BBA - General Subjects 1864 (2020) 129728

Contents lists available at ScienceDirect

BBA - General Subjects

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

The structure – Activity correlation in the family of dicationic imidazolium surfactants: Antimicrobial properties and cytotoxic effect

Alexandra D. Voloshina[a], Syumbelya K. Gumerova[a], Anastasiia S. Sapunova[a], Natalia V. Kulik[a], Alla B. Mirgorodskaya[a,*], Alla A. Kotenko[b], Tatiana M. Prokopyeva[b], Vasilii A. Mikhailov[b], Lucia Ya Zakharova[a], Oleg G. Sinyashin[a]

[a] Arbuzov Institute of Organic and Physical Chemistry, FRC Kazan Scientific Center, Russian Academy of Sciences, Arbuzov str., 8, Kazan 420088, Russia
[b] L.M. Litvinenko Institute of Physical Organic Chemistry and Coal Chemistry, 70 R. Luxemburg St., 83114 Donetsk, Ukraine

ARTICLE INFO

Keywords:
Dicationic imidazolium surfactants
Antimicrobial activity
Cytotoxicity
Hemolysis
Resistance
Apoptosis

ABSTRACT

Background: The development of new effective microbicide surfactants and the search for the structure–biological activity relationship is an important and promising problem. Surfactants containing imidazolium fragment attract attention of researchers in the field of chemotherapy, because these compounds often exhibit high antimicrobial activity. The aim of this work is to identify the newly synthesized surfactants from the viewpoint of their potential usefulness in pharmacology and medicine. For this purpose, a detailed study of antimicrobial, hemolytic and cytotoxic activity of dicationic alkylimidazolium surfactants of the m-s-m (Im) series with a variable length of a hydrocarbon tail (m = 10, 12) and a spacer fragment (s = 2, 3, 4) was carried out.

Methods: Aggregation of surfactants in solutions was estimated by tensiometry and conductivity. Antimicrobial activity was determined by the serial dilution technique. Cytotoxic effects of the test compounds on human cancer and normal cells were estimated by means of the multifunctional Cytell Cell Imaging system. Cell Apoptosis Analysis was made by flow cytometry.

Results: The test compounds show high antimicrobial activity against a wide range of test microorganisms and do not possess high hemolytic activity. Importantly, some of them display a bactericidal activity comparable to ciprofloxacin fluoroquinolone antibiotic against Gram-positive bacteria, including methicillin-resistant strains of *S. aureus* (MRSA). The cytotoxicity of the compounds against normal and tumor human cell lines has been tested as well, with cytotoxic effect and selectivity strongly controlled by structural factor and kind of cell line. Superior results were revealed for compound 10–4-10 (Im) in the case of HuTu 80 cell line (duodenal adenocarcinoma), for which IC_{50} value at the level of doxorubicin and a markedly higher selectivity index (SI 7.5) were demonstrated. Flow cytometry assay shows apoptosis-inducing effect of this compound on HuTu 80 cells, through significant changes in the potential of mitochondrial membrane.

Major conclusions: Antibacterial properties are shown to be controlled by alkyl chain length, with the highest activity demonstrated by surfactants with decyl tail, with the length of the spacer fragment showing practically no effect. The results indicate that the mechanism of cytotoxic effect of the compounds can be associated with the induction of apoptosis via the mitochondrial pathway.

General significance: Selectivity against pathogenic microorganisms and low toxicity against eukaryotic cells allow considering dicationic imidazolium surfactants as new effective antimicrobial agents. At the same time, high selectivity against some cancer cell lines indicates the prospect of their using as components of new anticancer drugs.

1. Introduction

Due to the widespread occurrence of multidrug-resistant infectious agents, there is a demand to search for safe chemical compounds, which will form the basis of new drugs. Surfactants containing imidazolium fragment attract attention of researchers in the field of chemotherapy, because these compounds often exhibit high antimicrobial activity [1–6]. Functionalization of imidazolium surfactants with various

* Corresponding author.
E-mail address: mirgorod@iopc.ru (A.B. Mirgorodskaya).

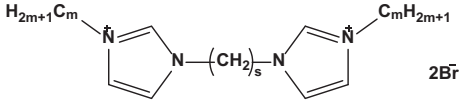
https://doi.org/10.1016/j.bbagen.2020.129728
Received 8 June 2020; Received in revised form 28 August 2020; Accepted 3 September 2020
Available online 06 September 2020

substituents makes it possible to modify their hydrophilic-lipophilic balance and aggregation properties, as well as to tune their biological activity. Dicationic imidazolium surfactants are of a particular interest [7–9]. They represent a large class of geminal surfactants with two hydrophobic tails and two positively charged head groups covalently linked by a spacer fragment. Gemini surfactants display superior properties to corresponding monocationic analogues: they more effectively reduce the surface tension at the phase boundary, have an order of magnitude lower critical micelle concentration (CMC), possess high solubility, and high wetting and solubilization effects [10–15]. These properties of dicationic surfactants predetermine their broad application as solubilizers and adjuvants, nanocontainers and nanoreactors, as well as non-viral vectors for gene delivery into cells of a living organism. Dicationic surfactants also show antimicrobial effect, which is maximum in compounds with a tail length C10-C12 [16,17]. In contrast, monocationic amphiphiles, whose antimicrobial activity shows a positive trend with an increasing the carbon chain length, reach maximum activity in case of tetra- or hexadecyl compounds, above which a so-called 'cut off effect' is observed [3,4,18,19].

The structure of head group and spacer fragment plays an important role in the aggregation behavior and properties of gemini surfactants [20–22]. There is a large body of recent literature data on the aggregation behavior of imidazolium dicationic surfactants in solutions, in which CMC values and adsorption parameters are determined [1,23,24]. There is a few information about their solubilization effect and complexation with proteins and DNA [1,25–29]. By contrast, an information on the antimicrobial properties of such compounds is fragmentary. However, some data on dicationic imidazolium surfactants indicate their significant potential. Thus, the study of 3,3′(α,ω-dioxaalkyl)-bis(1-alkylimidazolium) chlorides showed the high efficiency of this surfactants against *Staphylococcus aureus, Enterococcus faecalis, Acinetobacter baumannii, Escherichia coli, Klebsiella pneumoniae, Enterobacter cloacae, Pseudomonas aeruginosa, Candida krusei, and Candida albicans* [30,31]. In addition, antimicrobial effect was demonstrated for a series of new dicationic imidazolium surfactants containing amide group in the spacer fragment, which depends on the length of hydrophobic tail. High antifungal activity of these compounds against *Candida albicans,* one of the main opportunistic pathogens that cause a wide range of human diseases, should particularly be noted [32].

In this regard, the development of new effective microbicides based on dicationic imidazolium surfactants and the search of structure–-biological activity relationship is an important and promising problem. Taking into account the wide use of amphiphilic compounds in cancer chemotherapy for the preparation of nanoparticles with target agents and antitumor drugs [33–36], the investigation of antimicrobial properties of imidazolium surfactants should be extended by their cytotoxicity study.

The aim of this work is to identify new bioactive surfactants that can be used in pharmacology and medicine. For this purpose, a detailed study of antimicrobial, hemolytic and cytotoxic activity of a number of dicationic alkylimidazolium surfactants of the m-s-m (Im) series with a variable length of the hydrophobic radical and a varying distance between the head groups was carried out (Fig. 1).



m-s-m (Im), where m=10,12; s=2; 3; 4

Fig. 1. The structural formula of dicationic alkylimidazolium surfactants.

2. Materials and methods

2.1. Alkylimidazoles and bis-imidazolium salts. General remarks

Alkylimidazoles and bis-imidazolium salts were made according to Scheme 1.

This scheme is known from literature [37,38], but some details look incomplete or controversial. That is why detailed synthetic description provided for representative examples. Structure and purity of final and intermediary compounds were confirmed by ^{1}H NMR and IR spectra, and appropriate results of elemental analysis.

Step 1. N-Dodecylimidazole.

In a high form beaker (250 ml), imidazole (8.19 g; 0.120 M) was dissolved in dimethylsulfoxide (50 ml), and coarsely ground potassium hydroxide (12.89 g; 0.23 M) was added under stirring. Turbid mixture was heated at 50–60 °C and stirred until sediment became compact. Dodecyl bromide (25.06 g; 0.1005 M; approx. 24.1 ml) was added from dropping funnel over approx. Half an hour at 60–65 °C under intense stirring (at least 300 rpm). Heating was stopped and stirring was continued for an hour. The mixture was diluted with water up to 150 ml. Top layer was separated with a funnel, dissolved in methylene dichloride (50 ml), and washed with cold water (2 × 25 ml). Organic layer was separated, and volatiles were immediately distilled of on water bath, and, then, under reduced pressure. The residue was dissolved in acetonitrile (50 ml) and filtered. Solvent was removed under vacuum (20–30 mmHg). The crude product is yellowish oil; yield 23.22 g (9.82 × 10^{-2} M; 97%). After distillation in vacuo (130–135 °C/ 0.1 mmHg) - colorless liquid, solidified at freezing, mp 11–12 °C. ^{1}H NMR (400 MHz, CDCl3, δ, ppm): 7.45 (s, 1H), 7.05(s, 1H), 6.90 (s, 1H), 3.92 (t, J = 7.1 Hz,2H), 1.80–1.75 (m, 2H), 1.33–1.22 (m, 18H), 0.88 (t, J = 6.8 Hz, 3H).

