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Evaluation of antimicrobial activity of synthesized 9*H*-alkylcarbazole and 10*H*-alkylphenothiazine derivatives on the cells of *Salmonella enterica* ser. Typhimurium, *Saccharomyces cerevisiae*, and *Candida albicans*

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10*H*-substituted phenothiazine and 9*H*-substituted carbazole derivatives are important because of a very wide range of applications and especially in medical chemistry due to their pharmacological activities. In this study, we synthesized 9*H*-alkylcarbazole and 10*H*-alkylphenothiazine derivatives with various lengths of alkyl chains and evaluated their antimicrobial and efflux inhibiting activities on the cells of *Salmonella enterica* ser. Typhimurium, *Saccharomyces cerevisiae*, and *Candida albicans*. Results of our study revealed that an increased length of alkyl chains of the carbazoles increased the accumulation of efflux indicator tetraphenylphosphonium (TPP^+) ions. Cells of *S. enterica* efflux mutant ΔTolC had a considerable susceptibility to the synthesized compounds. The compounds exerted synergy with fluconazole against *S. cerevisiae* yeast. Efflux pump mutant ΔPdr5 was hypersensitive to the investigated carbazole and phenothiazine derivatives. The inhibitory effect of the compounds with a shorter alkyl chain (10-methyl-10*H*-phenothiazine and 9-methyl-9*H*-carbazole) was the highest for *Candida albicans cells*.

Keywords: phenothiazine, carbazole, tetraphenylphosphonium ions, minimal inhibitory concentration, efflux pumps, *Salmonella enterica*, *Saccharomyces cerevisiae*, *Candida albicans*

INTRODUCTION

Antimicrobial resistance is a worldwide problem in human and veterinary medicine. An extensive use of antimicrobials leads to the spread of resistant bacteria in animals and humans (Michael et al., 2014). Antimicrobial drugs from both hospital and agricultural sources can persist in soil or aquatic environments, and these compounds may affect the treatment of human diseases (Allen et al., 2010). The appearance of multiple resistant bacteria of human and animal origin is accompanied by co-contamination of the environment apparently leading to a great health concern (Kossow et al., 2017).

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Microorganisms have the remarkable ability to pump antimicrobials out of cells. This protective feature is the most widespread form of resistance to many classes of antimicrobials. Over the years, several solutions to solve this problem have been proposed (Brüssow, 2017). One of such solutions is efflux pump inhibitors, which could be therapeutic agents for restoration of sensitivity to antibiotics. Efflux pump inhibitors act against multidrug resistant efflux pumps with different substrates and they are expected to not only reverse resistance to a single drug but also to clinically beneficial antimicrobials. Some antimicrobial derivatives could act as enhancers of antibiotic efficiency. They may not have any antimicrobial properties alone, but when used with bacteria-resistant drug they could enhance the effect of the drug (Opperman et al., 2015). One of the best-studied peptidomimetic compounds – phenylalanine-arginine β-naphthylamide (PaβN) – was originally described in 1999 and characterized further in 2001 as a broad-spectrum efflux pump inhibitor (Lomovskaya et al., 2001), but as it possesses detrimental side effects, it was not introduced into clinical practice (Bolla, Brune, 2017).

Another relevant problem is resistance to antifungal compounds. New directions of treatment of fungal infections are needed, especially for infections caused by the yeast of the *Candida* family. ABC (ATP-binding cassette) transporters of pleiotropic drug resistance subfamily, like Cdr1p of *C. albicans*, are the most frequent cause of resistance to antifungal agents.

Contemporary attempts to find effective enhancers were relatively unsuccessful against Gram-negative bacteria, so it is important to continue the search for new effective compounds.

Phenothiazines are the oldest synthetic antipsychotic drugs, which do not have analogues in the world of natural compounds. Phenothiazine was first synthesized by August Bernthesen in 1883 and for many years it was used in veterinary medicine as an anthelmintic drug. Having an amino alkyl side chain connected to nitrogen atom, these compounds are important in medicinal chemistry (Clausen et al., 2017; Carsuo et al., 2008; Conchon et al., 2008; Bouaziz et al., 2015; Indumati et al., 2012; Zhang et al., 2010; Kantevari et al., 2011; Asche, Demeunynck, 2007; Hieda et al., 2014; Humphries et al., 2016; Kaur et al., 2012; Rennison et al., 2016; Pluta, Morak-Młodawska, Jeleń, 2011; Sarmiento et al., 2011; Warman et al., 2013; Spengler et al., 2016; Liu et al., 2009; Mosnaim et al., 2006; Hou et al., 2019).

Investigation of substituted 10*H*-phenothiazines has had a strong growth during the recent years because of wide range of applications (Table). Carbazoles are also a large and interesting group of organic compounds: its moiety is frequent in structures of numerous drugs such as listed in the Table.

Table. **Frequency of 9*H*-carbazole and 10*H*-phenothiazine moiety in the drugs**

Drug type	Carbazole	Phenothiazine
Antibacterial	(Clausen et al., 2017; Carsuo et al., 2008; Conchon et al., 2008; Bouaziz et al., 2015; Indumati et al., 2012)	(Pluta et al., 2011)
Antifungal	(Zhang et al., 2010)	(Sarmiento et al., 2011)
Antitubercular	(Kantevari et al., 2011)	(Warman et al., 2013)
Anticancer	(Asche, Demeunynck, 2007)	(Spengler et al., 2016)
Antioxidant	(Hieda et al., 2014)	(Liu et al., 2009)
Antidiabetic	(Humphries et al., 2016)	(Mosnaim et al., 2006)
Antipsychotic, sedatives	(Kaur et al., 2012)	(Hou et al., 2019)
Anthelmintic	(Rennison et al., 2016)	(Smith, 1942)
Antiviral	(Gulza et al., 2014)	(Mucsi et al., 2001)
Anti-inflammatory	(Nalli et al., 2016)	(Sharma et al., 2005)

A slight change in the structure of these compounds causes distinguishable differences in their biological activities (Jaszczyszyn et al., 2012). Applied as neuroleptic drugs, phenothiazine derivatives easily cross the blood-brain barrier, since they exhibit a strong affinity to lipid bilayers of the cell membranes in neurons and other lipid-rich tissues as the phenothiazine ring possesses a high degree of lipophilicity (Seelig et al., 1994). Depending on the structure of substituents in the side chain, the intensity of the neuroleptic action of phenothiazine derivatives could be ranked as follows: piperazine group > piperidine group > aliphatic chain (Jaszczyszyn et al., 2012). Among the candidates for effective anti-MDR drugs, phenothiazines are worth further studying, because they are strong inhibitors of the Pgp transport function and exhibit several cancer chemopreventive actions (Jaszczyszyn et al., 2012).

