Antifungal activity of gemini quaternary ammonium salts

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A series of gemini quaternary ammonium chlorides and bromides with various alkyl chain and spacer lengths was synthesized. The most active compounds against fungi were chlorides with 10 carbon atoms within the hydrophobic chain. Among these compounds were few with no hemolytic activity at minimal inhibitory concentrations. None of the tested compounds were cytotoxic and mutagenic. Cationic gemini surfactants poorly reduced the adhesion of microorganisms to the polystyrene plate, but inhibited the filamentation of Candida albicans. One of the tested compounds eradicated C. albicans and Rodotorula mucilaginosa biofilm, what could be important in overcoming catheter-associated infections. It was also shown that gemini surfactants enhanced the sensitivity of C. albicans to azoles and polyenes, thus they might be potentially used in combined therapy against fungi.

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1. Introduction

Surfactants as surface-active compounds can interact with the cellular membranes of microorganisms and in consequence be good antimicrobial agents (Shirai et al., 2006, 2009; Hoque et al., 2012; Grenier et al., 2012). On the other hand surfactants easily absorb at liquid/solid interphases, coat surfaces and protect them from adhesion of microorganisms (McCarron et al., 2007).

The diseases caused by fungal colonization have become a big problem due to ever growing strain resistance (Pfaller, 2012). For this reason the antifungal activity of many known and commonly used antifungals such as fluconazole is still decreasing and the solution to this problem could be finding new active compounds and working out new strategies of extermination of pathogenic fungi.

Candida albicans is the most well known opportunistic pathogen which lives in yeast or filamentous form. C. albicans biofilms are the most difficult form to eradicate due to e.g., a polymeric matrix, which strongly protects mycelium against penetration by drugs (Tournu and Van Dijck, 2012). Infections, like fungemia or meningitis caused by Rhodotorula sp. are less common, however they might occur if the patient is immunosuppressed. A saprophytic form of this fungus has been collected from skin, vaginal and respiratory tracts, but a colonizing form has been found in catheters, contact lenses, bronchoscopes and hemodialysis machines, where it can create biofilms (Savin et al., 2008). Thus adhesion, the first step to biofilm formation seems to be a good moment to apply antifungal compounds. Surfactants are perfect compounds for diminishing the biofilm creation risk.

Gemini surfactants are a class of amphiphilic compounds built from two classic surfactant moieties (of two hydrophobic tails and two cationic head groups per molecule) bound together by a spacer group. In comparison with corresponding single chain surfactants (mono-QASs), these surfactants (gemini-QASs, also called bis-QASs) are more efficient in lowering surface tension and have much lower critical micelle concentration (CMC) (Lachowicz et al., 1992, 1995; Fuhrhop and Wong, 2004). Due to their higher surface activity they have excellent dispersion stabilization and soil clean-up properties (Conte et al., 2005). These compounds are widely used as effective emulsifiers and dispersing agents (Schnell et al., 2008). Moreover, they appear to be excellent for creating complexes with DNA and are effective in mediating transfection. Due to their construction, DNA carrier molecules built from gemini surfactants are able to deliver genes to cells of almost any DNA molecule size (Mc Gregor et al., 2001; Pullmannova et al., 2012; Kim et al., 2011).

Single tail single head cationic surfactants show good antimicrobial activity, however they exhibit hemolytic activity (Shalez et al., 2001; Vieria and Carmona-Riberio, 2006). Amino acid based surfacants from the cationic guanidine group have strong antimicrobial activity and are less toxic to human cells and more environmentally friendly (Moran et al., 2004). Cationic gemini surfactants based on arginine have a very low critical micelle concentration (CMC) and high antimicrobial activity, but are toxic for human cells (Perez et al., 2002; Castillo et al., 2004). Colomer and co-workers (2011) synthesized a series of gemini, lysine-based surfactants and tested their antimicrobial and hemolytic activity. Generally the
antibacterial activity of lysine derivatives was lower than arginine-based compounds. Moreover, single-chain lysine surfactants were less hemolytic than corresponding gemini amphiphiles.

In this work we describe the biological activity of the series of gemini quaternary ammonium salts having betaine based ester type alkyl chain arrangements – their hemolytic activity, cytotoxicity and mutagenic potential as well as their activity against the growth, adhesion and biofilm formation of two pathogenic fungi – Candida albicans and Rhodotorula mucilaginosa.