Step 2. 1,2-bis(3′-dodecylimidazolium-1′-yl)ethane dibromide.

Freshly prepared solution of N-dodecylimidazole (2.93 g, 1.24 10^{-2} M) in tetrahydrofuran (2 ml), and 1,2-dibromoethane (0.98 g; 5.2 10^{-2} M) were placed in closed vessel, and mixture was heated up to 45–50 °C for ten days. After cooling, reaction mixture was diluted with acetone (25 ml) and thoroughly mashed. Non-dissolved part was filtered, washed with acetone (2 × 5 ml) and dried. White powder was obtained; yield 2.48 g (5.78 × 10^{-3} M; 72%). Crude product was recrystallized from isopropanol (20 ml). ^{1}H NMR (400 MHz, $CDCl_3$, δ, ppm): 10.34 (s, 2H), 8.86 (s, 2H), 7.14 (s, 2H), 5.31 (s, 4H), 4.15 (t, J = 7.5 Hz, 4H), 1.95–1.87 (m, 4H), 1.40–1.24 (m, 36H), 0.88 (t, J = 6.5 Hz, 6H). Anal.Calcd for $C_{32}H_{60}Br_2N_4$: C, 58.18; H, 9.15; N, 8.48; Br 24.19. Found: C, 57.98; H, 9.56; N, 8.39; Br 23.80. IR (KBr, ν, cm^{-1}): 3400 br, 3020, 2910, 2840, 1570, 1560, 1465, 1445, 1160, 850, 799, 710, 640, 620.

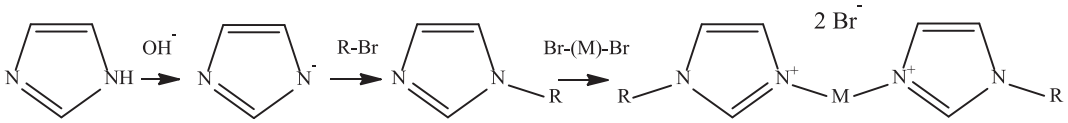
Synthetic procedures, melting points, spectral data for other dibromides under investigation summated in Supplementary Info.

2.2. Surface tension and conductivity measurements

Surface tension measurements were performed by the ring detachment method using KRUSS K6 tensiometer. Specific conductivities were measured with Inolab Cond 720 conductometer. Experimental temperatures were maintained at 25 ± 0.1 °C, unless otherwise indicated. All experiments were accurate within ± 4%.

2.3. Antimicrobial activity

Antimicrobial activity of the bis-imidazolium salts was determined by the serial dilution technique in Muller Hinton Broth 2 [39]. The cultures used for testing included Gram-positive bacteria: *Staphylococcus aureus ATCC 6538P FDA 209P*, *Bacillus cereus ATCC 10702 NCTC 8035*; Gram-negative bacteria: *Escherichia coli ATCC 25922*, *Pseudomonas aeruginosa* ATCC 9027, and fungi: *Aspergillus niger* BKMF-1119, *Trichophyton mentagrophytes* var. gypseum 1773, and *Candida albicans*



Scheme 1. The synthesis of bis-imidazolium salts

ATCC 10231; methicillin-resistant strains of *S. aureus* (MRSA) was obtained from hospital patients with chronic tonsillitis in the Republican Clinical Hospital (Kazan, Russia). The bacterial load was 3.0×10^5 cfu/ml. The results were recorded every 24 h for 5 days. Cultures were incubated at 37 °C. The experiment was repeated three times. The dilutions of the compounds were prepared immediately in nutrient media; 5% DMSO was added for better solubility and the test strains were not inhibited at this concentration. The minimum inhibitory concentration (MIC) was defined as the minimum concentration of a compound that inhibits the growth of the corresponding test microorganism. The growth of bacteria as well as the absence of the growth due to the bacteriostatic action of bis-imidazolium salts were recorded. To determine minimal bactericidal concentration (MBC), an aliquot of the bacterial culture was transferred onto Mueller-Hinton agar in a 10-cm Petri dish and incubated for 24 h at 37°C. MBC was the minimal concentration of the tested compound at which bacterial colonies were not detected indicating that the bacteria were killed with an efficiency of > 99.9% [40,41].

2.4. LIVE/DEAD BacLightTM bacterial viability method

To count live and dead cells, *Stapylococcus aureus* 209 P (control and test samples) were stained with a commercial dye mix of the *LIVE/DEAD BacLightTM Bacterial Viability* kit for 30 min in the dark. Then, the suspension was mixed with an equal volume of 0.5% low-melting agarose. The preparations were analyzed using a Nikon Eclipse Ci-S fluorescence microscope (Nikon, Japan). Cell counting in a given scan volume was carried out differentially according to red and green fluorescence.

2.5. Hemolytic activity

Hemolytic activity of the bis-imidazolium surfactants was estimated by comparing the optical density of a solution containing the test compound with that of blood at 100% hemolysis. A 10% suspension of human red cells was used as an object of investigation. Red cells with heparin was washed three times with physiological saline (0.9% NaCl) solution, centrifuged for 10 min at 800 rpm, and resuspended in physiological saline (0.9% NaCl) solution to a concentration of 10%. The concentrations of the compounds that corresponded to the MIC values for the bacterial test strains were prepared in physiological saline (0.9% NaCl) solution (supplemented with 5% DMSO), and 4.5 ml of the compound at the corresponding dilution was added to 0.5 ml of a 10% suspension of erythrocytes. The samples were incubated for 1 h at 37 °C and centrifuged for 10 min at 2000 rpm. Release of hemoglobin was controlled by measuring the optical density of the supernatant on Microplate reader Iinvitrologic (Russia) at $\lambda = 540$ nm. The control sample corresponding to zero hemolysis (blank experiment) was prepared by adding 50 μl of 10% red blood cell suspension to 450 μl of physiological saline solution (0.9% NaCl). The control sample corresponding to 100% hemolysis was prepared by adding of 50 μl of 10% red blood cell suspension to 450 μl of distilled water.

2.6. Cytotoxicity assay

Cytotoxic effects of the test compounds on human cancer and normal cells were estimated by means of the multifunctional Cytell Cell Imaging system (GE Health Care Life Science, Sweden) using the Cell Viability Bio App which precisely counts the number of cells and evaluates their viability from fluorescence intensity [42]. Two fluorescent dyes that selectively penetrate the cell membranes and fluoresce at different wavelengths were used in the experiments. DAPI is able to penetrate intact membranes of living cells and colors nuclei in blue and Propidium iodide dye penetrates only dead cells with damaged membranes, staining them in yellow. IC_{50} was calculated using an online tool: MLA—"Quest Graph™ IC50 Calculator." AAT Bioquest, Inc., 25 July 2019, https://www.aatbio.com/tools/ic50-calculator. DAPI and propidium iodide were purchased from Sigma. The M-Hela clone 11 human, epithelioid cervical carcinoma, strain of Hela, clone of M-Hela; human breast adenocarcinoma cells (MCF-7); human duodenal cancer cell line (HuTu 80); from the Type Culture Collection of the Institute of Cytology (Russian Academy of Sciences) and Chang liver cell line (Human liver cells) from N. F. Gamaleya Research Center of Epidemiology and Microbiology were used in the experiments. The cells were cultured in a standard Eagle's nutrient medium manufactured at the Chumakov Institute of Poliomyelitis and Virus Encephalitis (PanEco company) and supplemented with 10% fetal calf serum and 1% non-essential amino acids. The cells were plated into a 96-well plate (Eppendorf) at a concentration of 1×10^5 cells/ml, 150 μl of medium per well, and cultured in a CO_2 incubator at 37 °C. Twenty-four hours after seeding the cells into wells, the compound under study was added at a preset dilution, 150 μl to each well. The dilutions of the compounds were prepared immediately in nutrient media; 5% DMSO that does not induce inhibition of cells at this concentration was added for better solubility. The experiments were repeated three times. Intact cells cultured in parallel with experimental cells were used as a control.

2.7. Flow cytometry assay

2.7.1. Cell culture

M-Hela cells at 1×10^6 cells/ well in a final volume of 2 ml were seeded into 6-well plates. After 24 h of incubation, various concentrations of compound 10-4-10 (Im) were added to wells.