Derivatives of carbazole and phenothiazine are considered as potential multidrug resistance (MDR) efflux pump inhibitors (Rodrigues et al., 2011). It is very important to discover molecules which could inhibit the efflux and to understand the mechanism of inhibition (Cuestas et al., 2012). Here we explore a possibility to use phenothiazine and carbazole derivatives as susceptibility enhancers to antimicrobials, which can act as competitive substrates in efflux of the drugs.

MATERIALS AND METHODS

Bacteria, yeast, and chemicals

Salmonella enterica ser. *typhimurium* strain SL1344 wild type (WT) and ΔTolC mutant with Δ*tolC* channel deletion were obtained from Prof. Seamus Fanning (Institute of Food and Health, University College Dublin, Ireland). *Saccharomyces cerevisiae* strains W303-1a (MATa) and ΔPdr5 (MATa, *pdr5*:HIS3) were obtained from Prof. Chuang-Rung Chang (National Tsing Hua University, Taiwan). *Candida albicans* ATCC 10231 were obtained from Dr Eglė Lastauskienė (Institute of Biosciences, Vilnius University).

10*H*-Phenothiazine 98%, 9*H*-carbazole 95%, 1-bromoethane 98%, iodomethane 99%, 1-bromohexane 98%, tetrabutylammonium hydrogensulfate 97%, potassium hydroxide 96%, potassium carbonate 96%, chloramphenicol 98% were purchased from Aldrich (St. Louis, MO) and used as received without additional purification. Polymyxin B (PMB) sulphate, Gramicidin D (GD), Luria-Bertani broth (LB) came from Sigma (St. Louis, MO), tetraphenylphosphonium (TPP^+) chloride from Fluka (St. Gallen, Switzerland), and ethylene diamine tetraacetic acid (EDTA), HCl and glucose – from Sharlau (Barcelona, Spain). Tris(hydroxymethyl) aminomethane (Tris) was obtained from Roth (Karlsruhe, Germany), ethidium bromide and fluconazole from Acros Organics (New Jersey, USA). Yeast extract and bacteriological peptone were from Oxoid (Hampshire, England), RPMI medium 1640 from Merck (WGK, Germany). PAβN (phenylalanine-arginine β-naphthylamide) was synthesized as described in Sutkuviene et al. (2013).

Instrumentation for the synthesized compounds

^{1}H NMR spectra of the synthesized compounds were recorded using a Bruker Ascend 400 (400 MHz) apparatus using chloroform-d. Mass spectra were obtained on Waters ZQ spectrometer. Synthesis reactions of 9*H*-carbazole and 10*H*-phenothiazine compounds were monitored by thin-layer chromatography (TLC) on pre-coated plastic sheets with 0.25 mm Merck silica gel 60F-254. Column chromatography of synthesized carbazole and phenothiazine compounds was carried out using Merck silica gel 60 (230–240 Mesh). Melting points were determined on a Stuart SMP11 apparatus (Bibby Scientific, Staffordshire, UK). TPP^+ selective electrodes (Daugelavicius et al., 1997) were connected to the potential-amplifying system based on an ultralow input bias current operational amplifier AD549JH (Analog Devices, Norwood, Ma, USA). The amplifying system was connected to a computer through PowerLab 8/35 logger (ADInstruments, Oxford, UK). Agar salt bridges were used for indirect connection of

reference electrodes (Orion model 9001; Thermo Fisher Scientific, USA) with cell suspensions in the vessels. The representative sets of curves from three independent series of measurements are presented in the Fig. 4.

Synthesis of *N*-alkylphenothiazine and *N*-alkylcarbazole derivatives

General procedure of synthesis and analysis of phenothiazine derivatives

Compounds **1–3** were synthesized by the N-alkylation method (Simokaitiene et al., 2006). 10*H*-Phenothiazine (1.00 g, 5 mmol) was dissolved in 10 mL of dry toluene. Then potassium carbonate (0.346 g, 2.5 mmol), potassium hydroxide (0.842 g, 15 mmol), tetrabutylammonium hydrogensulfate (0.02 g, 0.06 mmol) and iodomethane (1.065 g, 7.5 mmol) or 1-bromoethane (0.817 g, 7.5 mmol) or 1-bromohexane (1.238 g, 7.5 mmol) were added, respectively (Fig. 1). The reaction mixture was refluxed for 12 hours. After TLC monitoring, the reaction mixture was cooled down at room temperature and filtered. The solvent was removed under reduced pressure on a rotary evaporator. Compounds were recrystallized from ethanol and additionally purified by column chromatography (eluent: ethylacetate-hexane, 1:3). Yields, melting points, and spectral data of these compounds are given bellow.

*10-Methyl-10H-phenothiazine (**1**)*

White solid. M.p: 99°C; Yield: 65%; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.21–7.12 (m, 4H, Ar), 6.96–6.90 (m, 2H, Ar), 6.82 (d, 2H, Ar, J = 8 Hz), 3.38 (s, 3H, Ar-$\underline{CH_3}$); MS (ESI, m/z): 214.08 $[M+H]^+$, 199.02.

*10-Ethyl-10H-phenothiazine (**2**)*

White solid. M.p: 102°C; Yield: 79%; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.18–7.08 (m, 4H, Ar), 6.96–6.82 (m, 4H, Ar), 1.42 (t, 3H, Ar-CH_2-$\underline{CH_3}$, J = 6.9 Hz), 3.93 (b.s, 2H, Ar-$\underline{CH_2}$-CH_3); MS (ESI, m/z): 228.10 $[M+H]^+$, 199.02.