2. Materials and methods

2.1. Materials

Chloroacetyl chloride (98%), bromoacetyl bromide (≥98%), 1-hexanoyl (98%), 1-octanoyl (≥99%), 1-decanoyl (99%), 1-tetradecanoyl (97%), N,N,N,N-tetramethylthylene-diamine (99%), N,N,N,N′-tetramethyl-1,3-propanediamine (99%), dichloromethane (pure p.a.), acetonitrile (pure p.a.), sodium hydrogen carbonate (pure p.a.) and magnesium sulfate anhydrous (pure p.a.) were all purchased from Sigma–Aldrich.

2.2. Synthesis of gemini quaternary ammonium salts

A series of cationic gemini surfactants was synthesized by a two-step procedure, as reported before (Tehrani-Bagha et al., 2012), constituting the appropriate n-alkyl α-chloro- or α-bromoacetates (ABr/CIA) synthesis and in the next step–quaternization of N,N,N′-tetramethyl-1,3-propanediamine or N,N,N,N′-tetramethyl-1,3-propanediamine with ABr/CIA (for the structure see Fig. 1, abbreviated as TMEG-n Br/Cl or TMPG-n Br/Cl; n = 6, 8, 10, 12, 14).

2.3. Synthesis of n-alkyl-α-halo-acetates (general procedure)

n-Alkyl α-bromo/chloro-acetates were synthesized in a reaction of n-alkanoles (1-hexan, 1-octan, 1-decan, 1-dodecanol or 1-tetradecanol) with chloroacetyl chloride (or bromoacetyl bromide) in dichloromethane as a solvent. Thus, 0.5 mol of the respective primary alcohol, dissolved in 400 cm³ of dichloromethane, was stirred under reflux and 0.7 mol of chloroacetyl chloride/bromoacetyl bromide in 100 cm³ of dichloromethane was added stepwise, while the hydrobromide/hydrochloride formed was trapped in a NaOH solution. The reaction mixture was refluxed for an additional 8 h, then cooled and neutralized with sodium hydroxide, and washed with water several times and dried over anhydrous magnesium sulphate.

2.4. Quaternization of tetramethyl-diamines with n-alkyl-α-halo-acetates (general procedure)

After the solvent evaporation, n-alkyl-α-halo-acetates were achieved in an 80% yield and immediately used for the quaternization step of N,N,N′,N′′-tetramethylhexadecylamines or N,N,N′,N′′-tetramethyl-1,3-propanediamine. In the latter step 0.1 mol of the diamine in acetonitrile was heated at 80 °C and 0.2 mol of a given alkyl α-bromoacetate (or α-chloroacetate) also in acetonitrile was added drop wise to the reaction mixture. The mixture was refluxed further for 30 h at 80 °C, then cooled in a refrigerator and the precipitated product was filtered off (yield 25–40%). The crude products were recrystallized from a hexane/chloroform solvent system with (v/v) ratio dependent on the compound and purity of the product. The best effect of the process was obtained using procedure as follow. Crude product was dissolved in appropriate volume of chloroform under reflux and then n-hexane was added drop wise until the beginning of precipitation was observed. Solution was cooled to the room temperature and kept several days in refrigerator. Crystallized product was filtered off, dried and its purity was checked (sharp m.p., 1H NMR spectra). If the purity was not satisfying crystallization was repeated. The scheme of 2-step synthesis of gemini surfactants is shown in Fig. 2.

The surfactants were purified by repeated crystallization until no impurities could be detected by NMR spectra. The chemical structure of compounds was determined by their 1H NMR (Bruker Avance 300 MHz, CDCl3, internal standard TMS, δ ppm). The results indicate that the gemini quaternary ammonium bromides/chlorides are at least 98 mole% pure, as was confirmed by the very narrow range of melting points. The 1H NMR spectra (shown as examples) are described as follows:

TMEG-10Cl: 0.86 [6H, t, J = 5.7 Hz, 2−(CH2)]; 1.25–1.40 [28H, m, 2−(CH2)]; 1.66–1.79 [4H, m, 2−(CH2)]; 3.774 [12H, s, 2−(N−CH2)]; 4.17 [4H, t, J = 6.9 Hz, 2−(N−CH2)]; 4.69 [4H, s, 2−(N−CH2)]; 4.83–4.93 [4H, m, 2−(O−CH2)];