2.7.2. Cell apoptosis analysis

The cells were harvested at 2000 rpm for 5 min and, then, washed twice with ice-cold PBS, followed by resuspension in binding buffer 100 μl. Next, the samples were incubated with 0.35 μl of annexin V-Alexa Fluor 647 and 0.1 μl of propidium iodide for 40 min at room temperature in the dark. Finally, the cells were analyzed by flow cytometry (Guava easy Cyte, MERCK, USA).

The experiments were repeated three times.

2.7.3. Mitochondrial membrane potential

The cells were harvested at 2000 rpm for 5 min and, then, washed twice with ice-cold PBS, followed by resuspension in JC-10 (10 μg/ml) and incubation at 37 °C for 10 min. After the cells were rinsed three times and suspended in PBS, the JC-10 fluorescence was observed by flow cytometry.

2.7.4. Statistical analysis

IC50 was calculated using an online tool: MLA-"Quest Graph™ IC50

Table 1
Antimicrobial activity of dicationic alkylimidazolium surfactants[a].

Test compounds	Minimum bacteriostatic concentration (MIC), μM					Minimum fungistatic concentration (MIC), μM		
	Sa	*Bc*	*Ec*	*Pa*	MRSA-*Sa*	*Tm*	*Ca*	*An*
10–2-10 (Im)	0.8 ± 0.06	1.5 ± 0.1	12.9 ± 1.0	25.8 ± 2.2	3.1 ± 0.1	51.6 ± 4.7	6.5 ± 0.4	206 ± 18.2
10–3-10 (Im)	1.5 ± 0.1	1.5 ± 0.1	6.2 ± 0.5	12.6 ± 0.8	3.1 ± 0.1	51.0 ± 4.5	6.2 ± 0.3	202 ± 16.8
10–4-10 (Im)	1.4 ± 0.1	1.4 ± 0.1	3.0 ± 0.2	12.3 ± 0.9	6.2 ± 0.4	49.5 ± 3.8	6.2 ± 0.3	–
Im-10[b]	1000 [3]		500 [3]				500 [3]	
12–2-12 (Im)	5.9 ± 0.4	11.8 ± 1.1	23.6 ± 1.8	23.6 ± 1.7	3.0 ± 0.1	23.6 ± 1.9	23.6 ± 1.9	–
12–3-12 (Im)	5.8 ± 0.3	11.6 ± 0.9	23.1 ± 1.6	23.1 ± 1.8	5.8 ± 0.3	46.2 ± 3.1	23.1 ± 1.7	–
12–4-12 (Im)	11.3 ± 0.9	11.3 ± 0.7	22.7 ± 1.4	22.7 ± 1.7	5.6 ± 0.3	182 ± 11.5	22.7 ± 1.6	–
12–2-12 (Me)[c]	9.7 [44]		81[44]	325[44]				–
Ciprofloxacin	0.7 ± 0.05	1.4 ± 0.1	0.7 ± 0.05	0.7 ± 0.05	340 ± 29			
Norfloxacin	7.5 ± 0.5	24.4 ± 2.1	4.7 ± 0.02	12.1 ± 1.1				
Ketoconazole						7.3 ± 0.5	7.3 ± 0.5	

Test compounds	Minimum bactericidal concentration (MBC), μM					Minimum fungicidal concentration (MFC), μM		
10–2-10 (Im)	1.5 ± 0.1	3.1 ± 0.2	25.8 ± 2.1	51.6 ± 4.2	6.4 ± 0.5	206 ± 17.4	6.5 ± 0.4	–
10–3-10 (Im)	1.5 ± 0.1	3.1 ± 0.2	12.6 ± 1.1	12.6 ± 1.2	6.2 ± 0.5	202 ± 15.3	6.2 ± 0.3	–
10–4-10 (Im)	3.0 ± 0.2	3.1 ± 0.2	6.2 ± 0.4	12.3 ± 1.1	6.2 ± 0.4	395 ± 28.1	6.2 ± 0.3	–
12–2-12 (Im)	5.9 ± 0.4	11.8 ± 0.9	94.4 ± 8.7	94.4 ± 7.5	24.4 ± 1.8	94.4 ± 8.6	23.6 ± 1.8	–
12–3-12 (Im)	5.8 ± 0.3	23.1 ± 1.8	23.1 ± 1.9	23.1 ± 1.8	23.1 ± 1.7	46.2 ± 3.6	26.2 ± 3.9	–
12–4-12 (Im)	11.3 ± 0.8	11.3 ± 0.8	22.7 ± 1.7	45.4 ± 3.6	11.4 ± 0.7	182 ± 12.4	22.7 ± 1.9	–
Ciprofloxacin	0.7 ± 0.9	1.4 ± 0.1	0.7 ± 0.05	0.7 ± 0.06	–			
Norfloxacin	7.5 ± 0.6	24.4 ± 2.1	24.4 ± 2.3	49.0 ± 4.2				
Ketoconazole						7.3 ± 0.5	7.3 ± 0.5	

[a] Average of three values measured; ± standard deviation (SD); − means non-active;
[b] Im-10 - 1-Decyl-3-methylimidazolium chloride;
[c] 12–2-12 (Me) - ethylene-1,2-bis(dimethyldodecylammonium) dibromide.

Calculator." AAT Bioquest, Inc., 25 July 2019, https://www.aatbio.com/tools/ic50-calculator [43].

The cytometric results were analyzed by the Cytell Cell Imaging multifunctional system using the Cell Viability BioApp and Apoptosis BioApp application. The data in tables and graphs are given as the mean ± standard error.

3. Results and discussion

3.1. Antimicrobial activity of test compounds

Table 1 summarizes results on the antibacterial activity of geminis under study. The compounds were tested for antibacterial (bacteriostatic and bactericidal) activity against a number of Gram-positive *S. aureus 209P (Sa), B. cereus* 8035 *(Bc)* and Gram-negative bacteria *E. coli* F-50 *(Ec), Pseudomonas aeruginosa* 9027 (*Pa*) including methicillin-resistant strains of *S. aureus* (MRSA-*Sa*). Antifungal activity was studied on *Trichophyton mentagrophytes* var. *gypseum* 1773, *Aspergillus niger* 1119 and *Candida albicans* 10,231.

Typically, the primary information on the antimicrobial properties of newly synthesized compounds can be derived from their screening assessment aimed at the determining the MIC value, i.e. the lowest concentration that completely inhibits the visible growth and proliferation of microorganisms [39,44]. It follows from the data in Table 1 that the compounds have a broad spectrum of antimicrobial activity. Moreover, imidazolium surfactants with decyl radical showed higher activity than their dodecyl analogues with respect to all bacterial strains including MRSA-*Sa* and the yeast-like fungus *C. albicans*. It should be noted that all compounds studied have amphiphilic character and form aggregates beyond the CMC, with the functional properties of monomeric and aggregated molecules markedly differing. To elucidate whether surfactants in monomer or aggregated form contribute to the antimicrobial effect, aggregation threshold needs to be determined. To this end, CMC values were examined by methods of tensiometry and conductometry (Figs. S1, S2), which are summarized in Table 2 along with literature data. The comparison of the values in Tables 1 and 2

Table 2
CMC values of gemini imidazolium surfactants investigated by different techniques.

Surfactant	CMC, mmol/l	
	Surface tension method	Conductivity method
10–2-10 (Im)	4.0	4.3
10–3-10 (Im)	5.0, 3.37[a]	5.0, 4.60[a]
10–4-10 (Im)	4.2, 4.50 [a]	5.6, 5.07[a]
12–2-12 (Im)	0.55[a]	0.64 [a]
12–3-12 (Im)	0.53, 0.53 [a]	0.70, 0.61[a]
12–4-12 (Im)	0.65, 0.72 [a]	0.83, 0.73 [a]

[a] From [1].

makes it possible to conclude that in all cases, the active surfactant concentration turned out to be lower than CMC; i.e., these substances are active in non-aggregated state. The dicationic imidazolium surfactants are significantly more active than their monocationic analogues [3], as well as dicationic surfactants with ammonium head group [45] (see Table 1).

Although the MIC value is the primary characteristics of antimicrobial activity, it gives few information, whether this drug belongs to bacteriostatic or bactericidal agents. Importantly, bacteriostatic and bactericidal drugs are assumed to differ in mechanism of action [46]. Therefore, minimal bactericidal concentration (MBC) was further determined as the lowest concentration of antimicrobial agents needed to kill the bacteria. Values MBC and MIC complement each other, and their relation may serve as a criterion for the attribution of the compound tested to either bacteriostatic or bactericidal agents. Antibacterial agents are usually considered as bactericidal if MBC is not more than four times higher compared to MIC [46]. This case is observed for the series 10-s-10(Im) and 12-s-12(Im); therefore, these compounds were attributed to bactericidal agents (the same is observed in the case of fungicidal activity). Noteworthy, dicationic surfactants of 10-s-10(Im) series are of particular interest from the viewpoint of their potential as novel antimicrobial agents, since their MBC values in

relation to Gram-positive bacteria including MRSA and MFC against *C. albicans* are markedly lower compared to commercial preparation. All test compounds did not show high antimicrobial activity against *T. mentagrophytes* var. *gypseum 1773* and *A. niger 1119* (please see Table 1).

