antifungal activity, but compounds possessing strong electron donating groups (dimethylamino, diethylamino – **Comps VII, VIII** and **IX**) are the most active (especially **Comps VIII** and **IX; Comp VIII** is preferable to **Comp VII** as the *N*-alkyl chain C_8H_{17} is preferable to C_6H_{13}).

Comps IV, **VI**, **X**, **XII**, and **XIII** with long alkyl chains ($C_{18}H_{37}$–$C_{20}H_{41}$) revealed high hydrophobicity and low water solubility as well as their logP values were >6. According to the Lipinski's rule of 5, logP should be <5 for drug-likeness of a molecule. It coincides with obtained experimental data: these compounds did not show any fungicidal activity at the concentrations of <64 μg/ml. Due to this, these compounds were excluded from further analysis.

In case of fluconazole, MIC_{50} for the cells of ATCC10231 and mutant strains varied from <0.06 to 0.5 μg/ml, whereas MIC_{90} for all strains was in the range of 0.06 to 1 μg/ml. Cells of the

SV1 strain demonstrated the same sensitivity to fluconazole as ATCC10231 cells (Table 3). Similar sensitivity of *C. albicans* cells to fluconazole has been reported by other 29-31]. Our results showed that *mdr1*Δ strain had higher resistance to fluconazole compared with *cdr1*Δ, *cdr2*Δ, and *cdr1*Δ*cdr2*Δ strains. It is known [32] that Cdr1 pump in *C. albicans* has a higher impact on fluconazole resistance than Cdr2 pump. This was confirmed by our results that cells without Cdr1 pump showed lower $MIC_{50/90}$ for fluconazole than cells bearing Cdr1 pump, but lacking Cdr2 pump (Table 3). *cdr1*Δ and *cdr1*Δ*cdr2*Δ strains showed a significant increase in sensitivity to all styrylpyridinium compounds mentioned above, suggesting that these compounds can be substrates of Cdr1 or Cdr2 pumps and competitive inhibitors of fluconazole efflux.

3.4. Antifungal activity of styrylpyridinium compounds in combination with fluconazole

Fluconazole is known as a first-choice drug to treat *C. albicans* infections. It is effective, well tolerated, with predictable pharmacokinetics and can be used in different groups of patients, even children [33]. However, *C. albicans* can develop resistance to fluconazole through various mechanisms. Therefore, combined antifungal treatment is considered as a relevant tool. One of our goals was to investigate a possible synergy between styrylpyridinium compounds and fluconazole against *C. albicans*. For this purpose, the lowest non-active concentration of the compounds against ATCC10231 and SV1 cells were tested in combination with various concentrations of fluconazole.

Comps I, **II**, **III**, **V**, **VIII**, and **XI** in combination with fluconazole demonstrated the ΣFIC values of 0.19–0.5, indicating significant synergism against ATCC10231, SV1, *cdr1*Δ, *cdr2*Δ, and *mdr1*Δ cells (Table 4). A ΣFIC value of ≤ 0.5 was interpreted as synergy, whereas ΣFIC values between 0.5 and 1.0 were interpreted as additive [34].

The most relevant synergism was achieved in case of SV1 strain, combining fluconazole with **Comp XI** which resulted in ΣFIC of 0.19. Significant synergism between **Comp IX** and fluconazole was obtained studying cells of ATCC10231, SV1 and *cdr2*Δ strains.

In cases when *cdr1*Δ*cdr2*Δ strain cells demonstrated the highest sensitivity to all tested compounds and fluconazole alone, no synergism of fluconazole in combination with styrylpyridinium compounds was observed (Table 4). Moreover, fluconazole had no synergistic effect with **Comps VII** and **IX** against *cdr1*Δ and *mdr1*Δ strains.

There are many studies available on combinations of antifungal drugs with synthetic or natural molecules. Namely, it has been reported that quercetin, a dietary flavonoid, in combination with fluconazole acts synergistically against vulvovaginal *C. albicans*. Combination of these drugs has ability to reduce adhesion and down-regulate expression of *ALS1*, *ALS3*, *HWP1*, *SUN41*, *UME6*, and *ECE1* genes responsible for biofilm formation [35]. Pure polyphenol curcumin I with fluconazole or amphotericin B synergistically triggers the generation of reactive oxygen species and induces apoptosis, which could be reversed by antioxidant ascorbic acid [36]. The advantages of combined treatment are the increased activities of both compounds resulting in a reduced number of resistant microorganisms and the lower toxicity of both compounds [37].