*10-Hexyl-10H-phenothiazine (**3**)*

Yellow solid. M.p: 72°C; Yield: 75%; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 7.18–7.10 (m, 4H, Ar), 6.96–6.82 (m, 4H, Ar), 3.84 (s, 2H, Ar-$\underline{CH_2}$-$(CH_2)_4$-CH_3), 1.80 (p, 2H, Ar-CH_2-$\underline{CH_2}$-$(CH_2)_3$-CH_3, J = 7.4 Hz), 1.43 (p, 2H, Ar-$(CH_2)_2$-$\underline{CH_2}$-$(CH_2)_2$-CH_3, J = 7.3 Hz), 1.35–1.18 (m, 4H, Ar-$(CH_2)_3$-$\underline{CH_2}$-$\underline{CH_2}$-CH_3), 0.87 (t, 3H, Ar-$(CH_2)_5$-$\underline{CH_3}$, J = 6.9 Hz); MS (ESI, m/z): 284.15 $[M+H]^+$, 199.02.

General procedure of synthesis and analysis of N-alkylcarbazole derivatives

Compounds **4–6** were synthesized by the N-alkylation method (Simokaitiene et al., 2006). 9*H*-Carbazole (1.00 g, 6 mmol) was dissolved in 15 mL of dry toluene. Then potassium carbonate (0.4299 g, 3 mmol), potassium hydroxide (1.0037 g, 18 mmol)), tetrabutylammonium hydrogensulfate (0.02 g, 0.06 mmol) and iodomethane (1.2819 g, 9 mmol) or 1-bromoethane (0.9870 g, 9.1 mmol) or 1-bromhexane (1.4493 g, 8.8 mmol) were added into the reaction mixture, respectively. The reaction mixture was refluxed for 12–14 hours. After TLC monitoring, the reaction mixture was cooled down at room temperature and filtered. The solvent was removed under reduced pressure on a rotary evaporator. Compounds were recrystallized from ethanol and additionally purified by column chromatography (eluent: ethylacetate-hexane, 1:3). Yields, melting points, and spectral data of these compounds are given bellow.

*9-Methyl-9H-carbazole (**4**)*

White solid. M.p: 89°C; Yield: 59%; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.10 (d, 2H, Ar, J = 7.8 Hz), 7.51–7.46 (m, 2H, Ar, J = 8.1 Hz), 7.41 (d, 2H, Ar, J = 8.1 Hz), 7.25–7.21 (m, 2H, Ar), 3.86 (s, 3H, Ar-$\underline{CH_3}$); MS (ESI, m/z): 182.05 + $[M+H]^+$, 167.05.

*9-Ethyl-9H-carbazole (**5**)*

White solid. M.p: 68°C. Yield: 46%; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.12 (d, 2H, Ar, J = 7.7 Hz), 7.49–7.45 (m, 2H, Ar), 7.42 (d, Ar, J = 8.1 Hz), 7.25–7.21 (m, 2H, Ar), 4.40 (q, 2H, Ar-$\underline{CH_2}$-CH_3, J = 7.2 Hz), 1.45 (t, 3H, Ar-CH_2-$\underline{CH_3}$, J = 7.2 Hz); MS (ESI, m/z): 196.04 + $[M+H]^+$, 167.98.

*9-Hexyl-9H-carbazole (**6**)*
Yellow solid. M.p: 61°C. Yield: 47%; ^{1}H NMR ($CDCl_3$, 400 MHz, δ, ppm): 8.11 (d, 2H, Ar, *J* = 7.8 Hz), 7.48–7.44 (m, 2H, Ar), 7.41 (d, 2H, Ar, *J* = 8.1 Hz), 7.25–7.20 (m, 2H, Ar), 4.31 (t, 2H, -CH_2-, *J* = 7.3Hz), 1.90 (p, 2H, -CH_2-, *J* = 7.6Hz), 1.43–1.26 (m, 6H, -$(CH_2)_3$-), 0.88 (t, 3H, -CH_3 *J* = 7.0 Hz); MS (ESI, m/z): 252.11 + $[M+H]^+$, 167.98.

Determination of antimicrobial activity to the synthesized compounds
To evaluate the antimicrobial activity of the synthesized compounds, we applied the broth dilution method. First, *S. enterica* SL1344 or ΔTolC cells were cultivated in fresh LB medium with aeration at 37°C for 18 hours to OD_{600} of 1. *S. cerevisiae* cells were cultivated in 10 mL fresh yeast extract peptone dextrose (YPD) medium (1% yeast extract, 2% peptone, 2% glucose) at 30°C for 18 h. The procedure involved serial two-fold dilutions of the antimicrobial compounds in a liquid growth medium in 96-well microtitration plates. Dilutions of our synthesized compounds were performed starting from concentration of 150 μM of ethanolic stock solution. Each well was inoculated with a microbial inoculum. The bacterial cells were inoculated to obtain concentration 5×10^5 and 1–5 10^3 cfu/mL for the yeast. Microplates were incubated without agitation at 37°C for bacteria and 30°C for yeast. The turbidity of the cell suspensions was measured using TECAN GENios Pro™ (Männedorf, Switzerland) plate reader after 16–20 hours of incubation for bacteria and after 48 hours for yeast. The plate was shaken 5 s before each registration point. Representative sets of at least three independent measurements are presented in the following chapter.

Electrochemical measurements
To monitor the interaction of the synthesized compounds with bacteria, overnight culture of *S. enterica* SL1344 cells was diluted 1:50 in fresh LB medium and grown with aeration at 37°C to OD_{600} of 1. The cells were collected by centrifugation at 4°C for 10 min at 3000 × g (Heraeus Megafuge 16R, Thermo Fisher Scientific, Walthman, Ma, USA). Pelleted cells were re-suspended in 100 mM Tris/HCl (pH 8.0) to obtain 1/150 of the original cell culture volume, kept on ice and used within 4 h. The measurements of TPP^+ concentration were performed simultaneously in two vessels. 2.5×10^9 cfu/ml were added to thermostated (37°C) and magnetically stirred reaction vessels containing 5 mL of Tris/HCl, pH 8.0 supplemented with 3 μM TPP^+ (Daugelavicius et al., 1997). The solutions of **1–6** compounds in ethanol were used. Representative sets of at least three independent measurements are presented below.