In general, antimicrobial effect of cationic surfactants has a complicated mechanism, with a variety of factors contributing [18,20,47,48]. Both, the structural characteristics of surfactants and kind and structure of microorganisms should be taken into account. Two different groups of bacteria, Gram-positive and Gram-negative demonstrate different sensitivity toward the cationic surfactants. This is due to the fact that these families of bacteria differ in the structure of their cell walls. Unlike with Gram-positive bacteria that have inner cytoplasmic membrane neighboring with cell wall, Gram-negative bacteria have additional outer membrane, which prevents the access of foreign membranotropic compounds, thereby increasing the resistance of cell against antimicrobial agents including surfactants [49].

These structural specificities are responsible for the differences in the values of MIC and MBC between these two groups of bacteria. Similarly, for gemini imidazolium surfactants under study, the active concentration determined for Gram-positive bacteria (*S. aureus, B. Cereus*) are few lower compared to those for Gram-negative bacteria (*E. coli* F-50*, Pseudomonas aeruginosa)*. Meanwhile, all these values are comparable with the reference preparations (please, see Table 1).

3.2. Hemolytic activity

An important characteristic in evaluating the biological activity of new chemical compounds is their mammalian cell cytotoxicity. Therefore, m-s-m (Im) dicationic alkylimidazolium surfactants were tested for cytotoxicity against human red blood cells (hemolytic activity). Fig. 2 and Table S 1 show the degree of hemolysis of human erythrocytes caused by the compounds in the concentration range of 0.8–50 μM.

The MIC and MBC values of the test compounds fall within this range. It follows from these data that surfactants with a decyl hydrophobic substituent turned out to be less toxic than their dodecyl analogues. Gramicidin S antibiotic, with a bacteriostatic and bactericidal effect at 0.8–12.5 μM, was used as a reference preparation. It is known that Gramicidin S results in erythrocyte hemolysis and thus cannot be used for intravenous administration. Nevertheless, due to the sufficiently high antimicrobial activity, it is widely used in medicine for external treatment of purulent wounds and abscesses and as a part of complex preparations for the treatment of the pharynx and oral cavity [50,51]. Dicationic alkylimidazolium surfactants in the concentration range under study possess lower hemolytic activity than Gramicidin S antibiotic (Fig. 2, Table S2). Hemolysis of m-s-m (Im) compounds at concentrations of ≤12.5 μM is less than 50% and they may be of interest for further studies with living objects.

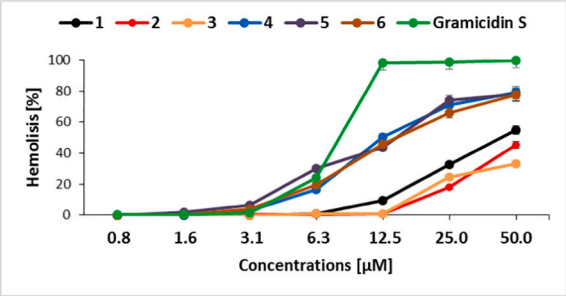


Fig. 2. Hemolytic activity of dicationic alkylimidazolium surfactants: 1–10–2-10(Im), 2–10–3-10(Im), 3–10–4-10(Im), 4–12–2-12(Im), 5–12–3-12(Im), 6–12–4-12(Im).

3.3. Effect of compound 10–2-10 (Im) on alteration of cell wall permeability of S. aureus

It is of special interest to elucidate elementary mechanisms of antimicrobial effect of amphiphilic compounds studied. The methods using fluorescent dyes that selectively penetrate membranes are the most reliable methodological approaches for identification of viable bacterial cells in vitro [52]. One of these methods, *LIVE/DEAD BacLightTM Bacterial Viability* (Invitrogen) (a method of vital staining of bacteria), is widely used for qualitative and quantitative analyses of viable cells in populations. This method indicates the state of the bacterial population of *Staphylococcus aureus* after exposure to antimicrobial agents. It involves two dyes that emit at different wavelengths. The low molecular weight dye SYTO 9 emits in the green spectral range and could penetrate intact membranes. The high molecular weight dye propidium iodide, which emits in the red spectral range, penetrates only the cells with damaged membranes. As a result, living cells are stained in green and dead cells are stained in red.

Fig. 3 shows the images of a bacterial population of *Staphylococcus aureus* after exposure to the leader compound 10–2-10 (Im) at bacteriostatic and bactericidal concentrations. Using fluorescence microscopy, the ratio of living to dead cells before and after exposure to the compound was calculated due to selective staining with fluorophores. It can be seen that the number of dead cells (stained in red) increases compared to the control with an increase in the concentration of compounds. Assessment of the quantitative effect of compound 10–2-10(Im) on the cells is given in Fig. 4. It can be seen that the bacterial cytoplasmic membrane starts to destabilize upon the cultivation of bacteria in the presence of the test compound at its MIC; i.e., that is, the number of cells stained in red increases in the *S. aureus* population. Significant changes in the permeability of *S. aureus* cytoplasmic membranes were observed with an increase in the concentrations of compounds to MBC and higher.

These results suggest that the specific mechanism of action of the test compounds is associated with the damage to bacterial cytoplasmic membrane. The hydrophobic part of the molecule (alkyl chain) presumably assists in the cell membrane penetration resulting in its damage and replacement of extracellular fluid by intracellular, which leads to cell lysis and death [30]. According to current concepts antimicrobial activity of cationic surfactants is mainly mediated through their integration with the lipid membranes [53,54], which results in anomalous changes in the membrane functions conjugated with their native permeability, i.e. transport of substances, energy transformation, etc. Under the higher surfactant concentration exceeding CMC values, dual effect can occur due to micelle formation. On the one hand, cooperative positively charged aggregates show enhanced electrostatic affinity toward cell membrane and may serve as a 'depot' maintaining surfactant concentration needed. On the other, beyond the definite threshold, solubilization of lipid components of membranes can occur accompanied by irreversible changing the structure of proteins and their functionality [48,55–57]. The structure of charged species, i.e. head groups and counterions influences the antimicrobial activity, e.g. surfactants with cyclic head groups demonstrate the higher effect compared to acyclic analogs. However, the key factor is alkyl chain length responsible for the 'cut off' effect assuming the occurrence of the optimal alkyl chain length. In the case of monocationic imidazole-containing surfactants, maximal activity is achieved for hexadecyl derivatives [3,4,19]. For gemini surfactants studied herein, decyl derivatives show the highest antimicrobial effect, whereas spacer length plays the minor role. These dicationic imidazoloim surfactants appeared to surpass their monocationic counterparts and ammonium analogs in the activity.

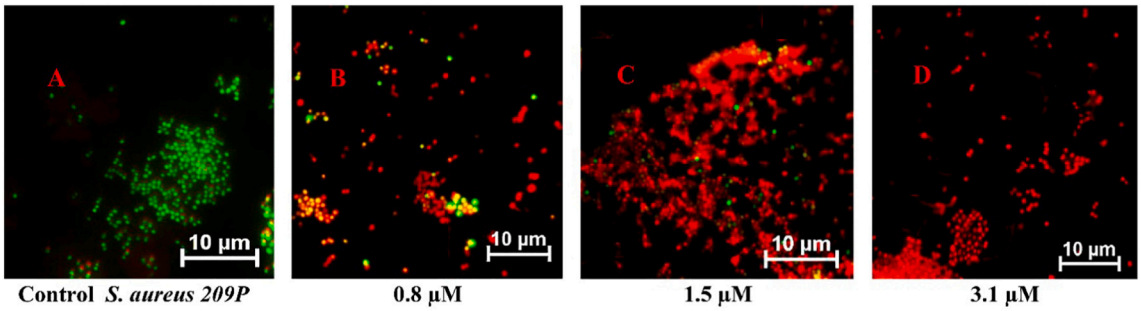


Fig. 3. The effect of dicationic surfactant on the permeability and integrity of the cytoplasmic membrane of *Staphylococcus aureus 209p* using fluorescence microscopy: (A) image of the control sample; (B) image of the test sample treated with compound 10–2-10(Im) at a bacteriostatic concentration of 0.8 μM; (C) image of the test sample treated with compound 10–2-10(Im) at a bactericidal concentration of 1.5 μM; (D image of the prototype treated with compound 10–2-10(Im) at a concentration doubled by MBC(2 × MBC), i.e. 3.1 μM.