3.5. Effects of styrylpyridinium compounds on C. albicans respiration

In order to know how fast the compounds enter *C. albicans* cells and interact with mitochondria, we monitored the induced changes in cell respiration. For this, we measured the concentration of dissolved oxygen in an RPMI-1640 medium in the presence of styrylpyridinium compounds (Fig. 2).

In the absence of compounds in the medium, the added cells immediately started intensive respiration, and after 6 min, only 25% of the initial dissolved oxygen concentration was left in the medium. Similar intensity of respiration was obtained after the addition of fluconazole (Fig. 2). In the presence of **Comps I** and **XI,** the initial rate of respiration was lower, and during the first 6 min, the concentration of dissolved oxygen decreased to 50% or 40%, respectively (Fig. 2).

In the presence of other compounds, lower stable dissolved oxygen concentrations in the medium were achieved. At equilibrium state, the dissolved oxygen concentration varied from 40% (**Comp II**) to 70% (**Comp III**). In the presence of **Comp IX,** the rate of *C. albicans* respiration gradually decreased and the dissolved oxygen concentration was approaching to the initial one. At the end of experiments, solid $Na_2S_2O_4$ was added (~20 mM), and the concentration of dissolved oxygen dropped to 0 (Fig. 2).

Summarizing the results on the effects of the compounds on SV1 cell respiration, we can conclude that **Comp IX** strongly inhibited respiration, while **Comp XI** had the smallest effect on this process. Other compounds only slowed down respiration to a different extent.

3.6. Viability of CHO-K1 cells after treatment with styrylpyridinium compounds

Fungi share similar cellular and biochemical characteristics with other eukaryotic cells, and therefore, fungicidal agents may adversely affect mammalian cells [38]. However, it was demonstrated that usage of sodium nitroprusside or salicylhydroxamic acid [39] resulted in the efficiently inhibited respiration of *C. albicans* without causing damage to mammalian cells. Consequently, we evaluated the cytotoxicity of the synthesized styrylpyridinium compounds. To explore cytotoxicity, a 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)-based assay was applied, which shows the ability of metabolically active (live) cells to reduce tetrazolium salt of XTT, leading to the formation of orange formazan compounds.

Comp XI had the weakest effect on the viability of CHO-K1 cells. After treatment with MIC_{90} (16 μg/ml), which was required to inhibit the growth of SV1 cells, a 30% decrease in the viability of CHO-K1 cells was recorded (Fig. 3).

Comp IX possessed the highest toxicity: at MIC_{50} and MIC_{90} concentrations diminished viability by 70%, and a 100% inhibition of CHO-K1 cell growth was registered at 2 μg/ml concentration of this compound (Fig. 3A). In addition, **Comp III** at fungicidal concentrations (up to 8 μg/ml) affected CHO-K1 cells by decreasing viability to 10%. **Comps I**, **II**, **V**, **VII,** and **VIII** at MIC_{50} concentrations reduced CHO-K1 cell viability to 30%–40%, and MIC_{90} concentrations of **Comps I** and **VIII** decreased the viability of mammalian cells to 10% and 0%, respectively. **Comps II**, **V**, and **VII** at MIC_{90} concentrations reduced viability to 35%–55%, respectively (Fig. 3A). Styrylpyridinium compounds in combination with fluconazole had no considerable effect on the growth of CHO-K1 cells compared with the effect of compounds on the cell viability when tested separately (Fig. 3B).

In summary, the evaluation of cytotoxicity showed the same tendency as respiration measurements: **Comp IX** was the most toxic, and **Comp XI** at MIC_{90} concentration (16 μg/ml) demonstrated the lowest effect on the viability of CHO-K1 cells.