TMEG-12Cl: 0.86 [6H, t, J = 6.6 Hz, 2−(CH2)]; 1.43–1.41 [36H, m, 2−(CH2)]; 1.66–1.80 [4H, m, 2−(O−CH2)]; 3.63 [12H, s, 2−(N−CH2)]; 4.17 [4H, t, J = 6.8 Hz, 2−(N−CH2)]; 4.73 [4H, s, 2−(N−CH2)]; 4.84–4.92 [4H, m, 2−(O−CH2)];

TMEG-14Cl: 0.86 [6H, t, J = 6.8 Hz, 2−(CH2)]; 1.24–1.37 [44H, m, 2−(CH2)]; 1.66–1.79 [4H, m, 2−(O−CH2)]; 3.54 [12H, s, 2−(N−CH2)]; 4.18 [4H, t, J = 6.9 Hz, 2−(N−CH2)]; 4.48 [4H, s, 2−(N−CH2)]; 4.89–4.99 [4H, m, 2−(O−CH2)];

TMEG-10Cl: 0.85 [6H, t, J = 6.6 Hz, 2−(CH2)]; 1.24–1.48 [28H, m, 2−(CH2)]; 1.61–1.65 [4H, m, 2−(O−CH2)]; 2.68–2.89 [2H, m, 2−(N−CH2)]; 3.63 [12H, s, 2−(N−CH2)]; 3.97 [4H, t, J = 6.9 Hz, 2−(N−CH2)]; 4.15 [4H, s, 2−(N−CH2)]; 4.83–4.99 [4H, m, 2−(O−CH2)];

TMEG-12Cl: 0.86 [6H, t, J = 6.6 Hz, 2−(CH2)]; 1.24–1.49 [36H, m, 2−(CH2)]; 1.61–1.66 [4H, m, 2−(O−CH2)]; 2.71–2.81 [2H, m, 2−(N−CH2)]; 3.89 [4H, t, J = 6.9 Hz, 2−(N−CH2)]; 4.15 [4H, s, 2−(N−CH2)]; 4.84–4.93 [4H, m, 2−(O−CH2)];

TMEG-14Cl: 0.86 [6H, t, J = 6.6 Hz, 2−(CH2)]; 1.24–1.41 [44H, m, 2−(CH2)]; 1.62–1.86 [44H, m, 2−(O−CH2)]; 2.59–2.78 [2H, m, 2−(N−CH2)]; 3.57 [12H, s, 2−(N−CH2)]; 3.86 [4H, t, J = 6.9 Hz, 2−(N−CH2)]; 4.15 [4H, s, 2−(N−CH2)]; 4.76–4.91 [4H, m, 2−(O−CH2)];

2.5. Strains

In the present study, the following fungal strains were used: Candida albicans ATCC 90028, Candida parapsilosis IHEM 3270, Rhodotorula mucilaginosa IHEM 18459 and Saccharomyces cerevisiae Σ1278b.
2.6. Minimal inhibitory concentration (MIC)

To establish the antifungal activity of the tested compounds, minimal inhibitory concentration (MIC) on 96-well polystyrene microtiter plates (Sarstedt) was determined. Strains were incubated with compounds in the range of concentrations 10–800 µM. Plates were incubated for 48 h at 37 °C in YPG medium (1% yeast extract Difco, 1% peptone Difco, 2% glucose) and optical density was measured using a microplate reader at A590nm (ASYS UVM 340 Biogenet) according to Clinical and Laboratory Standards Institute (2008), 3rd ed. M27-A3. Negative and growth control wells did not contain surfactants.

2.7. Cytotoxicity assay

For cytotoxicity measurements alamarBlue Assay (Sigma) was used. The S. cerevisiae Σ1278b strain was incubated for 12 h with or without a given compound. AlamarBlue (resazurin) was added in an amount equal to 10% of the volume in the well. The plate was incubated for 4 h in the dark. Conversion of resazurine to a reduced form (pink color) was observed.

2.8. Hemolysis

Gemini-QAS were tested for hemolytic activity, as described by Falkinham III et al., 2012. 5 mL of sheep blood was centrifuged to obtain morphic elements (2500 rpm, 15 min), washed 3 times in PBS (pH 7.4) and resuspended in PBS. The compound at various concentrations was mixed with 100 µL of erythrocytes and incubated for 1.5 h at 37 °C. Absorbance was measured at λ = 540 nm. As positive and negative controls, PBS and 1% SDS (respectively) were used. This test was repeated at least three times.