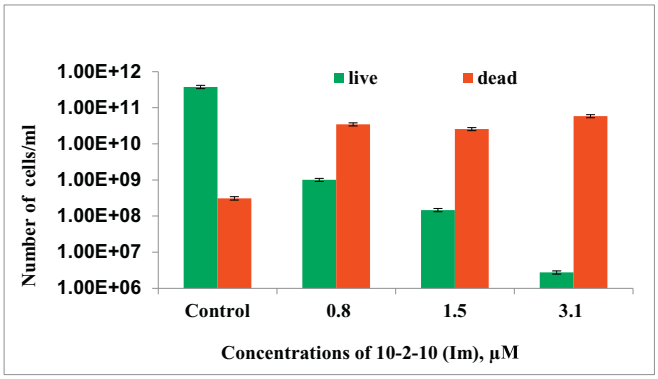


Fig. 4. Quantitative assessment of the effect of the compound 10–2-10 (Im) on the permeability of the *S. aureus* cytoplasmic membrane.

3.4. Cytotoxicity of test compounds toward cancer and normal human cell lines

Due to the fact that amphiphilic compounds have recently been widely used in cancer chemotherapy for delivery of target agents and antitumor drugs, we studied the cytotoxic activity of the dicationic imidazolium surfactants against normal and cancer human cell lines. Studies of the effect of compounds on normal (non-tumor) cell lines would assess the safety of the test compounds on the human body, while discovery of selectivity against cancer cells would enhance cytotoxic properties of the delivered antitumor agents. Compounds of the m-s-m(Im) series were tested for cytotoxicity against normal (Chang liver - human normal liver cells) and human tumor cell lines (M-Hela - epithelioid cervical carcinoma; MCF-7 – human breast adenocarcinoma cells; HuTu 80 - human duodenal adenocarcinoma). The tested compounds showed high activity against cancer cell lines and moderate cytotoxicity against normal cells. As can be seen, cytotoxic effect depends both on hydrophobicity of surfactants and spacer length, with the specificity displayed toward the cell line. The most significant results were obtained in the case of compound 10–4-10(Im) showing selective cytotoxicity toward duodenal adenocarcinoma cell line (HuTu 80) and cervical carcinoma cell line (M-Hela), which were comparable with the doxorubicin (the reference drug). Selectivity of compounds for cancer cells is an important criterion to assess the cytotoxic effect. For this purpose, the selectivity index (SI) was calculated as the ratio between the IC_{50} value for normal cells and the IC_{50} value for cancer cells. The values for the test compounds are given in Table 3.

The compounds with SI ≥ 3 are usually highly selective [58]. In this regard, it can be considered that compound 10–4-10(Im) are highly selective toward HuTu 80 and M-Hela cell lines. The SI values for these lines were 7.3 and 3.9, respectively. Doxorubicin and tamoxifen reference drugs were far inferior to the leading compounds in selectivity.

3.5. Induction of apoptotic effects by test compounds

One of the main mechanisms of the cytotoxic activity of compounds is the induction of apoptosis (programmed cell death), which eliminates significantly damaged or dead cells [59]. Now, apoptosis is one of the key mechanisms addressed in the development of new antitumor drugs.

With reference to the above mentioned, the study of the apoptosis-inducing ability of the leader compound (10–4-10 (Im)) with a selective cytotoxic effect is of a particular interest. Apoptotic effects were evaluated using the annexin V kits, which can bind phosphatidylethylserine. There is a minimum content of phosphatidylserine on the surface of healthy cell membranes; therefore, interaction of annexin V with these cells is marginal. When there is apoptosis, phosphatidylserine molecules appear on the surface of the cells and could interact with the protein. This interaction results in the increase in the fluorescence intensity of apoptogenic cells, which is detected by a flow cytometer.

According to the flow cytometry data (Figs. 5, 6), a dose-dependent induction of apoptosis is observed as a result of 24-h incubation of HuTu 80 cells with 10–4-10(Im) dicationic surfactant. With an increase in the concentration of the compound, an increase in the number of cells at the irreversible late apoptosis stage is observed; 31.37 and 41.89% at the concentrations of the compounds of 5 and 10 μM, respectively. These results are consistent with the literature data, which show the cytotoxic potential of imidazolium surfactants through induction of apoptosis [60].

3.6. Effects on the mitochondrial membrane potential ($\Delta\psi m$) by the lead compound

There are two mechanisms for the induction of apoptosis: the extrinsic pathway, through death receptors, and the internal pathway mediated by mitochondria. The extrinsic pathway triggers apoptosis in response to external stimuli, during which specific ligands bind at 'death' receptors on the surface of the cell membrane. These receptors are typically members of the Tumor Necrosis Factor Receptor (TNFR) gene family, such as TNFR1 or FAS. TNFR-associated death domain (TRADD) and Fas-associated death domain (FADD) trigger the activation of the zymogenic forms of caspases −8 and − 10, which leads to downstream caspases activation and the formation of the so-called death-inducing signal complex (DISC). As a result of these processes, the essential substrates for cell viability are cleaved, thereby causing cell death. [61].

In case of mitochondrial apoptotic pathway, cell death occurs as a result of irreparable DNA damage. For this reason, the cell triggers an intrinsic apoptotic cascade. The internal pathway of apoptosis induction is followed by a disruption of the mitochondrial membrane, which leads to a decrease in its potential, which is a key indicator of the state of cells [62].

Table 3
In vitro cytotoxic effects (μM) and selectivity index values (SI) of dicationic alkylimidazolium surfactants[a].

Test compound	Cancer cell lines						Normal cell lines
	M-Hela		MCF_7		HuTu 80		Chang liver
	IC_{50}	SI	IC_{50}	SI	IC_{50}	SI	
10–2-10 (Im)	41.6 ± 3.3	0.6	25.7 ± 2.3	1.0	8.4 ± 0.5	3.0	25.5 ± 2.1
10–3-10 (Im)	37.4 ± 3.1	1.1	25.5 ± 2.1	1.5	8.5 ± 0.7	4.6	39.1 ± 3.2
10–4-10 (Im)	6.8 ± 0.5	3.9	25.3 ± 2.4	1.0	3.6 ± 0.2	7.3	26.4 ± 2.5
12–2-12 (Im)	13.6 ± 1.2	1.2	25.7 ± 2.2	0.6	16.6 ± 1.4	1.0	16.7 ± 1.4
12–3-12 (Im)	12.8 ± 1.2	1.2	19.2 ± 1.6	0.7	6.3 ± 0.4	2.0	12.6 ± 1.1
10–4-12 (Im)	12.6 ± 1.0	1.0	25.2 ± 2.1	0.5	10.8 ± 0.8	1.2	12.9 ± 1.2
Doxorubicin	3,0 ± 0.1	1.0	3,0 ± 0.2	1.0	3.0 ± 0.1	1.0	3,0 ± 0.2
Tamoxifen	28 ± 2.5	1.5	25 ± 2.2	1.7	–		42.1 ± 3.5

[a] The experiments were repeated for three times. The results are expressed as the mean ± standard deviation (SD).

The possibility of apoptosis through the mitochondrial pathway was assessed by flow cytometry using the JC-10 fluorescent dye (in the Mitochondria Membrane Potential Kit). In normal cells, JC-10 is accumulated in the mitochondrial matrix, where it forms red-fluorescent aggregates. However, in apoptotic and necrotic cells, JC-10 diffuses from mitochondria, turns into a monomeric form, and emits green fluorescence, which is detected by a flow cytometer [62].

A decrease in the mitochondrial membrane potential of HuTu 80 cells was detected after treatment with compound 10–4-10 (Im), which become more marked with an increase in its concentration. It is clear from the results in Fig. 7, that the number of green-emissive cells increases with an increase in the concentration of the test compound, and vice versa, the content of red-emissive aggregates decreases (Fig. 8).

Thus, the data analysis showed that the leader compound (10–4-10(Im)) has apoptogenic activity against the cancer cell line (HuTu 80) and the induction of apoptosis most likely occurs through the internal mitochondrial pathway.

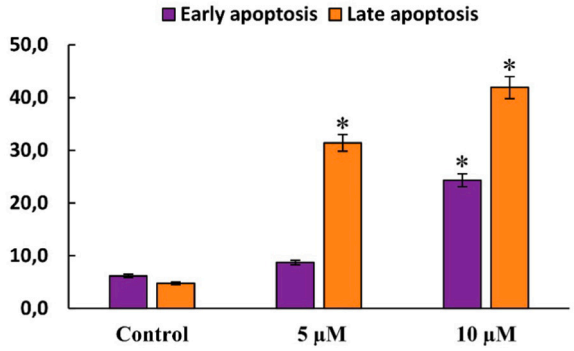


Fig. 6. Representative histograms for the number of cells (% of the total) at the early and late stages of apoptosis for the control and experimental groups after treatment with 10–4-10(Im). The values are presented as mean ± SD (n = 3): (*) $P > 0.01$ compared to control.