RESULTS AND DISCUSSION

Synthesis of N-alkylphenothiazine and *N*-alkylcarbazole compounds
N-alkylphenothiazines (**1–3**) and N-alkylcarbazoles (**4–6**) were synthesized by the reaction of 10*H*-phenothiazine/9*H*-carbazole with alkyl (ethyl, methyl, hexyl) iodide or bromide in the presence of tetrabutylamonium hydrogensulfate, potassium carbonate, potassium hydroxide and dry toluene, stirred at 110°C for 12–14 hours (Fig. 1). The ^{1}H NMR of compounds **1** and **4** showed the singlets at δ 3.38 and 3.86 ppm, respectively, showing the presence of the CH_3 group. This clearly indicates the formation of 10-methyl-10*H*-phenothiazine (**1**) and 9-methyl-9*H*-carbazole (**4**). Compound **2** showed a triplet at δ 1.42 ppm and a broad singlet at δ 3.93 ppm, compound **5** showed quadruplet at δ 4.40 ppm and triplet at 1.45 ppm showing the presence of CH_2 and CH_3 groups. This indicates the formation of 10-ethyl-10*H*-phenothiazine (**2**) and 9-ethyl-9*H*-carbazole (**5**). Spectra of compound **3** showed peaks at δ 3.84, 1.80, 1.43, 1.35–1.18, 0.87 ppm showing the presence of all CH_2 and CH_3 groups present in 10-hexyl-10*H*-phenothiazine (**3**). Spectra of compound **6** showed peaks at δ 4.31, 1.90, 1.43–1.26, 0.88 ppm showing the presence of all CH_2 and CH_3 groups present in 9-hexyl-9*H*-carbazole (**6**). All other aromatic protons were observed at expected regions. Furthermore, mass spectra data are in accordance with the expected structure of the obtained compounds.

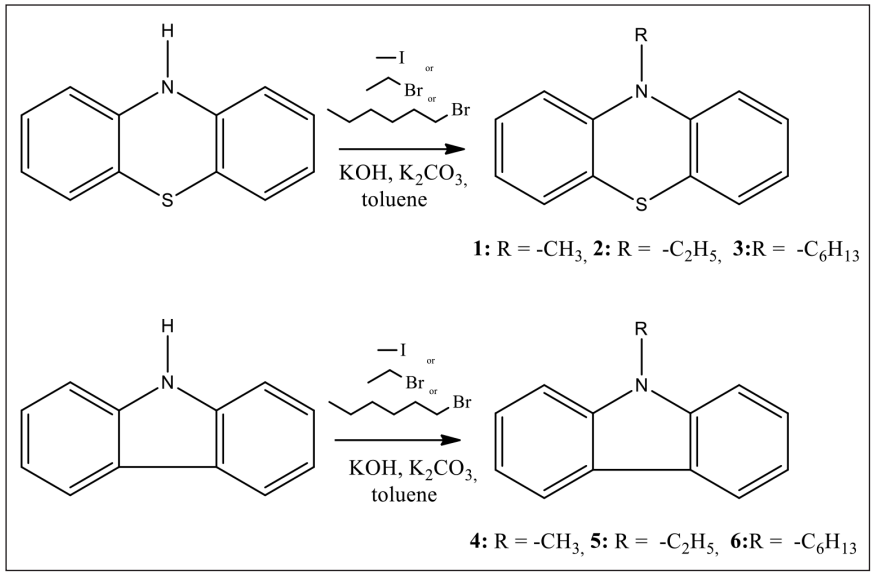

Fig. 1. Synthesis scheme and structures of compounds **1**–**6**

Evaluation of antimicrobial activity of the synthesized materials

Determination of susceptibility of S. enterica cells with N-alkyl phenothiazines (1–3) and N-alkyl carbazoles (4–6)

Susceptibility of *S. enterica* wild type SL1344 and ΔTolC mutant cells to the synthesized compounds was determined. *S. enterica* ΔTolC cells lack the channel-forming porin TolC, an important component of RND family efflux pumps. The experiments were conducted in the 96-well plates, the initial concentration of compounds **1–6** was 150 μM (Fig. 2). The screening results showed that the presence of compounds **1–6** in the LB medium did not affect the growth of wild type cells, but ΔTolC mutant cells were more sensitive to these compounds. Higher sensitivity of ΔTolC cells was chosen because it possesses higher sensitivity to the experimental compounds. This difference occurred because of the nonfunctioning RND type efflux pumps in ΔTolC mutant cells.

RND family pump inhibitor phenylalanyl-arginyl-B-naphtylamide (PAβN) was used for deeper exploration of efflux. Screening results showed that in the presence of PaβN, the synthesized compounds at concentration of 37.5 μM decreased the optical density of bacteria suspension indicating the negative effect on the viability of both *S. enterica* cells types. 9-Hexyl-9*H*-carbazole (**6)** with the longest N-alkyl chain was the most active against ΔTolC mutant cells**.** Therefore, we can conclude that compounds **1–6** are RND efflux pump substrates. The data presented above are in correlation with the data presented by other scientists. It is known that PAβN could bind to AcrB binding sites. The results suggest how an inhibitor, even when it is pumped out itself, can reduce the pumping of other substrates.

PAβN inhibited the efflux of other drugs by binding to the bottom of the distal binding pocket, the so-called hydrophobic trap, and also by interfering with the binding of other drug substrates to the upper part of the binding pocket. However, its mechanism of inhibition is not clear (Kinana et al., 2016).

In the other part of our research, chloramphenicol (Cm), which is known as substrate of MDR efflux pumps (Sun et al., 2014), was used. The combined system of the synthesized carbazole and phenothiazine compounds with Cm was used to determine if activity of the MDR efflux pump substrate could be enhanced.

The concentration of compounds **1–6** was 20 μM as a result of the previous experiments (Fig. 2 A, B). The solutions of this concentration did not influence the growth of the cells, but the growth was decreased by the combination of PAβN and compounds **1–6**.