2.9. Ames’ test

Two strains of Salmonella Typhimurium, TA98 and TA100, deficient in the synthesis of histidine, were used according to method proposed by Ames et al., 1975. The tested compound at given concentration and 100 µL of bacterial culture (10^8 cell/mL) were added to 2 mL of top agar and spread on the plate with minimal Davis media. The mixture without tested compound was used as negative control. Plates were incubated for 48 h at 37 °C and colonies were counted. As positive control cisplatin was used in the concentration 5 µg per plate. The mutagenic ratio (MR) – ratio of the number of revertants grown in the presence of tested compound to the number of spontaneously appeared revertants (on the negative control) was calculated. Mutagenic ratio equal or higher than 2.0 determines the mutagenic potential of tested compound. Mutagenic ratio lower than 1.7 indicates lack of mutagenic activity. Values of MR between 1.7 and 2.0 designate the compound as potential mutagen. The test was repeated three times with similar results.

2.10. The influence of gemini surfactants on Candida albicans sensitivity to azoles and polynes

To determine the influence of the tested gemini-QAS on C. albicans sensitivity to antifungals (azoles and polynes), C. albicans were diluted in YPG on microtiter plates. Gemini cationic surfactants at final concentrations of 1/2 MIC, and itraconazole and fluconazole at final concentrations of 5 and 150 µg/mL, respectively, were applied. From the polyene group 0.3 µM amphotericin B was used. Compounds were added separately or in combinations: gemini surfactant-azoles or gemini surfactants-amphotericin B. Cells were incubated at 28 °C for 24 h and viability was measured using a microplate reader at A590nm (ASYS UVM 340 Biogenet). Negative and growth control wells did not contain surfactants. This test was repeated at least three times.

2.11. Effect of gemini quaternary ammonium salts on the adhesion and biofilm removal

C. albicans and R. mucilaginosa were grown on a YPG medium at 30 °C for 24 h. The cells were then washed once with phosphate-buffered saline (PBS, pH 7.4) and diluted in fresh YPG medium to a final concentration of 5 × 10^6 cells/mL. Gemini surfactants were added to a 96-well flat-bottom polystyrene plate (Sarstedt) at concentrations: 10–240 µM, and the plate was incubated for 2 h at 37 °C with shaking. Then, the plate was washed with distilled water and the tested strains were added to the final culture volume, 100 µL in every well. The plate was incubated at 37 °C for 2 h to induce germination. Non-adherent cells were removed by several washes with water. Adherent germ tube forms were stained with 0.1% crystal violet for 5 min and washed three times with distilled water. Next, the 150 µL of isopropanol-0.02 N HCl and 50 µL of 0.25% SDS were added to each well to dissolve the crystal violet. The absorbance of each well was measured using a microplate reader at A590 nm (ASYS UVM 340 Biogenet). Assays were carried out twice in three replicates.

In the case of biofilm formation, the assay was performed as described above, only the plates were initially incubated with C. albicans and R. mucilaginosa cells for 12 h at 37 °C, washed and incubated with tested gemini-QAS. After incubation, non-adhered cells were removed by several washes. Wells were stained with crystal violet and the absorbance of each well was measured as described above. The assay was performed twice in three replicates.
Table 1

Minimal inhibitory concentration (MIC) of gemini quaternary ammonium salts.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Minimal inhibitory concentrations [µM] of gemini surfactants for tested strains:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>TMEG-6 Cl</td>
<td>800</td>
</tr>
<tr>
<td>TMEG-8 Cl</td>
<td>160</td>
</tr>
<tr>
<td>TMEG-10 Cl</td>
<td>10</td>
</tr>
<tr>
<td>TMEG-12 Cl</td>
<td>320</td>
</tr>
<tr>
<td>TMEG-12 Br</td>
<td>500</td>
</tr>
<tr>
<td>TMEG-14 Cl</td>
<td>320</td>
</tr>
<tr>
<td>TMPG-10 Cl</td>
<td>20</td>
</tr>
<tr>
<td>TMPG-12 Cl</td>
<td>160</td>
</tr>
<tr>
<td>TMPG-12 Br</td>
<td>240</td>
</tr>
</tbody>
</table>

2.12. Filamentous growth

To investigate whether gemini surfactants impact *C. albicans* filamentation, the morphology of this strain was observed after 2, 4, 6 and 24 h incubations at 37 °C in YPG medium with a given compound at final concentrations of MIC, 1/2 and 1/4 MIC. An AXIO Imager A2 (ZEISS) microscope was used for observations. Scale bar = 10 µm.