4. Conclusion

The antimicrobial, hemolytic and cytotoxic activities of dicationic imidazolium surfactants with a variable length of a hydrophobic group and spacer fragment have been studied. It has been shown that the nature of a hydrophobic tail is the main structural factor affecting the antimicrobial activity of the compounds, while the length of the spacer fragment has practically no effect on their biological properties. The compounds have displayed high antimicrobial activity against a wide range of test microorganisms and did not have high hemolytic activity. In the case of Gram-positive bacteria, compounds of the 10-s-10 (Im) series have shown bactericidal activity at the level of the ciprofloxacin fluoroquinolone antibiotic including methicillin-resistant strains of *S. aureus*, while fungicidal activity against *C. albicans* was comparable with Ketoconazole. The mechanism of the antibacterial effect of the dicationic surfactants is associated with damage to the cytoplasmic membrane. The highest antimicrobial activity has been observed in the case of surfactants containing decyl hydrophobic tail.

Cytotoxicity study of dicationic imidazolium surfactants against normal and tumor human cell lines has demonstrated a selective cytotoxic effect of compound 10–4-10 (Im) against HuTu 80 duodenal adenocarcinoma, the mechanism of which is presumably apoptosis induction via the mitochondrial pathway.

Importantly, MBC and MFC of the leader compounds are markedly

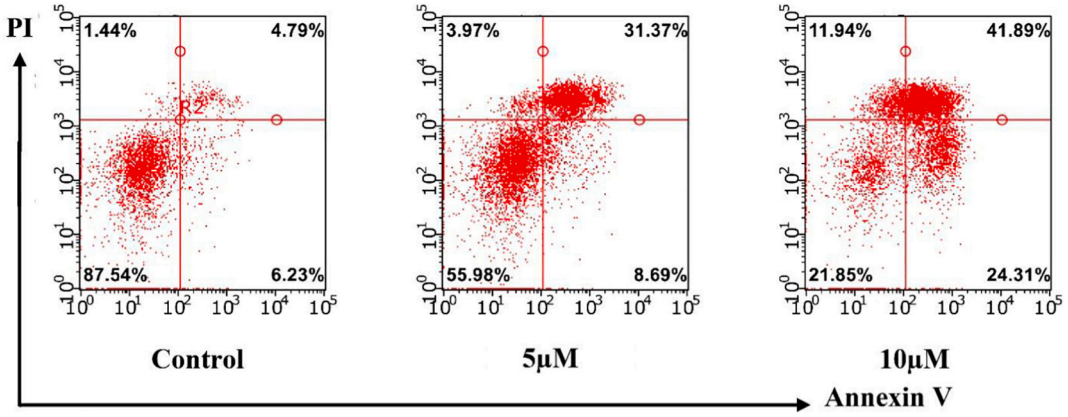


Fig. 5. Apoptosis induction in HuTu 80 cells incubated with 5 and 10 μM of compound 10–4-10 (Im) for 24 h. Quantification of dot plots expressed as percentage of total cells (mean ± SD, $n = 3$).

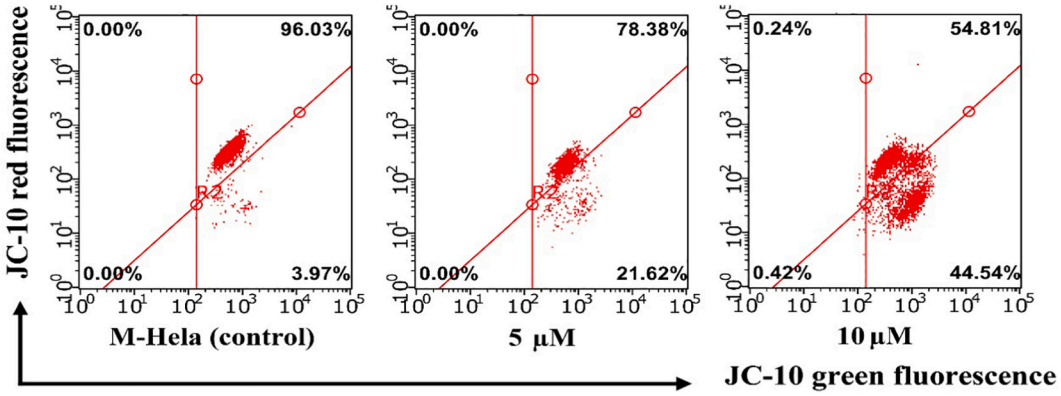


Fig. 7. Flow cytometry analysis of M-Hela cells treated with 10–4-10 (Im).

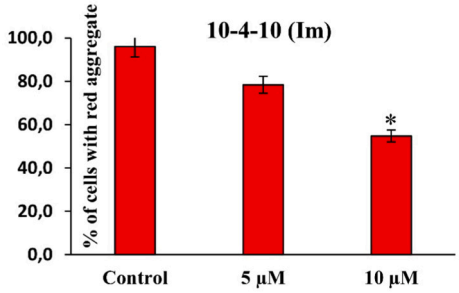


Fig. 8. Quantitative measurement of % cells with red aggregates. Values are presented as mean ± SD (n = 3): (*) P > 0.01 compared to control.

lower than concentrations corresponding their cytotoxic effect toward the normal Chang liver cells. The selective activity against pathogenic microorganisms in combination with low toxicity toward eukaryotic cells allow imidazolium geminis to be considered as novel effective antimicrobial agents. In addition, these amphiphiles exhibit selective effect toward some tumor cell lines, which opens perspectives for their use as components of antitumor drugs.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work is supported by the Russian Science Foundation (grant № 19-73-30012).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbagen.2020.129728.

References

[1] A. Bhadani, T. Misono, S. Singh, K. Sakai, H. Sakai, M. Abe, Structural diversity, physicochemical properties and application of imidazolium surfactants: recent advances, Adv. Colloid Interf. Sci. 231 (2016) 36–58, https://doi.org/10.1016/j.cis.2016.03.005.

[2] M.T. Garcia, I. Ribosa, L. Perez, A. Manresa, Micellization and antimicrobial properties of surface-active ionic liquids containing cleavable carbonate linkages, Langmuir 33 (26) (2017) 6511–6520, https://doi.org/10.1021/acs.langmuir.7b00505.

[3] J. Łuczak, Ch. Jungnickel, I. Łącka, S. Stolte, J. Hupka, Antimicrobial and surface activity of 1-alkyl-3-methylimidazolium derivatives, Green Chem. 12 (2010) 593–601, https://doi.org/10.1039/B921805J.

[4] D. Demberelnyamba, K.-S. Kim, S. Choi, S.-Y. Park, H. Lee, Ch.-J. Kim, I.-D. Yoo, Synthesis and antimicrobial properties of imidazolium and pyrrolidinonium salts, J. Bioorg. Med. Chem. 12 (2004) 853–857, https://doi.org/10.1016/j.bmc.2004.01.003.

[5] D.A. Kuznetsova, D.R. Gabdrakhmanov, L.R. Ahtamyanova, S.S. Lukashenko, A.M. Kusova, Yu.F. Zuev, A.D. Voloshina, A.S. Sapunova, N.V. Kulik, D.M. Kuznetsov, I.R. Nizameev, M.K. Kadirov, L. Ya, Zakharova, novel self-assembling systems based on imidazolium amphiphiles with cleavable urethane fragment for construction of soft nanocontainers for biomedicine application, J. Mol. Liq. 298 (2020) 111961, https://doi.org/10.1016/j.molliq.2019.111961025.

[6] D. Lopes-de-Campos, C. Nunes, B. Sarmento, S. Jakobtorweihen, S. Reis, Metronidazole within phosphatidylcholine lipid membranes: new insights to improve the design of imidazole derivatives, Eur. J. Pharm. Biopharm. 129 (2018) 204–214, https://doi.org/10.1016/j.ejpb.2018.05.036.

[7] R. Kamboj, S. Singh, A. Bhadani, H. Kataria, G. Kaur, Gemini imidazolium surfactants: synthesis and their biophysiochemical study, Langmuir 28 (2012) 11969–11978, https://doi.org/10.1021/la300920p.

[8] A. Pinazo, R. Pons, M. Bustelod, M.Á. Manresa, C. Morán, M. Raluy, L. Pérez, Gemini histidine based surfactants: characterization; surface properties and biological activity, J. Mol. Liq. 289 (2019) 111156, https://doi.org/10.1016/j.molliq.2019.111156.

[9] A. Shaheen, A.W. Mir, R. Arif, A.L. Wani, Synthesis, micellization behavior and cytotoxic properties of imidazolium based gemini surfactants, Colloid Interface Sci. 36 (2020) 100257, https://doi.org/10.1016/j.colcom.2020.100257.

[10] F.M. Menger, J.S. Keiper, Gemini surfactants, J. Angew, Chem. Int. Ed. Engl. 112 (2000) 1906–1920, https://doi.org/10.1002/1521-3773(20000602)39:11<1906::AID-ANIE1906>3.0.CO;2-Q.

[11] R. Zana, Dimeric and oligomeric surfactants. Behavior at interfaces and in aqueous solution: a review, Adv. Colloid Interf. Sci. 97 (2002) 205–253, https://doi.org/10.1016/S0001-8686(01)00069-0.