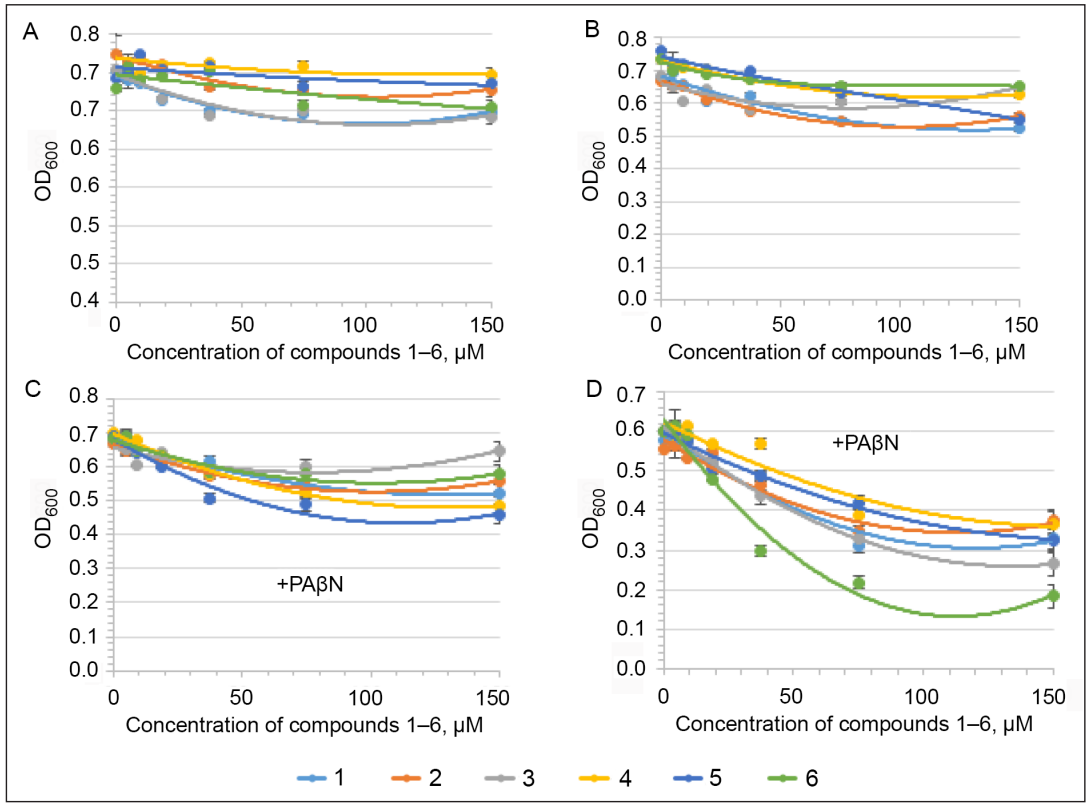

Fig. 2. Impact of compounds **1–6** and efflux inhibitor PAβN on the growth of *S. enterica* SL1344 (**A** and **C**) and ΔTolC (**B** and **D**) cells. The experiments were performed in LB-Medium. The cells were incubated for 20 hours at 37°C. In **B** and **D** the medium contained 32 μM PAβN. The initial cell concentration was 5×10^5 cfu/mL

To determine if synthesized compounds could influence the growth of the cells in composition with antibiotic – Cm, we performed the following experiments.

The results of these experiments showed that the synthesized compounds increase the efficiency of Cm on the cells of both strains (Fig. 3). The growth inhibition results showed that mutant cells were more sensitive compared to WT cells.

Our results indicate that both phenothiazines (compounds **1–3)** and carbazoles (compounds **4–6)** enhanced the sensitivity of *S. enterica* cells to Cm. The effect of the synthesized compounds was more expressed on ΔTolC mutant, i.e., the cells with nonfunctioning RND type of efflux pumps. The sensitivity effect did not depend much on the compounds, but we could still conclude that the sensitivity of the cells for phenothiazine having longer alkyl chain are higher comparing with the phenothiazine having the shortest chain.

Comparing carbazole and phenothiazine compounds for the wild type cells, phenothiazines have a higher impact for the cell sensitivity to chloramphenicol. This effect is evident for both types of the cells except in the case of ΔTolC.

We applied electrochemical measurements to determine the interaction of the synthesized compounds with another efflux pump substrate – tetraphenylphosphonium (TPP^+). Potentiometric measurements allowed us to follow the permeability, depolarization of plasma membrane and efflux activity in real time. TPP^+ accumulates inside the cells due to plasmamembrane voltage (ΔΨ) and releases out of the cells after depolarization of inner membrane. TPP^+ selective electrode did not show potential change when the measurements

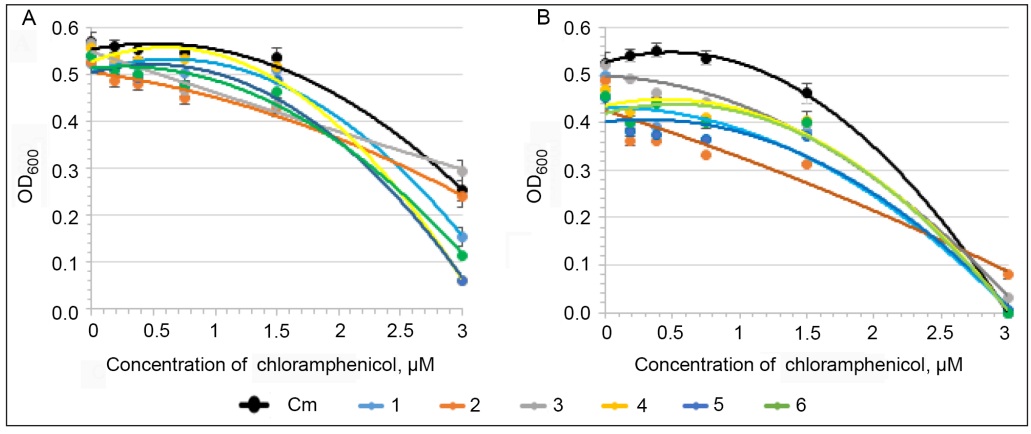

Fig. 3. Effects of chloramphenicol and the synthesized compounds on the growth of *S. enterica* SL1344 (A) and ΔTolC (B) cells. The experiments were performed in LB-Medium. The cells were grown in 96-well plates for 20 hours at 37°C. Concentration of compounds **1–6** was 20 μM. The initial cell concentration was 5×10^5 cfu/mL

were performed with TPP^+ and synthesized compounds mixtures without bacteria. These experiments show that there is no interaction between TPP^+ and synthesized compounds.