3. Results

3.1. Determination of minimal inhibitory concentration (MIC)

Among gemini quaternary ammonium salts with shorter spacers the most active compound against the tested fungi was chloride, with 10 carbon atoms within the alkyl chain (TMEG-10 Cl) (Table 1). This compound exhibited antifungal activity at low concentrations (10–40 µM) against non-pathogenic (*S. cerevisiae*) and pathogenic (*C. albicans*, *C. parapsilosis* and *R. mucilaginosa*) fungi. Shortening and elongation of the hydrocarbon chain caused the decrease in antifungal activity. The comparison of bromide and chloride with the same alkyl chain length revealed that the compound with the chloride counterion has a stronger fungicidal effect.

The group of compounds with longer spacers included three gemini surfactants, and the most active against fungi was chloride with 10 carbon atoms within the hydrocarbon chain (TMEG-10 Cl). Elongation of the alkyl chain caused the decrease in antifungal effect and chlorides were more active against fungal cells than bromides (TMPG-12 Cl, TMPG-12 Br) (Table 1).

3.2. Hemolytic activity of gemini surfactants

Gemini surfactants with the lowest MIC values were tested for their hemolytic activity. It was shown that the compound with 8 carbon atoms within the alkyl chain and the shorter spacer (TMEG-8 Cl) did not exhibit hemolytic activity at MIC, at a three times higher concentration (Fig. 3). Elongation of the hydrophobic chain (to 10 carbon atoms) did not cause hemolysis at minimal inhibitory concentration, however with the increase of concentration some disintegration of erythrocyte membrane was observed. Chloride with the longer spacer (TMPG-10 Cl) caused the strongest hemolysis. 10 µM of this compound slightly lysed erythrocytes, but 80 µM (MIC for *C. albicans*) effected in the high degree of hemolysis (60%) (Fig. 3).

3.3. Cytotoxic activity of gemini surfactants

The cytotoxicity of gemini surfactants with the strongest antifungal activity was tested with resazurin solution (AlamarBlue). No cytotoxic effect against mitochondrial metabolism manifested with the change of the well color from blue to pink. Our results showed that none of the studied compounds was cytotoxic at the concentrations equal and lower than MIC.

3.4. Mutagenic potential of gemini surfactants

The mutagenicity of the most active gemini-QAS–TMEG-10 Cl and TMPG-10 Cl, measured by Ames’ Test showed that none of the tested compounds exhibit mutagenic potential. The ratio of the revertant number to negative control (MR) was elevated in the case of TMEG-10 Cl for the TA98 strain (1.6), however it never reached 2.0 (Table 2).

3.5. Influence of cationic gemini surfactants on *C. albicans* sensitivity to azoles and polyenes

*C. albicans* is a fungal pathogen causing skin and systemic infections. Azoles (e.g., fluconazole, itraconazole) and polyenes (e.g., amphotericin B) are the most commonly used in their treatment. Pathogenic strains can acquire resistance to these antibiotics by e.g., active efflux of the drug by the plasma membrane transporters MFS and ABC.

Our study showed that gemini surfactants enhanced fungicidal the effect of azoles at lower concentrations than when the antibiotics were applied solely. Azoles caused a drop of about 40% in *C. albicans* viability, and the addition of gemini surfactant significantly

Table 2

<table>
<thead>
<tr>
<th>Tested compound</th>
<th>Concentration [µM]</th>
<th>TA98</th>
<th>TA100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colony number</td>
<td>MR&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Colony number</td>
</tr>
<tr>
<td>PC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&gt;400</td>
<td>&gt;11.0</td>
<td>&gt;400</td>
</tr>
<tr>
<td>NC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>34 ± 7.8</td>
<td>1.6</td>
<td>241.5 ± 2.1</td>
</tr>
<tr>
<td>TMEG-10 Cl</td>
<td>20</td>
<td>56 ± 8.5</td>
<td>196 ± 16.9</td>
</tr>
<tr>
<td>TMEG-12 Cl</td>
<td>40</td>
<td>33 ± 2.8</td>
<td>192 ± 12.7</td>
</tr>
<tr>
<td>TMEG-10 Br</td>
<td>20</td>
<td>27 ± 2.8</td>
<td>97 ± 2.8</td>
</tr>
</tbody>
</table>