[12] R. Sharma, A. Kamal, M. Abdinejad, R. Kumar Mahajan, H.-B. Kraatz, Advances in the synthesis, molecular architectures and potential applications of gemini surfactants, Adv. Colloid Interf. Sci. 248 (2017) 35–68, https://doi.org/10.1016/j.cis.2017.07.032.

[13] M.H. Mondal, A. Roy, S. Malik, A. Ghosh, B. Saha, Review on chemically bonded geminis with cationic heads: second-generation interfactants, J. Res. Chem. Intermed. 42 (2016) 1913–1928, https://doi.org/10.1007/s11164-015-2125-z.

[14] A.B. Mirgorodskaya, L. Ya Zakharova, E.I. Khairutdinova, S.S. Lukashenko, O.G. Sinyashin, Supramolecular systems based on gemini surfactants for enhancing solubility of spectral probes and drugs in aqueous solution, Colloids Surf. A Physicochem. Eng. Asp. 510 (2016) 33–42, https://doi.org/10.1016/j.colsurfa.2016.07.065.

[15] I. Badea, S. Wettig, R. Verrall, M. Foldvari, Topical non-invasive gene delivery using gemini nanoparticles in interferon-gamma-deficient mice, Eur. J. Pharm. Biopharm. 65 (3) (2007) 414–422, https://doi.org/10.1016/j.ejpb.2007.01.002.

[16] K. Taleb, M. Mohamed-Benkada, N. Benhamed, S. Saidi-Besbes, Y. Grohens, A. Derdour, Benzene ring containing cationic gemini surfactants: synthesis, surface properties and antibacterial activity, J. Mol. Liq. 241 (2017) 81–90, https://doi.org/10.1016/j.molliq.2017.06.008.

[17] E. Obłak, A. Piecuch, A. Krasowska, J. Luczyński, Antifungal activity of gemini quaternary ammonium salts, J. Microbiol. Res. 168 (2013) 630–638, https://doi.org/10.1016/j.micres.2013.06.001.

[18] P. Balgavý, F. Devínsky, Cut-off effects in biological activities of surfactants, J. Colloid Interface Sci. 66 (1996) 23–63, https://doi.org/10.1016/0001-8686(96)00295-3.

[19] D.A. Kuznetsova, D.R. Gabdrakhmanov, S.S. Lukashenko, A.D. Voloshina, A.S. Sapunova, N.V. Kulik, I.R. Nizameev, M.K. Kadirov, R.R. Kashapov, L. Ya, Zakharova Supramolecular systems based on cationic imidazole-containing amphiphiles bearing hydroxyethyl fragment: aggregation properties and functional activity, J. Mol. Liq. 289 (2019) 111058, https://doi.org/10.1016/j.molliq.2019.111058.

[20] L. Ya, T.N. Pashirova Zakharova, S. Doktorovova, A.R. Fernandes, E. Sanchez-Lopez, A.M. Silva, S.B. Souto, E.B. Souto, Cationic surfactants: self-assembly, structure-activity correlation and their biological applications, Int. J. Mol. Sci. 20 (22) (2019) 5534, https://doi.org/10.1016/j.ijpharm.2019.118953.

[21] D.R. Gabdrakhmanov, E.A. Vasilieva, M.A. Voronin, D.A. Kuznetsova, F.G. Valeeva,

A.B. Mirgorodskaya, S.S. Lukashenko, V.M. Zakharov, A.R. Mukhitov, D.A. Faizullin, V.V. Salnikov, V.V. Syakaev, Sh.K. Latypov, Yu.F. Zuev, L. Ya Zakharova, Soft nanocontainers based on hydroxyethylated geminis: role of spacer in self-assembling, solubilization, and complexation with oligonucleotide, J. Phys. Chem. C 124 (2020) 2178–2192, https://doi.org/10.1021/acs.jpcc.9b10079.

[22] M. Suhail, A. Kumar, A. Khan, A. Naeem, S.F. Badshah, Surfactants and their role in pharmaceutical product development: an overview, J. Pharma Pharm. 6 (2) (2019) 72–82, https://doi.org/10.15436/2377-1313.19.2601.

[23] C. Ren, F. Wang, Z. Zhang, H. Nie, N. Li, M. Cui, Synthesis, surface activity and aggregation behavior of gemini imidazolium surfactants 1,3-bis(3-alkylimidazolium-1-yl)propane bromide, Colloids Surf. A Physicochem. Eng. Asp. 467 (2015) 1–8, https://doi.org/10.1016/j.colsurfa.2014.11.031.

[24] M. Ao, P. Huang, G. Xu, X. Yang, Y. Wang, Aggregation and thermodynamic properties of ionic liquid-type gemini imidazolium surfactants with different spacer length, J. Colloid Polym. Sci. 287 (2009) 395–402, https://doi.org/10.1007/s00396-008-1976-x.

[25] W. Gospodarczyk, M. Kozak, Interaction of two imidazolium gemini surfactants with two model proteins BSA and HEWL, Colloid Polym. Sci. 293 (2015) 2855–2866, https://doi.org/10.1007/s00396-015-3671-z.

[26] T. Zhou, G. Xu, M. Ao, Y. Yang, C. Wang, DNA compaction to multi-molecular DNA condensation induced by cationic imidazolium gemini surfactants, Colloids Surf. A Physicochem. Eng. Asp. 414 (2012) 33–40, https://doi.org/10.1016/j.colsurfa.2012.08.060.

[27] T. Tian, Q. Kang, T. Wang, J. Xiao, L. Yu, Alignment of nematic liquid crystals decorated with gemini surfactants and interaction of proteins with gemini surfactants at fluid interfaces, J. Colloid Interface Sci. 518 (2018) 111–121, https://doi.org/10.1016/j.jcis.2018.02.027.

[28] L. Casal-Dujat, P.C. Griffiths, C. Rodriguez-Abreu, C. Solans, S. Rogerse, L. Perez-Garcia, Nanocarriers from dicationic bis-imidazolium amphiphiles and their interaction with anionic drugs, J. Mater. Chem. B 1 (2013) 4963–4971, https://doi.org/10.1039/C3TB20289E.

[29] T. Zhou, A. Llizo, P. Li, C. Wang, Y. Guo, M. Ao, L. Bai, C. Wang, Y. Yang, G. Xu, High transfection efficiency of homogeneous DNA nanoparticles induced by imidazolium gemini surfactant as nonviral vector, J. Phys. Chem. C 117 (2013) 26573–26581, https://doi.org/10.1021/jp4061363.

[30] Ł. Pałkowski, J. Błaszczyński, A. Skrzypczak, J. Błaszczak, K. Kozakowska, J. Wróblewska, S. Kożuszko, E. Gospodarek, J. Krysiński, R. Słowiński, Antimicrobial activity and SAR study of new gemini imidazolium-based chlorides, J. Chem. Biol. Drug Design. 83 (2014) 278–288, https://doi.org/10.1111/cbdd.12236.

[31] Ł. Pałkowski, J. Błaszczyński, A. Skrzypczak, J. Błaszczak, A. Nowaczyk, J. Wroblewska, S. Kożuszko, E. Gospodarek, R. Słowiński, J. Krysiński, Prediction of antifungal activity of gemini imidazolium compounds, Biomed. Res. Int. (2015), https://doi.org/10.1155/2015/392326 (Article ID 392326).

[32] L. Wang, H. Qin, L. Ding, S. Huo, Q. Deng, B. Zhao, L. Meng, T. Yan, Preparation of a novel class of cationic gemini imidazolium surfactants containing amide groups as the spacer: their surface properties and antimicrobial activity, J. Surfactant Deterg. 17 (2014) 1099–1106, https://doi.org/10.1007/s11743-014-1614-1.

[33] I. Brigger, C. Dubernet, P. Couvreur, Nanoparticles in cancer therapy and diagnosis, Adv. Drug Deliv. Rev. 54 (2002) 631–651, https://doi.org/10.1016/s0169-409x(02)00044-3.

[34] S. Ansell, S. Johnstone, P. Tardi, L. Lo, S. Xie, Y. Shu, T. Harasym, N. Harasym, L. Williams, D. Bermudes, B. Liboiron, W. Saad, K. Prud'homme, L. Mayer, Modulating the therapeutic activity of nanoparticle delivered paclitaxel by manipulating the hydrophobicity of prodrug conjugates, J. Med. Chem. 51 (2008) 3288–3296, https://doi.org/10.1021/jm800002y.

[35] S. Aryal, C.-M. Hu, V. Fu, L. Zhang, Nanoparticle drug delivery enhances the cytotoxicity of hydrophobic-hydrophilic drug conjugates, J. Mater. Chem. 22 (2012) 994–999, https://doi.org/10.1039/C1JM13834K.