In the process of electrochemical measurements *S. enterica* ser. Typhimurium SL1344 cells were added to Tris/HCl buffer and EDTA was used to permeabilize the bacterial outer membrane (OM). Due to low permeability of the OM to lipophilic compounds and activity of the efflux pumps, *Salmonella enterica* cells bind low amounts of TPP^+ ions. To increase the influx of TPP^+ ions, EDTA was used.

The cells accumulated TPP^+ after EDTA addition and equilibrium distribution of this indicator ion was achieved in 3 min (Fig. 4A). TPP^+

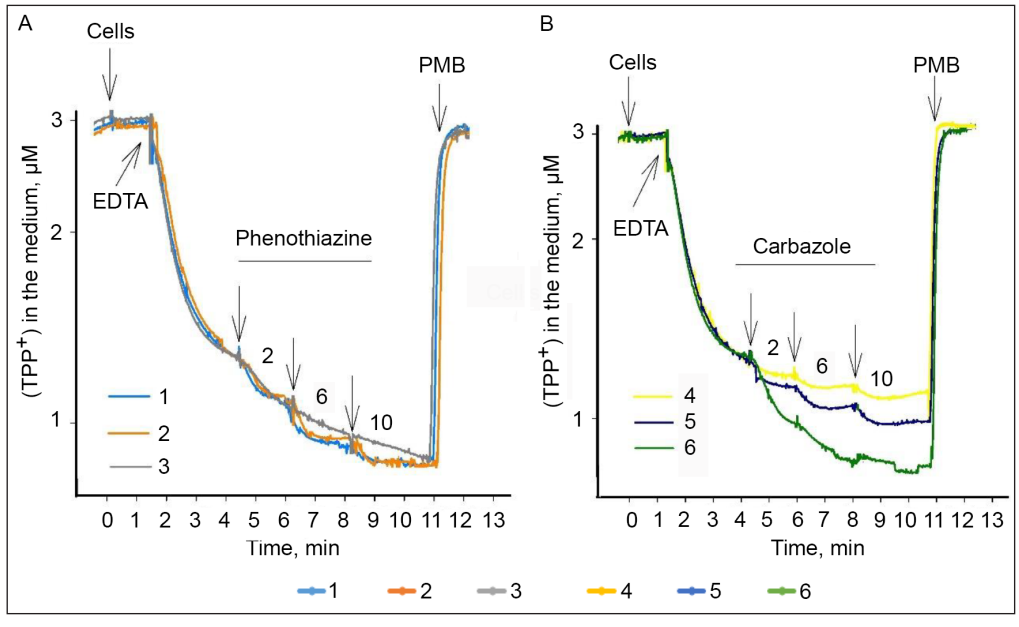

Fig. 4. Influence of phenothiazines and carbazoles on the accumulation of TPP^+ ions in *S. enterica* SL1344 cells. The experiments were performed in 100 mM Tris/HCl buffer, pH 8, containing 0.1% glucose, at 37°C. Cells were added to OD_{600} of 1, EDTA – to 0.1 mM, polymyxin B (PMB) – to 100 μg/ml. Concentrations of compounds **1–6** in μM are indicated in the figures

accumulation remained constant over the time without adding synthesized compounds. Increasing concentrations of the synthesized phenothiazines induced additional uptake of TPP^+. 10-Methyl-10*H*-phenothiazine (**1**) and 10-ethyl-10*H*-phenothiazine (**2**) additions induced stronger uptake of lipophilic TPP^+ cation than 10-hexyl-10*H*-phenothiazine (**3**), whereas efficiency of efflux inhibition of compounds **4–6** depended on the length of the alkyl chain. The results of the experiments with carbazoles (Fig. 4B) showed that compounds **4** and **5**, bearing methyl and ethyl chains, had a weaker effect on TPP^+ accumulation than 9-Hexyl-9*H*-carbazole (**6**) with the longest alkyl chain. The results demonstrate that efflux-inhibiting efficiency of carbazoles correlate with the length of N-alkyl chain in the molecules. Polycationic antibiotic PMB was used to permeabilize the outer membrane of Gram-negative bacteria and depolarize the cytoplasmic membrane at high concentrations. In order to evaluate the total amount of accumulated TPP^+, we used 100 μg/ml addition of PMB which caused the depolarization of cytoplasmic membrane and TPP^+ ions were released back to the incubation medium.

Determination of susceptibility of *S. cerevisiae*, *C. albicans* cells with N-alkylphenothiazines (1–3) and N-alkylcarbazoles (4–6)

Susceptibility of *S. cerevisiae* wild type W303-1a (Fig. 5A) cells and ΔPdr5 (Fig. 5B) mutant to the synthesized phenothiazines and carbazoles was tested (Fig. 5A, B). The ΔPdr5 cells do not