4. Conclusions

The present study demonstrated that newly and previously synthesized styrylpyridinium compounds (**Comp I-III**, **V**, **VII-IX**, and **XI**) with *N*-alkyl chains not longer than $C_{12}H_{25}$ are effective against all tested *C. albicans* cells. Their logP values were <5, in accordance with the Lipinski's rule of 5. Compounds possessing logP more than 6 (according the Molinspiration method) or 9 (according to PerkinElmer Information method) do not have antifungal activity. At fungicidal concentrations, **Comp XI** with the CN- group as a substituent was the least toxic to

mammalian cells, showed the most effective synergism with fluconazole, and only slightly inhibited the respiration of *C. albicans*. This compound fungicidally acted against all tested yeast strains, but the highest concentration was required to inhibit the growth of SV1 cells with increased *CDR1*, *CDR2*, and *MDR1* gene expression. These results suggest that **Comp XI** could be a substrate of efflux pumps. **Comp IX** exhibited the strongest fungicidal properties and effectively blocked the respiration of SV1 cells. However, the toxicity of this compound to mammalian cells was also rather high. Studied compounds have polar surface area less than 90 A^2 and such molecules can permeate membranes.

Summarizing these findings, delocalization of the positive charge of the pyridinium moiety of **Comp IX**, in conjugation with strong electron-donating diethylamino group at the phenyl group (push-pull mechanism), results in more even distribution of electrons and diminution of polarity. **Comp XI** with a strong electron-withdrawing CN group does not promote interaction with the cationic *N* atom of pyridinium. The results of our study indicate that styrylpyridinium could be a promising candidate in the development of new antifungal drugs. Therefore, our next step will be a comprehensive evaluation of styrylpyridinium compounds acting as inhibitors of efflux pumps.

Funding

This research was funded by the European Social Fund under "The Development of Competences of Scientists, other Researchers and Students through Practical Research Activities" measure (No. 09.3.-LMT-K-712-02-0200) under grant agreement with the Research Council of Lithuania (LMTLT). Support of the Latvian group project NFI/R/2014/051 is acknowledged.

Acknowledgments

We wish to thank Prof. Dominique Sanglard (Institute of Microbiology, University of Lausanne and University Hospital Center, Lausanne, Switzerland) for kindly providing *Candida albicans* mutant strains, and Laboratory of Microbiology (Republican Panevezys Hospital,

Lithuania) for providing clinical isolate strain SV1. Also, we thank Dr. Z. Kalme and I. Bruvere for the performance of some steps of synthesis.

Author contributions

SV designed and performed biological experiments of this work and prepared the manuscript. NK and SS participated at the susceptibility evaluation of compounds on *Candida* cells. LB was responsible for the outset of the chemical part. LK was responsible for the synthesis of a major part of compounds. BV was responsible for compounds analysis, structure determination, and the final chemical part of the project. GD was the leader of the chemical part of the project, responsible for the analysis of the structure-activity relationship of compounds. RD participated in designing of the experiments, revised the manuscript, leader of the team. All authors have read and approved the final manuscript.

Competing financial interests

The authors declare no competing financial interests.

References

1. Enoch, D.A., et al., *The Changing epidemiology of invasive fungal infections.* Methods Mol Biol, 2017. **1508**: p. 17-65.
2. Yenisehirli, G., et al., *In Vitro Susceptibilities of Candida albicans isolates to antifungal agents in Tokat, Turkey.* Jundishapur J Microbiol, 2015. **8**(9): p. e28057.
3. Brown, A.J., et al., *Metabolism impacts upon Candida immunogenicity and pathogenicity at multiple levels.* Trends Microbiol, 2014. **22**(11): p. 614-22.
4. Achkar, J.M. and B.C. Fries, *Candida infections of the genitourinary tract.* Clin Microbiol Rev, 2010. **23**(2): p. 253-73.
5. Mayer, F.L., D. Wilson, and B. Hube, *Candida albicans pathogenicity mechanisms.* Virulence, 2013. **4**(2): p. 119-28.
6. Cuenca-Estrella, M., *Antifungal drug resistance mechanisms in pathogenic fungi: from bench to bedside.* Clin Microbiol Infect, 2014. **20 Suppl 6**: p. 54-9.