[36] D. Kuznetsova, D. Gabdrakhmanov, S. Lukashenko, L. Ahtamyanova, I. Nizameev, M. Kadirov, L. Zakharova, Novel hybrid liposomal formulations based on imidazolium-containing amphiphiles for drug encapsulation, Colloid Surf. B. 178 (2019) 352–357, https://doi.org/10.1016/j.colsurfb.2019.03.025.

[37] L. Wang, J. Liu, S. Huo, Q. Deng, T. Yan, L. Ding, C. Zhang, L. Meng, Q. Lu, Synthesis and surface properties of novel Gemini imidazolium surfactants, J. Surfactant Deterg. 17 (2014) 1107–1116, https://doi.org/10.1007/s11743-014-1615-0.

[38] M. Zhang, J. Chen, T. Gu, H. Qiu, Sh. Jiang, Novel imidazolium-embedded and imidazolium-spaced octadecyl stationary phases for reversed phase liquid chromatography, Talanta 126 (2014) 177–184, https://doi.org/10.1016/j.talanta.2014.03.057.

[39] M. Balouiri, M. Sadiki, S.K. Ibnsouda, Methods for in vitro evaluating antimicrobial activity: a review, J. Pharm. Anal. 6 (2016) 71–79, https://doi.org/10.1016/j.jpha.2015.11.005.

[40] A. Bogdanov, I. Zaripova, L. Mustafina, A. Voloshina, A. Sapunova, N. Kulik, V. Mironov, Synthesis and study of antimicrobial activity of water-soluble ammonium acylhydrazones based on new 1,ω-alkylenebis(isatins), Russ. J. Gen. Chem. 89 (7) (2019) 1368–1376, https://doi.org/10.1134/S107036321907003X.

[41] T. Dang, I. Nizamov, R. Salikhov, L. Sabirzyanova, V. Vorobev, T. Burganova, M. Shaidoullina, E. Batyeva, R. Cherkasov, T. Abdullin, Synthesis and characterization of pyridoxine, nicotine and nicotinamide salts of dithiophosphoric acids as antibacterial agents against resistant wound infection, Bioorg. Med. Chem. 27 (1) (2018) 100–109, https://doi.org/10.1016/j.bmc.2018.11.017.

[42] A. Voloshina, V. Semenov, A. Strobykina, N. Kulik, E. Krylova, V. Zobov, V. Reznik, Synthesis and antimicrobial and toxic properties of novel 1,3-bis(alkyl)-6-methyluracil derivatives containing 1,2,3- and 1,2,4-triazolium fragments, Russ. J. Bioorg. Chem. 43 (2017) 170–176, https://doi.org/10.1016/j.bmc.2018.11.017.

[43] M. Lisowska, M. Milczarek, J. Ciekot, J. Kutkowska, W. Hildebrand, A. Rapak, A. Miazek, An antibody specific for the dog leukocyte antigen DR (DLA-DR) and its novel methotrexate conjugate inhibit the growth of canine B cell lymphoma, Cancers. 11 (10) (2019) 1438, https://doi.org/10.3390/cancers11101438.

[44] A.S. Inacio, N.S. Domingues, A. Nunes, P.T. Martins, M.J. Moreno, L.M. Estronca, R. Fernandes, A.J.M. Moreno, M.J. Borrego, J.P. Gomes, W.L.C. Vaz, O.V. Vieira, Quaternary ammonium surfactant structure determines selective toxicity towards bacteria: mechanisms of action and clinical implications in antibacterial prophylaxis, J. Antimicrob. Chemother. 71 (2016) 641–654, https://doi.org/10.1093/jac/dkv405.

[45] A. Laatiris, M. El Achouri, M.R. Infante, Y. Bensouda, Antibacterial activity, structure and CMC relationships of alkanediyl α,ω-bis(dimethylammonium bromide) surfactants, Microbiol. Res. 163 (6) (2008) 645–650, https://doi.org/10.1016/j.micres.2006.09.006.

[46] G.L. French, Bactericidal agents in the treatment of MRSA infections–the potential role of daptomycin, J. Antimicrob. Chemother. 58 (2006) 1107–1117, https://doi.org/10.1093/jac/dkl393.

[47] M.E. Fait, L. Bakas, G.L. Garrote, S.R. Morcelle, M.C.N. Saparrat, Cationic surfactants as antifungal agents, Appl. Microbiol. Biotechnol. 103 (2019) 97–112, https://doi.org/10.1007/s00253-018-9467-6.

[48] C. Zhou, Y. Wang, Structure–*activity* relationship of *cationic surfactants* as *antimicrobial* agents, Curr.Opin. Colloid Interface Sci. 45 (2020) 28–43, https://doi.org/10.1016/j.cocis.2019.11.009.

[49] M.R.J. Salton, K.S. Kim, Structure', in: S. Baron (Ed.), Medical Microbiology, 4th ed., Univ of Texas Medical Branch, 1996.

[50] T. Mogi, K. Kita, Gramicidin S and polymyxins: the revival of cationic cyclic peptide antibiotics, J. Cell Mol. Life Sci. 66 (2009) 3821–3826, https://doi.org/10.1007/s00018-009-0129-9.

[51] M. Berditsch, S. Afonin, J. Reuster, H. Lux, K. Schkolin, O. Babii, D. Radchenko, I. Abdullah, N. William, V. Middel, U. Strähle, A. Nelson, K. Valko, A. Ulrich, Supreme activity of gramicidin S against resistant, persistent and biofilm cells of staphylococci and enterococci, Sci. Rep. 9 (2019) 1–15, https://doi.org/10.1038/s41598-019-54212-z.

[52] J. Alonso, S. Mascellaro, Y. Moreno, M. Ferrús, J. Hernández, Double-staining method for differentiation of morphological changes and membrane integrity of campylobacter coli cells, J. Environ. Microbiol. 68 (10) (2002) 5151–5154, https://doi.org/10.1128/AEM.68.10.5151-5154.2002.

[53] H. Heerklotz, Interactions of surfactants with lipid membranes, Q. Rev. Biophys. 41 (2008) 205–264, https://doi.org/10.1017/S0033583508004721.

[54] M.C. Jennings, K.P.C. Minbiole, W.M. Wuest, Quaternary ammonium compounds: an antimicrobial mainstay and platform for innovation to address bacterial resistance, ACS Infect. Dis. 1 (2015) 288–303, https://doi.org/10.1021/acsinfecdis.5b00047.

[55] C. Zhang, F.-Y. Su, J.-F. Zhang, S.-T. Yan, X.-H. Xing, Luciferase and fluorescent protein as dual reporters analyzing the effect of n-dodecyltrimethylammonium bromide on the physiology of Pseudomonas putida, Appl. Microbiol. Biotechnol. 93 (2012) 393–400, https://doi.org/10.1007/s00253-011-3663-y.

[56] E.F. Palermo, K. Kuroda, Chemical structure of cationic groups in amphiphilic polymethacrylates modulates the antimicrobial and hemolytic activities, Biomacromolecules. 10 (2009) 1416–1428, https://doi.org/10.1021/bm900044x.

[57] L. Huang, Y.-H. Xiao, X.-D. Xing, F. Li, S. Ma, L.-L. Qi, J.-H. Chen, Antibacterial activity and cytotoxicity of two novel cross-linking antibacterial monomers on oral pathogens, Arch. Oral Biol. 56 (2011) 367–373, https://doi.org/10.1016/j.archoralbio.2010.10.011.

[58] M. Ayoup, Y. Wahby, H. Abdel-Hamid, E. Ramadan, M. Teleb, M. Abu-Serie, A. Noby, Design, synthesis and biological evaluation of novel a-acyloxy carboxamides via Passerini reaction as caspase 3/7 activators, Eur. J. Med. Chem. 168 (2019) 340–356, https://doi.org/10.1016/j.ejmech.2019.02.051.

[59] V. Kaur, M. Kumar, A. Kumar, K. Kaur, V. Singh Dhillon, S. Kaur, Pharmacotherapeutic potential of phytochemicals: implications in cancer chemoprevention and future perspectives, Biomed. Pharmacother. 97 (2018) 564–586, https://doi.org/10.1016/j.biopha.2017.10.124.

[60] X. Li, C. Jing, W. Lei, J. Li, J. Wang, Apoptosis caused by imidazolium-based ionic liquids in PC12 cells, Ecotoxicol. Environ. Saf. 83 (2012) 102–107, https://doi.org/10.1016/j.ecoenv.2012.06.013.

[61] G. Pistritto, D. Trisciuoglio, C. Ceci, A. Garufi, G. D'Orazi, Apoptosis as anticancer mechanism: function and dysfunction of its modulators and targeted therapeutic strategies, Aging (Albany NY) 8 (4) (2016) 603–619, https://doi.org/10.18632/aging.100934.

[62] S. Perry, J. Norman, J. Barbieri, E. Brown, H. Gelbard, Mitochondrial membrane potential probes and the proton gradient: a practical usage guide, J. Biotech. 50 (2) (2011) 98–115, https://doi.org/10.2144/000113610.