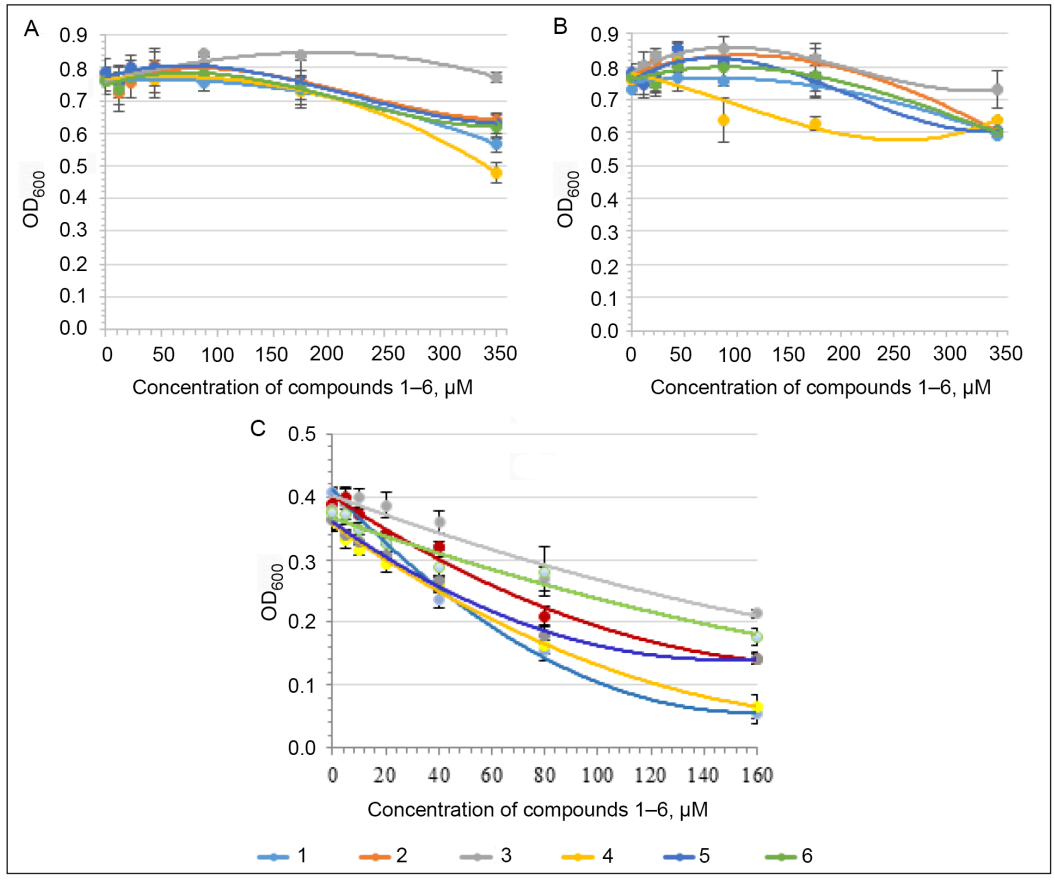

Fig. 5. Impact of compounds **1–6** on the growth of *S. cerevisiae* W303-1a (**A**), ΔPdr5 mutant cells (**B**) and *C. albicans* ATCC10231 (**C**). The screening was carried out in the 96-well plates, the initial concentration of compounds **1–6** was 350 μM (**A, B**) or 160 μM (**C**). Cells were grown in YPD (*S. cerevisiae*) or RPMI-1640 (*C. albicans*) media at 30°C (*S. cerevisiae*) or 37°C (*C. albicans*) for 24 h. The initial cell concentration was 1–5 × 10^3 cfu/mL

contain plasma membrane ABC transporter, which is involved in the resistance against xenobiotics (Belofsky et al., 2013). The synthesized compounds at the concentrations of up to 160 μM did not affect the growth of *S. cerevisiae* WT cells. Also, the efflux of mutant cells was not affected at the same range of concentrations and only compound **4** had a minor effect on the growth of mutant cells (Fig. 5A and 5B). The growth of *C. albicans* cells was more sensitive to the synthesized compounds, both phenothiazines and carbazoles (Fig. 5C). Methyl derivatives of the synthesized compounds had the strongest inhibitory effect, and hexyl derivatives the lowest.

To find out if carbazoles and phenothiazines can work as efflux inhibitors and enhance efficiency antifungal compounds, low concentrations of the synthesized compounds were tested in combination with the well-known drug fluconazole (Flu). This compound is widely used to treat a variety of fungal and yeast infections (Shi et al., 2018). It belongs to a class of antifungals called azoles which arrest the growth of the cells by several mechanisms. The best known mechanism includes overexpression or mutations in Erg11p (CYP51p); others change ergosterol metabolism and increase expression of energy-dependent drug efflux (Coste et al., 2007).

Although the synthesized derivatives of phenothiazines and carbazoles alone affected the growth of *S. cerevisiae* cells at concentrations higher than 180 μM, 20 μM of these compounds increased considerably the sensitivity of *S. cerevisiae* cells to Flu (Fig. 6). In the case of WT cells, the most efficient was

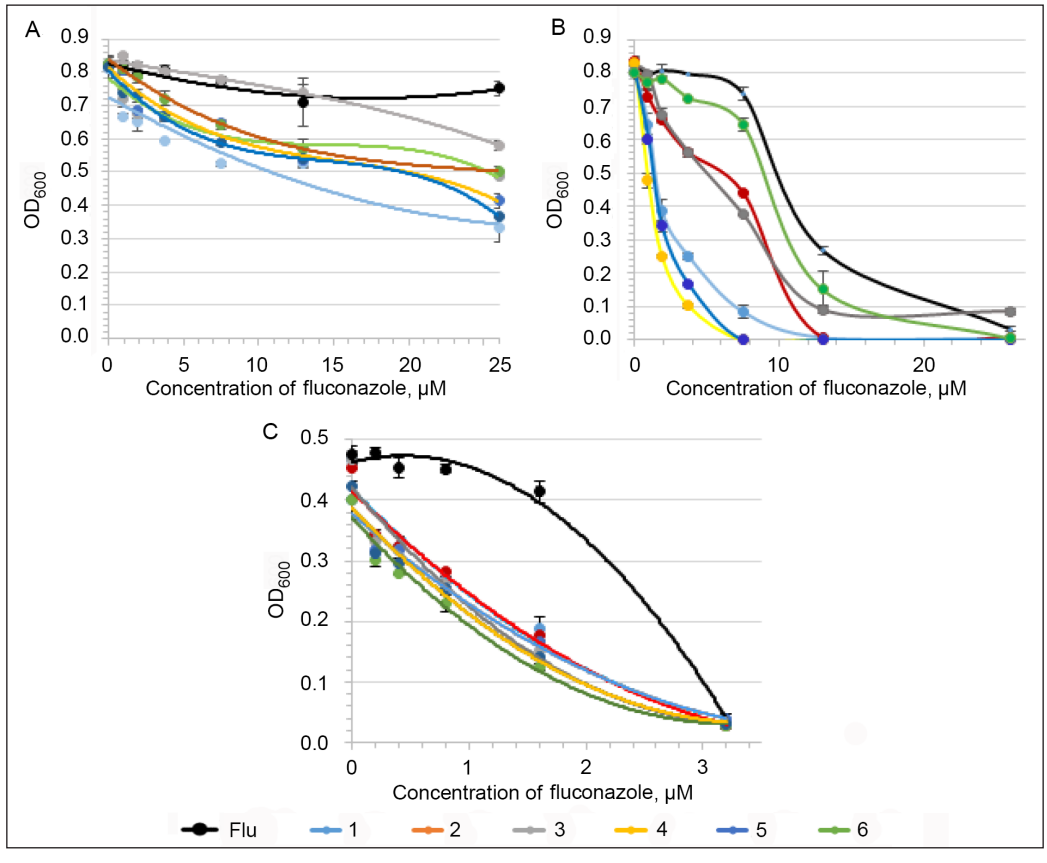

Fig. 6. Impact of fluconazole (Flu) and compounds **1–6** on the growth of *S. cerevisiae* W303-1a (**A**), ΔPdr5 (**B**), and *C. albicans* ATCC10231 (**C**). Cells were grown in YPD (*S. cerevisiae*) or RPMI-1640 (*C. albicans*) media at 30°C (*S. cerevisiae*) or 37°C (*C. albicans*) for 24 h. Concentration of compounds **1–6** was 20 μM in experiments with S. cerevisiae or 2.5 μM – with *C. albicans*. The initial cell concentration was 1–5 × 10^3 cfu/mL