7. Higgins, C.F., *Multiple molecular mechanisms for multidrug resistance transporters.* Nature, 2007. **446**(7137): p. 749-57.
8. Vargiu, A.V., et al., *Molecular mechanism of MBX2319 inhibition of Escherichia coli AcrB multidrug efflux pump and comparison with other inhibitors.* Antimicrob Agents Chemother, 2014. **58**(10): p. 6224-34.
9. Cui, J., et al., *Synergistic combinations of antifungals and anti-virulence agents to fight against Candida albicans.* Virulence, 2015. **6**(4): p. 362-71.
10. El-Naggar, M., et al., *Pyridine-ureas as potential anticancer agents: synthesis and In Vitro biological evaluation.* Molecules, 2018. **23**(6).
11. Shiraishi, Y., T. Inoue, and T. Hirai, *Local viscosity analysis of triblock copolymer micelle with cyanine dyes as a fluorescent probe.* Langmuir, 2010. **26**(22): p. 17505-12.
12. Krieg, R., et al., *Optimization of heterocyclic 4-hydroxystyryl derivatives for histological localization of endogenous and immunobound peroxidase activity.* Biotech Histochem, 2007. **82**(4-5): p. 235-62.
13. Dubur, G.Y., et al., *Fluorescent probes based on styrylpyridinium derivatives: optical properties and membrane binding.* J Biochem Biophys Methods, 1984. **10**(3-4): p. 123-34.
14. Kluska, M., et al., *New Applications and analysis of (E)-azastilbenes in environmental samples.* Crit Rev Anal Chem, 2019. **49**(5): p. 395-402.
15. Garcia, A., et al., *The voltage-sensitive dye RH421 detects a Na(+),K(+)-ATPase conformational change at the membrane surface.* Biochim Biophys Acta Biomembr, 2017. **1859**(5): p. 813-823.
16. Inshyn, D.C., S. V., Kovalska, V. B.; Yarmoluk, S. M., *Design of 4-(4-dialkylaminostyryl)-pyridinium dyes for fluorescent detection of amyloid fibrils. Biopolymers Cell, 2016. 32(4): p.289-299.* Biopolymers and Cell, 2016. **32**(4): p. 289-299.
17. Chanawanno, K., et al., *Synthesis, structure and in vitro antibacterial activities of new hybrid disinfectants quaternary ammonium compounds: pyridinium and quinolinium stilbene benzenesulfonates.* Eur J Med Chem, 2010. **45**(9): p. 4199-208.
18. Schelz, D.B., et al., *Dichloromethoxymethane as a reagent for synthesis of carbocyanine dyes with high-basicity heterocyclic end groups.* 1986. **7**(3): p. 187-213.
19. Mishra, B.K., et al., *Acid dissociation constants of N-alkyl p/o-hydroxy styryl pyridinium dyes in surfactant solutions.* Indian Journal of Chemistry - Section A Inorganic, Physical, Theoretical and Analytical Chemistry, 2001. **40**: p. 155-158.

20. Wyrzykiewicz, E., W. Prukala, and B. Kedzia, *Synthesis and antimicrobial properties of N-substituted derivatives of (E)-alpha-(or gamma)-azastilbenols.* Farmaco, 1994. **49**(2): p. 127-31.
21. Ster, D., et al., *Synthesis and mesophase behaviour of ionic liquid crystals.* Journal of Materials Chemistry, 2007. **17**(32): p. 3393-3400.
22. Turshatov, A.A., et al., *Molecular organization of an amphiphilic styryl pyridinium dye in monolayers at the air/water interface in the presence of various anions.* Langmuir, 2006. **22**(4): p. 1571-9.
23. Song, T., et al., *Encapsulation of dyes in metal-organic frameworks and their tunable nonlinear optical properties.* Dalton Trans, 2016. **45**(10): p. 4218-23.
24. Dey, N., S.K. Samanta, and S. Bhattacharya, *Heparin triggered dose dependent multi-color emission switching in water: a convenient protocol for heparinase I estimation in real-life biological fluids.* Chem Commun (Camb), 2017. **53**(9): p. 1486-1489.
25. Sanglard, D., et al., *Susceptibilities of Candida albicans multidrug transporter mutants to various antifungal agents and other metabolic inhibitors.* Antimicrob Agents Chemother, 1996. **40**(10): p. 2300-5.
26. Sanglard, D., et al., *Cloning of Candida albicans genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene.* Microbiology, 1997. **143 (Pt 2)**: p. 405-16.
27. Hall, M.J., R.F. Middleton, and D. Westmacott, *The fractional inhibitory concentration (FIC) index as a measure of synergy.* J Antimicrob Chemother, 1983. **11**(5): p. 427-33.
28. Peterson, E. and P. Kaur, *Antibiotic resistance mechanisms in bacteria: relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens.* Front Microbiol, 2018. **9**: p. 2928.
29. Rosana, Y., A. Yasmon, and D.C. Lestari, *Overexpression and mutation as a genetic mechanism of fluconazole resistance in Candida albicans isolated from human immunodeficiency virus patients in Indonesia.* J Med Microbiol, 2015. **64**(9): p. 1046-52.
30. Xu, J., et al., *Eucalyptal D enhances the antifungal effect of fluconazole on fluconazole-resistant Candida albicans by competitively inhibiting efflux pump.* Front Cell Infect Microbiol, 2019. **9**: p. 211.