<sup>a</sup> As positive control (PC) cisplatin (5 µg/plate) was used.
<sup>b</sup> As negative control (NC) bacterial dilution was added to top agar without tested compounds.
<sup>c</sup> Mutagenic ratio (MR)–see Ames’ Test in Section 2. The values are average from at least three tests.
enhanced fungicidal effect. The compound that decreased *C. albicans* viability on the highest level was TMEG-8 Cl, which caused around a 2-fold increase of *C. albicans* sensitivity (Fig. 4c). The compound with 10 carbon atoms within its alkyl chains (TMEG-10 Cl) showed a much weaker effect. It exhibited only 20% inhibition of *C. albicans* growth in the presence of itraconazole, and 40% in the case of fluconazole (Fig. 4a). The elongation of the spacer (TMPG-10 Cl) caused the increase of *C. albicans* sensitivity to azoles a 50% inhibition of *C. albicans* growth (Fig. 4b).

Amphotericin B, another tested antibiotic, belongs to the polyene class. It was shown that gemini surfactants with 10 carbon atoms within the alkyl chain, independently of spacer length,
strongly increased *C. albicans* sensitivity (about 10% of growth) to this antibiotic (Fig. 4d and e). The weakest fungidal effect with amphotericin B was observed for TMEG-8 Cl, since it caused only slight drop of *C. albicans* viability (Fig. 4f).

3.6. Effect of gemini surfactants on *C. albicans* and *R. mucilaginosa* adhesion

Due to their structure and properties gemini quaternary ammonium salts coat solid surfaces (e.g., plastic or silicone). The longer alkyl chains of the surfactants cause the compound to have a more hydrophobic nature. As a result of hydrophobic interactions, gemini-QAS might coat the surface and block the adhesion of microorganisms, which is the first stage of biofilm formation and the cause of infections, which are strongly resistant to treatment.

Our results showed that TMPG-10 Cl at high concentrations (240 μM for *C. albicans* and 160 μM for *R. mucilaginosa*) caused a 50% reduction in cell adhesion (Fig. 5c and d). 160 μM of gemini surfactant TMEG-10 Cl inhibited the *R. mucilaginosa* adhesion to 50% and *C. albicans* adhesion was 25% decreased by 240 μM of TMEG-10 Cl (Fig. 5a and b). TMEG-8 Cl did not inhibit the adhesion of strains (data not shown).

3.7. Effect of gemini surfactants on *C. albicans* filamentous growth

The ability of *C. albicans* to form filaments is one of the virulence determinants, thus the influence of gemini surfactants on this process was investigated. Microscopic observations showed that the tested compounds: TMEG-8 Cl, TMEG-10 Cl and TMPG-10 Cl inhibited *C. albicans* filamentation at concentrations equal to 1/4 MIC (Fig. 6). The reduction of filament formation was already occurring after 2 h incubation with the compounds, however complete inhibition of filamentation was noted only after 6 h incubation. Filamentation inhibition was observed also at 1/2 MIC and MIC of gemini surfactants with the repression of bud formation (data not shown).

Filamentous growth is one of the factors facilitating adhesion to host tissues and abiotic surfaces. The blockage of *C. albicans* filamentation by gemini-QAS might be one of the mechanisms conferring their antiadhesive activity.

3.8. Effect of gemini surfactants on *C. albicans* and *R. mucilaginosa* biofilm removal

Some fungal pathogens are able to form biofilm, composed of cells with altered metabolism and surrounded by an extracellular matrix, which makes the whole structure resistant to antifungals. The data indicates that monomeric quaternary ammonium salts are efficient in biofilm eradication, thus we decided to study the impact of gemini-QAS on fungal biofilm.

Our results showed (Fig. 7) that TMPG-10 Cl destroyed about 60% of biofilm formed by *C. albicans* and *R. mucilaginosa* at MIC (80 and 10 μM respectively) (Fig. 7f). A 5-fold increase in the concentration of this compound caused an 80% eradication of *R. mucilaginosa* biofilm, and the concentration of 240 μM removed the biofilm completely. In the case of *C. albicans* biofilm, the increase in compound concentration did not cause any further eradication of the biofilm (Fig. 7c and d).