10-Methyl-10*H*-phenothiazine (**1**). An even stronger Flu supporting effect of compound **1** was observed in experiments with efflux mutant. In general, in the lack of Pdr5 transporter *S. cerevisiae* cells were much more sensitive to Flu, but the synthesized compounds additionally increased the efficiency of this antifungal. In the case of the mutant strain, the antifungal efficiency of Flu was the highest in the presence of 10-Methyl-10*H*-phenothiazine (**1**) and also compounds **4** and **5**. In the case of opportunistic fungal pathogen *C. albicans*, the synthesized compounds enhanced the efficiency of Flu at the concentration of 2.5 μM almost equally.

CONCLUSIONS

In our experiments, the synthesized phenothiazine and carbazole derivatives (**1–6)** did not have strong bactericidal and fungal properties against the cells of *S. enterica*, *S. cerevisiae,* and *C. albicans*. However, *S. enterica* ΔTolC mutant cells were more sensitive to these compounds than wild type ones. Popular RND family efflux pump inhibitor PAβN increased the susceptibility of *S. enterica* WT cells to the synthesized phenothiazine derivatives and the inhibition level did not depend on the attached alkyl chain. However, in contrast to the synthesized phenothiazine derivatives, efficiency of the synthesized carbazoles was dependent on the length of the attached alkyl chain: 9-hexyl-9*H*-carbazole (**6**) was the most active. The results of experiments with antibiotic chloramphenicol confirmed that synthesized compounds acted as competitive inhibitors of efflux and increased the efficiency of antibiotics, especially in the case of *S. enterica* ΔTolC mutant strain. N-alkylphenothiazines increased the efficiency of the antibiotic, but the length of the chain did not produce a significant change on the efficiency.

Potentiometric measurements of the amount of cell accumulated TPP^+ confirmed the conclusion that the inhibitory efficiency of phenothiazines does not depend on alkyl chain length while the efficiency of carbazoles does: the increase in TPP^+ accumulation was the highest in the presence of 9-hexyl-9*H*-carbazole (**6**). Results of experiments with *S. enterica* cells indicated that phenothiazine and carbazole derivatives acted as competitive inhibitors in the case of TPP^+ efflux.

The following results of our experiments with the yeast cells showed that the synthesized compounds were non-toxic to the cells of *S. cerevisiae*, but slightly toxic to those of *C. albicans*. The data of *S. cerevisiae* ΔPdr5 mutant cells confirmed significantly the role of efflux in the interaction with phenothiazines and carbazoles. In the presence of the synthesized compounds, the cells of *S. cerevisiae* and especially of *C. albicans* were much more sensitive to fluconazole. We can conclude that the synthesized compounds acted as efflux inhibitors in yeast and they work synergistically with fluconazole.

Experiments with yeast cells revealed the dependence of the inhibitory activity of the compounds on the attached chain length: in the presence of fluconazole, the methyl groups possessing 10-methyl-10*H*-phenothiazine (**1**) and 9-methyl-9*H*-carbazole (**4**) had the strongest effect against *S. cerevisiae* cells. Combinations of fluconazole and compounds **1–6** showed high activity and effectively inhibited the growth of *C. albicans.*

This study demonstrated the differences in the efficiency of the synthesized compounds against yeast and gram-negative bacteria with evolved effective barriers.

As carbazole and phenothiazine derivatives have wide pharmaceutical activity, it could be considered as potential multidrug resistance (MDR) efflux pump inhibitors (Mahmood et al., 2016), but it requires further investigation.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interest.

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SALMONELLA ENTERICA SER. *TYPHIMURIUM, SACCHAROMYCES CEREVISIAE* IR *CANDIDA ALBICANS* LĄSTELIŲ SUSINTETINTŲ 9*H*-KARBAZOLO IR 10*H*-FENOTIAZINO DARINIŲ ANTIMIKROBINIO AKTYVUMO ĮVERTINIMAS

Santrauka

Šiame tyrime mes susintetinome įvairaus ilgio alkilo pakaitus turinčius 9*H*-karbazolo ir 10*H*-fenotiazino darinius bei įvertinome jų antimikrobinį ir daugiavaisčio atsparumo siurblius slopinantį poveikį *Salmonella enterica* ser. *Typhimurium*, *Saccharomyces cerevisiae* ir *Candida albicans* ląstelėms. Šie dariniai yra svarbūs dėl plataus jų pritaikymo spektro, ypač medicininėje chemijoje vertinamas jų farmakologinis aktyvumas. Mūsų tyrimo rezultatai atskleidė, kad didėjantis karbazolų alkilo grandinių ilgis padidino tetrafenilfosfonio (TPP^+) jonų kaupimąsi ląstelėje. *S. enterica* mutantinės ΔTolC ląstelės buvo jautresnės susintetintiems junginiams. Junginiai sustiprino flukonazolo poveikį *S. cerevisiae* mielių ląstelėse. Mutantinės padermės ΔPdr5 ląstelės buvo jautresnės tiriamiems karbazolo ir fenotiazino dariniams. Junginiai su trumpesne alkilo grandine (10-metil-10*H*-fenotiazinas ir 9-metil-9*H*-karbazolas) efektyviausiai slopino *Candida albicans* ląstelių gyvybingumą.

Raktažodžiai: fenotiazinas, karbazolas, tetrafenilfosfonio jonai, minimali slopinančioji koncentracija, išmetimo siurbliai, *Salmonella enterica*, *Saccharomyces cerevisiae*, *Candida albicans*