31. Sanglard, D. and A.T. Coste, *Activity of isavuconazole and other azoles against Candida clinical isolates and yeast model systems with known azole resistance mechanisms.* Antimicrob Agents Chemother, 2016. **60**(1): p. 229-38.
32. Vanden Bossche, H., *Mechanisms of antifungal resistance.* Rev Iberoam Micol, 1997. **14**(2): p. 44-9.
33. Popp, C., et al., *Competitive fitness of fluconazole-resistant clinical Candida albicans strains.* Antimicrob Agents Chemother, 2017. **61**(7).
34. Blanco, A.R., et al., *Efficacy of a fixed combination of tetracycline, chloramphenicol, and colistimethate sodium for treatment of Candida albicans keratitis.* Invest Ophthalmol Vis Sci, 2017. **58**(10): p. 4292-4298.
35. Gao, M., H. Wang, and L. Zhu, *Quercetin assists fluconazole to inhibit biofilm formations of fluconazole-resistant Candida albicans in In Vitro and In Vivo antifungal managements of vulvovaginal Candidiasis.* Cell Physiol Biochem, 2016. **40**(3-4): p. 727-742.
36. Sharma, M., et al., *Synergistic anticandidal activity of pure polyphenol curcumin I in combination with azoles and polyenes generates reactive oxygen species leading to apoptosis.* FEMS Yeast Res, 2010. **10**(5): p. 570-8.
37. Mukherjee, P.K., et al., *Combination treatment of invasive fungal infections.* Clin Microbiol Rev, 2005. **18**(1): p. 163-94.
38. Shrestha, S.K., A. Garzan, and S. Garneau-Tsodikova, *Novel alkylated azoles as potent antifungals.* Eur J Med Chem, 2017. **133**: p. 309-318.
39. Duvenage, L., et al., *Inhibition of classical and alternative modes of respiration in Candida albicans leads to cell wall remodeling and increased macrophage recognition.* MBio, 2019. **10**(1).

Figure legends

Scheme 1. General methods for the synthesis of *N*-alkyl styrylpyridinium dyes I–XIII. Reagents and conditions: (a) 4-methylpyridine, appropriate alkyl bromide or tosylate, MeCN, 24 h, 80 °C; (b) *N*-alkyl pyridinium bromide or tosylate, substituted benzaldehyde, MeOH, piperidine, reflux, 5 h.

Fig. 1. *CDR1*, *CDR2*, and *MDR1* gene expression in SV1 cells.

Fig. 2. Intensity of respiration of *C. albicans* SV1 cells. The concentration of dissolved oxygen was measured as described in Materials and methods. Concentrated yeast suspension was added to the vessel, containing 5 mL of RPMI-1640 medium (pH 7.0) and 32 μg/ml of the tested compound, to the final concentration of 1.0×10^8 cells/ml. Solid $Na_2S_2O_4$ was added to the vessel (~20 mM concentration).

Fig. 3. Effect of styrylpyridinium compounds and fluconazole on viability of CHO-K1 cells. CHO-K1 cells were grown 24 h in 96-well plates filled with DMEM medium in the presence of various concentrations of styrylpyridinium compounds (**A**), or serially diluted fluconazole (FLC) in combination with a constant concentration (0.25 μg/ml) of styrylpyridinium compounds (**B**). Viability of cells in the absence of tested compounds corresponds to 100%. The tested compounds were prepared as described above (chapter 2.5), were added to each well containing the attached cells, and were incubated at 37 °C for 24 h.