Shortening of the spacer length in the compound with 10 carbon atoms within the alkyl chain (TMEG-10 Cl) slightly decreased antibiofilm activity. It was shown that the MIC of this compound destroyed *C. albicans* and *R. mucilaginosa* biofilm by 50% and 40%, respectively. The complete eradication of *R. mucilaginosa* biofilm was observed at concentration of 100 μM (Fig. 7a and b). The antibiofilm activity was not observed for TMEG-8 Cl (data not shown).

4. Discussion

Gemini surfactants are a new class of amphiphilic compounds built from two classic surfactant moieties bound together by a special spacer group (Yoshimura et al., 2012; Buse et al., 2011; Zhou and Zhao, 2009; Tehrani-Bagha et al., 2012). These compounds appear...
to be excellent for creating complexes with DNA and to be effective in mediating transfection. Due to their construction, DNA carrier molecules built from gemini surfactants are able to deliver to cell genes of almost any DNA molecule size, which is impossible for viral gene delivery systems (Mc Gregor et al., 2001; Pullmannova et al., 2012).

Gemini quaternary ammonium salts exhibit stronger antibacterial and antifungal activity in comparison to mono-QAS (Lachowicz et al., 1992, 1995; Shirai et al., 2012; Fisher et al., 2012).

A series of gemini quaternary ammonium salts with different alkyl chain lengths (6–14 carbon atoms), spacer lengths and counterions (bromide or chloride) was synthesized. The data indicate that monomeric quaternary ammonium salts with 12 carbon atoms within the alkyl chain are the most active against yeast and gram-positive bacteria (Obļak and Krasowska, 2010; Obļak et al., 2002; Gilbert and Moore, 2005). Our results showed that among gemini-QAS chlorides with 10-carbon hydrophobic chains and shorter spacers (TMEG-10 Cl) had the strongest activity against yeast and pathogenic fungi. Elongation of the spacer (TMPG-10 Cl) slightly decreased the antifungal effect. Due to their strong activity against fungi, these compounds could be applied as potential fungicides. Our further studies concerned the compounds with the lowest MIC values (TMEG-10 Cl, TMEG-8 Cl and TMPG-10 Cl). It was shown that cationic gemini surfactants with shorter spacers exhibited weak hemolytic activity (about 15%). The compound with 8 carbons in the alkyl chain did not cause significant hemolysis even at concentration three times higher than MIC. Similar results were obtained for piridium-based gemini quaternary ammonium salts (Shirai et al., 2009), where the concentration causing 50% hemolysis was much higher for the surfactant with 8 carbons within the hydrocarbon chain in comparison with the remaining compounds. Among tested gemini surfactants, the compound with 10 carbon atoms within the alkyl chain did not show any significant hemolytic activity at MIC, however a 2-fold increase in concentration caused a high degree of erythrocyte membrane disruption. The elongation of the spacer (TMPG-10 Cl) also increased hemolytic activity (50% of hemolysis in MIC), which excludes the potential application of this compound as a drug in internal mycosis treatment. Potential
fungicides should not exhibit cytotoxic effects. Our preliminary results showed that gemini quaternary ammonium salts were not toxic for the mitochondrial metabolism of S. cerevisiae. Moreover, Ames’ test showed that studied surfactants had no mutagenic activity.

Common usage of antifungals raises multidrug resistance among microorganisms. Pathogenic fungi have developed numerous mechanisms of resistance, which allow them to survive in the presence of toxic agents (White et al., 1998). The drugs most commonly applied in mycosis treatment are azoles (e.g., ketoconazole, fluconazole), which inhibit the biosynthesis of ergosterol, one of the main lipids building the plasma membrane. The resistance to these drugs occurs via different mechanisms, e.g., the overexpression of ABC and MFS pumps that actively efflux antibiotics (Sanglard et al., 2009). Currently substances, which would enhance the antifungal activity of drugs (e.g., azoles) are being investigated (Nylasi et al., 2010; Ahmad et al., 2010; Kiraz et al., 2010). The results of our study showed that gemini-QAS strongly increased C. albicans (in planktonic form) sensitivity to azoles (fluconazole and intraconazole). Another popular class of antibiotic are polyenes (nystatin, amphotericin B), which (as amphiphilic drugs) intercalate into the plasma membrane, creating channels for cellular components (mainly K+ ions) and disrupting the proton gradient (te Welscher et al., 2012). Our studies regarding the influence of gemini-QAS on C. albicans sensitivity to amphotericin B showed that the combination of both compounds significantly decreased C. albicans viability. Gemini-QAS, similarly to polyenes, affect the plasma membrane, causing mono- and divalent cation, as well as ATP, leakage (Shirai et al., 2009; Palermo et al., 2011). The combined activity of these two classes of substances might strongly disrupt plasma membrane structure, decreasing the survival of fungal cells (Ramos et al., 1996).