Table 1. Styrylpyridinium compounds used in this study

Compounds	R1	R2	R3	R	X^-	logP /logP'	PSA, A^2	Reference
Comp I	H	OH	H	C_8H_{17}	Br^-	1.02/6.32 *8.87 #7.4 †6.30	23.24	[12]
Comp II	H	OH	H	$C_{10}H_{21}$	Br^-	2.03/7.42 *8.87 #7.4 †7.41	23.24	[19]
Comp III	H	OH	H	$C_{12}H_{25}$	Br^-	3.04/7.25	23.24	[20]
Comp IV	H	OMe	H	$C_{18}H_{37}$	Br^-	6.60/10.24	12.24	[21]
Comp V	OMe	OMe	H	$C_{12}H_{25}$	Br^-	3.16/7.21	21.47	this study
Comp VI	OMe	OMe	H	$C_{18}H_{37}$	Br^-	6.19/9.88	21.47	[22]
Comp VII	H	NMe_2	H	C_6H_{13}	$MeC_6H_4SO_3^-$	0.59/5.85	6.25	[13]
Comp VIII	H	NMe_2	H	C_8H_{17}	Br^-	1.60/6.95	6.25	[11]
Comp IX	H	NEt_2	H	$C_{12}H_{25}$	Br^-	4.37/8.74	6.25	this study
Comp X	$(CH_2)_3$	N	$(CH_2)_3$	$C_{18}H_{37}$	Br^-	7.62/10.96	6.25	this study
Comp XI	H	CN	H	$C_{12}H_{25}$	Br^-	3.27/7.39	26.8	this study
Comp XII	H	Ph	H	$C_{18}H_{37}$	Br^-	8.29/12.28	3.01	this study
Comp XIII	H	Ph	H	$C_{20}H_{41}$	Br^-	8.86/13.17	3.01	this study

*pKa; #logD; †acid

Table 2. *C. albicans* efflux pump mutant strains used in this study

Strain	Genotype	Reference
DSY448	*cdr1Δ::hisG-URA3 hisG/cdr1Δ::hisG*	[25]
DSY653	*cdr2Δ::hisG-URA3-hisG/cdr2Δ::hisG*	[26]
DSY465	*mdr1Δ::hisG-URA3-hisG/ mdr1Δ::hisG*	[25]
DSY654	*cdr1Δ::hisG/cdr1Δ::hisG cdr2Δ::hisG-URA3-hisG/cdr2Δ::hisG*	[26]

Table 3. *In vitro* activity of styrylpyridinium compounds against *C. albicans*

Compound	Strains												R^1-R^3	R	logP
	ATCC10231		**SV1**		***cdr1*Δ**		***cdr2*Δ**		***mdr1*Δ**		***cdr1*Δ*cdr2*Δ**				
	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}			
Fluconazole	0.15	0.5	0.15	0.5	<0.06	0.12	0.12	0.5	0.25	1	<0.06	0.06	-	-	-
Comp I	1	4	4	>16	0.5	4	1	5	2	6	0.5	4	4-OH	8	1.02
Comp II	0.5	2	6	8	0.25	1.5	0.5	2	0.75	3	0.5	1.5	4-OH	10	2.03
Comp III	1.5	2	7	8	0.5	1.5	0.5	1.6	0.75	1.5	0.75	1.4	4-OH	12	3.04
Comp IV	>64	>64	>64	>64	ND	ND	ND	ND	ND	ND	ND	ND	4-OMe	18	6.60
Comp V	0.5	1	3	4	1	2	1	3	1	2	1	1.5	$3,4\text{-}(OMe)_2$	12	3.16
Comp VI	>64	>64	>64	>64	ND	ND	ND	ND	ND	ND	ND	ND	$3,4\text{-}(OMe)_2$	18	6.19
Comp VII	4	8	4	16	1	1.8	1.5	8	1	2	1	1.5	$4\text{-}NMe_2$	6	0.59
Comp VIII	0.5	2	2	8	0.4	0.9	0.5	4	0.4	0.8	0.25	0.75	$4\text{-}NMe_2$	8	1.60
Comp IX	0.6	1	0.6	1	0.37	0.5	0.4	0.5	<0.25	0.25	<0.25	0.25	$4\text{-}NEt_2$	12	4.37
Comp X	>64	>64	>64	>64	ND	ND	ND	ND	ND	ND	ND	ND	Me_3NMe_3	18	7.62
Comp XI	2.8	8	4	>16	1	4	2	4	1	6	1	3	4-CN	12	3.27
Comp XII	>64	>64	>64	>64	ND	ND	ND	ND	ND	ND	ND	ND	4-Ph	18	8.29
Comp XIII	>64	>64	>64	>64	ND	ND	ND	ND	ND	ND	ND	ND	4-Ph	20	8.86