C. albicans is one of the most common human pathogens causing not only superficial mycosis but also systemic infections, especially dangerous for immunocompromised patients (Paller and Diekema, 2007). C. albicans may grow in different forms: yeast, pseudohyphal and hyphal. The switch between different types of growth is one of the virulence determinants, because it can lead to biofilm formation inside the host organism. The first stage in biofilm formation is the adhesion of fungal cells to the surface (e.g., intestinal tissue). During biofilm maturation the cells produce an extracellular matrix, composed of carbohydrates, proteins and phosphates, protecting biofilm from phagocytes and preventing drug penetration into the biofilm structure (Blankenship and Mitchell, 2006; Cuéllar-Cruz et al., 2012).

Biofilm formation is also common for Rhodotorula spp., that often colonize medical devices such as catheters or hemodialysis machines (Zaas et al., 2003). Gemini surfactants, as amphiphilic compounds, might coat theiotic and abiotic surfaces (due to hydrophobic interactions) and block the adhesion of microorganisms similarly to the action of some biosurfactants isolated from bacteria (Janek et al., 2012). Our results showed that gemini-QAS with 10 carbon atoms within the alkyl chain (with both shorter and longer spacers) inhibit the adhesion of R. mucilaginosa and C. albicans to the polystyrene surface at high concentrations. Shortening of the alkyl chain to 8 carbon atoms caused the decrease of antiadhesive activity, probably due to hydrophobic interactions being too weak. The application of gemini-QAS as adhesion blockers would reduce the risk of biofilm arising on the host tissues and medical devices.

The capacity for filamentous growth (hypha formation) is one of the virulence factors in C. albicans. Filamentation promotes fungal cell adhesion and biofilm maturation (Whiteway and Bachewich, 2007). The inhibition of hypha production lowers the risk of C. albicans infections (Messier and Grenier, 2011). To date numerous substances, which block the switch between yeast and filamentous forms were identified, examples being whey-derived fatty acids (linoleic, arachidonic) and capric acid isolated from Saccharomyces boulardi (Clement et al., 2007; Murzyn et al., 2010). Gemini quaternary ammonium salts are also effective inhibitors of C. albicans filamentous growth. Our results showed that chlorides with 10 and 8 carbons in the hydrocarbon chain almost completely inhibited filament formation at concentrations 4–fold lower than MIC. The usage of these compounds against C. albicans cells would significantly reduce their virulence, and biofilm formation.

The biofilm is extremely stable and resistant to numerous antimicrobial drugs (Ramage et al., 2005). Infections of medical devices (catheters, implants) by biofilm-forming microorganisms mean that the infected device needs to be removed and replaced. Monomeric quaternary ammonium salts are highly effective in bacterial biofilm eradication. It was shown that dimethylbenzylammonium chloride was strongly active against biofilm formed by Staphylococcus epidermidis. This compound penetrated inside the biofilm structure, but also changed the properties of the extracellular matrix, making it weaker and fluid (Davison et al., 2010).

Our results indicated that gemini quaternary ammonium salts exhibit strong activity against fungal biofilm. Chlorides with 10 carbon atoms within the alkyl chain (TMEG-10 Cl and TMPG-10 Cl) were highly reactive, especially against biofilm formed by R. mucilaginosa, because their minimal inhibitory concentrations eradicated about 50% of the generated biofilm.

Due to rising multidrug resistance among microorganisms, there is a strong need to search for new substances that would inhibit their growth and reduce the virulence. Currently studied gemini quaternary ammonium salts, due to their unique properties, could be good candidates for application as fungicides or disinfectants in order to overcome multidrug infections, often caused by biofilm formation.

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