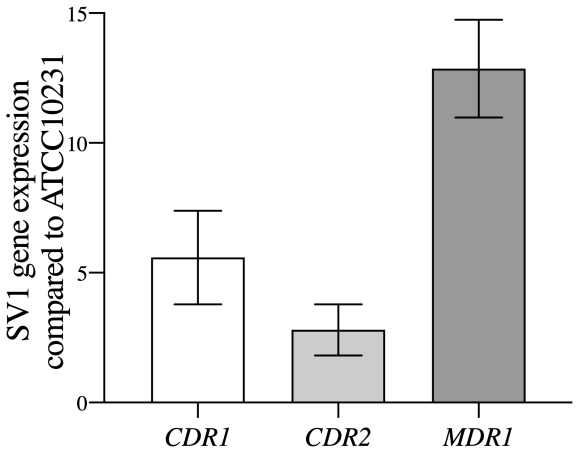
MIC_{50} = concentration (μg/ml) required to inhibit the growth of cells by 50%, MIC_{90} = concentration (μg/ml) required to inhibit the growth of cells by 90%, ND = not determined;

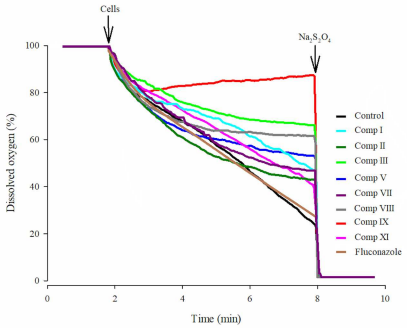
R - number of C atoms in *N*-alkyl chain.

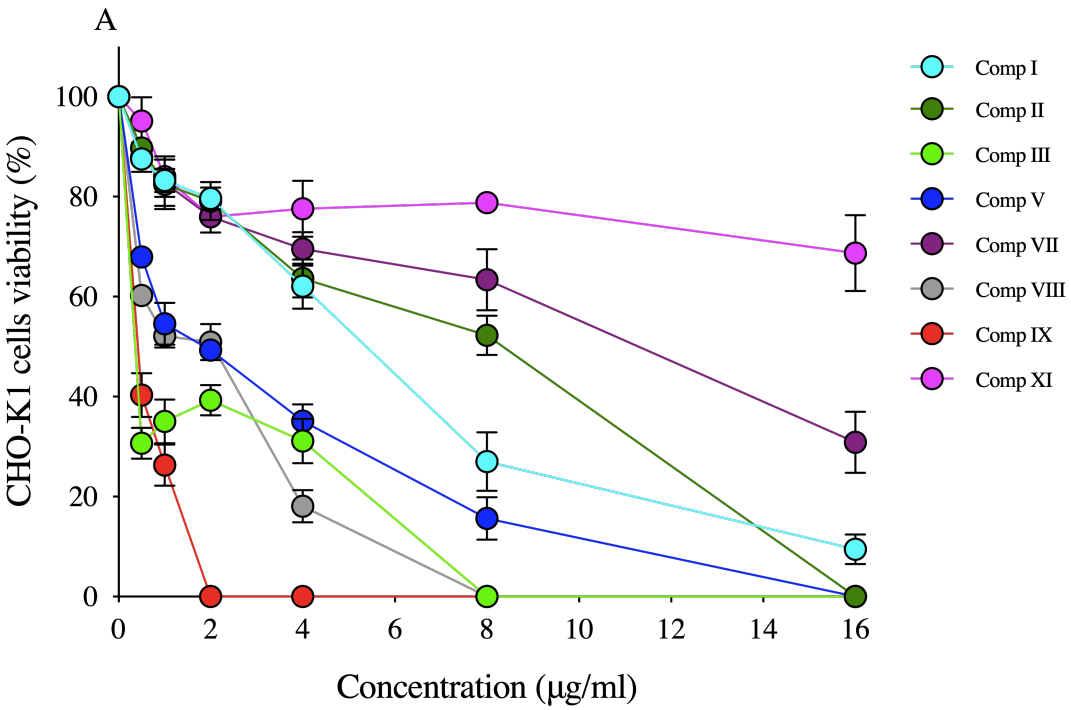
Table 4. Synergistic effects of styrylpyridinium compounds and fluconazole against *C. albicans*

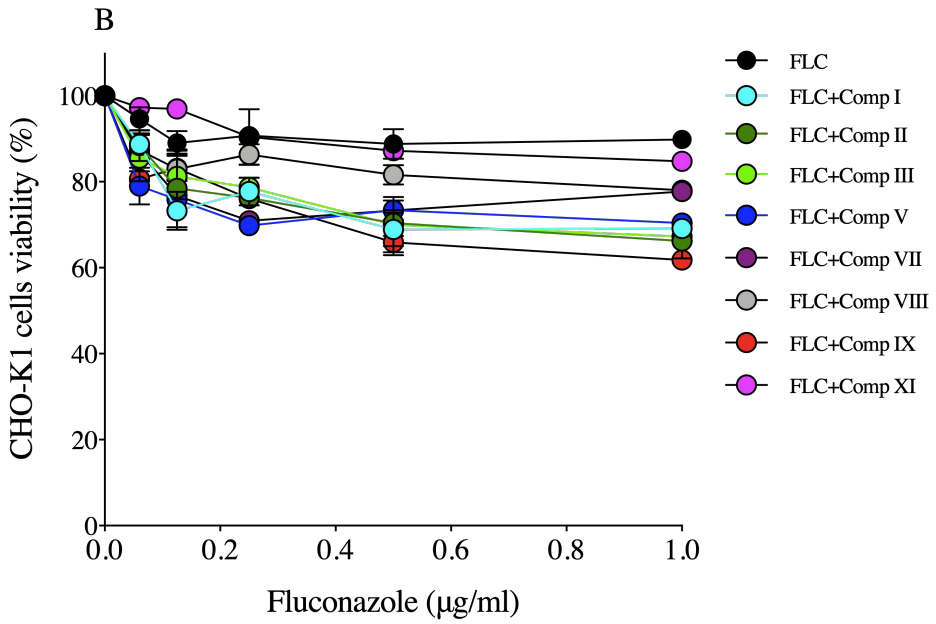
	Strains					
	ATCC10231	**SV1**	***cdr1*Δ**	***cdr2*Δ**	***mdr1*Δ**	***cdr1*Δ*cdr2*Δ**
Comp I	**0.5**	**0.38**	**0.5**	**0.45**	**0.45**	>0.5
Comp II	**0.5**	**0.31**	**0.41**	**0.38**	**0.41**	>0.5
Comp III	**0.38**	**0.31**	**0.41**	**0.4**	**0.42**	>0.5
Comp V	**0.5**	**0.38**	**0.31**	**0.29**	**0.25**	>0.5
Comp VII	**0.31**	**0.38**	>0.5	**0.5**	>0.5	>0.5
Comp VIII	**0.38**	**0.28**	**0.32**	**0.38**	**0.38**	>0.5
Comp IX	**0.38**	**0.38**	>0.5	**0.38**	>0.5	>0.5
Comp XI	**0.38**	**0.19**	**0.27**	**0.5**	**0.4**	>0.5

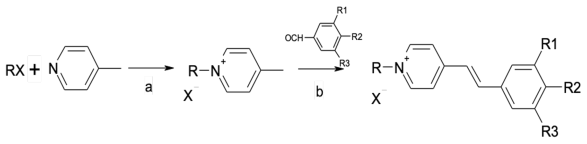
The results are presented as ΣFIC for the tested styrylpyridinium compounds, and the values in bold indicate significant synergism.










RX +
a
X⁻
OCH
R1
R2
R3
b
X⁻
R1
R2
R3