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The mode of antibacterial action of quaternary *N*-benzylimidazole salts against emerging opportunistic pathogens

Doris Crnčević [a], Lucija Krce [b], Linda Mastelić [a], Ana Maravić [c], Barbara Soldo [a], Ivica Aviani [b], Ines Primožič [d], Renata Odžak [a,*], Matilda Šprung [a,*]

[a] Department of Chemistry, Faculty of Science, University of Split, Ruđera Boškovića 33, Split, Croatia
[b] Department of Physics, Faculty of Science, University of Split, Ruđera Boškovića 33, Split, Croatia
[c] Department of Biology, Faculty of Science, University of Split, Ruđera Boškovića 33, Split, Croatia
[d] Department of Chemistry, Faculty of Science, University of Zagreb, Horvatovac 102a, Zagreb, Croatia

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ABSTRACT

Quaternary ammonium compounds (QACs) are antimicrobial agents displaying a broad spectrum of activity due to their mechanism of action targeting the bacterial membrane. The emergence of bacterial resistance to QACs, especially in times of pandemics, requires the continuous search for new and potent QACs structures. Here we report the synthesis and biological evaluation of QACs based on imidazole derivative, *N*-benzylimidazole. The antimicrobial activity was tested against a range of pathogenic bacteria and fungi, both ATCC and clinical isolates, showing varying activities ranging in minimal inhibitory concentrations (MICs) from as low as 7 ng/mL. The most promising compound, *N*-tetradecyl derivative (**BnI-14**), proved to be very potent against bacterial biofilms, even at sub-MIC doses, suggesting interference with the bacterial growth and/or division process. The **BnI-14** treatment induces bacterial membrane disruption, as observed by fluorescence spectroscopy and atomic force microscopy and it also binds to DNA indicating that bacterial membrane might not be the only cellular target of QACs. Most importantly, **BnI-14** exhibits low toxicity to healthy human cell lines, suggesting that *N*-benzylimidazolium-based QACs may be promising new antimicrobial agents.

1. Introduction

An ongoing COVID-19 pandemic, caused by a novel acute respiratory syndrome coronavirus 2 (SARS-CoV-2), raises global awareness about threats caused by infectious diseases [1]. At the first line of defense against such threats are compounds with powerful antibacterial and antiviral properties namely disinfectants or antiseptics [2]. Quaternary ammonium compounds (QACs) are antimicrobial and antiviral agents present in many commercial products [3,4]. The widespread use of these products and their prolonged retention in the environment rapidly led to the development of bacterial resistance. Today, as much as 83% of MRSA isolates are resistant to almost all commercial QACs [5] and it is expected that this number is even higher due to QACs overuse in the COVID-19 pandemic [6–8]. As the number of resistant bacteria grows and new pathogens emerge, there is an urgent need to develop new such compounds with wide spectrum activities against bacteria and viruses [9].

In recent years, several promising strategies for the development of new QACs have been proposed. These strategies include the development of QACs containing more than one positive ammonium center (e.g., bisQACs) [10–14] and QACs derived from the quaternization of natural products [15,16]. QACs with different heterocyclic core structures have been investigated not only for their antimicrobial activity [17–22], but also as potent antidotes for toxic organophosphorus compounds (e.g., chemical warfare agents) [23–25]. Apart from the permanent positive charge, all these studies revealed the importance of a long alkyl chain, which appeared to play a crucial structural role in bioactivity [3,26]. This was not surprising considering that QACs are amphiphilic compounds, thus the presence of an alkyl chain and its length are closely related to the bioactivity of such derivatives.

Natural products with imidazole are widely occurring nitrogen-containing heterocyclic compounds [22].

Derivatives of imidazole have been in the focus of scientific investigations due to their substantial biological potential resulting in

* Corresponding authors.
E-mail addresses: dcrncevic@pmfst.hr (D. Crnčević), lkrce@pmfst.hr (L. Krce), linbaz@pmfst.hr (L. Mastelić), amaravic@pmfst.hr (A. Maravić), barbara@pmfst.hr (B. Soldo), iaviani@pmfst.hr (I. Aviani), ines.primozic@chem.pmf.hr (I. Primožič), rodzak@pmfst.hr (R. Odžak), msprung@pmfst.hr (M. Šprung).

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diverse clinical applications [22,27]. However, the biological potential of one such derivative, benzylimidazole, has been poorly studied. So far, it has been shown that benzylimidazole derivatives have potent cardiotonic activity [28] and its *N*-*p*-substituted variants markedly increase the inhibition of parasite proliferation in the submicromolar range [29]. In addition, *N*-benzylimidazole derivatives are useful antifungal agents with superior activity and therefore these compounds have been used as agricultural bactericides against various plant pathogens and soil bacteria [30]. We and others have shown that *N*-benzylimidazole derivatives have promising antimicrobial potency [17,31,32] and that investigations of these compounds merit further attention.

Led by these findings and our previous studies of quaternized quinuclidine derivatives [16,19,20], we aimed to synthesized QACs containing *N*-benzylimidazole substituted with alkyl chains of 10, 12, and 14C atoms (**BnI-10**, **BnI-12,** and **BnI-14**). All the compounds were tested for their biological activity in addition to identifying their mechanism of action. Antimicrobial activity was evaluated against a range of yeast and mold strains, as well as several pathogenic bacteria. Minimal inhibitory concentrations (MICs) were determined against six Gram-negative and four Gram-positive strains. In addition, the activity of selected candidate that exhibited the best antimicrobial potential was further tested against bacteria in the form of cell suspensions and biofilms. The mode of action was investigated by atomic force microscopy (AFM) and propidium iodide uptake assay. Given the potential application as new antimicrobial agents, we investigated the cytotoxicity for selected candidate against representative healthy cell lines HEK293, RPE1, HaCaT, and tumor cell line HeLa.

2. Material and methods

2.1. Synthesis

N-Benzylimidazole and reagents for quaternization were commercially available (Alfa Aesar) and were used without further purifications. The dry organic solvents were used for the synthesis. The reaction progress was monitored by thin layer chromatography using DC-Alufolien Aluminiumoxide 60 F_{254} plates (Merck) with 5:1 and 9:1 chloroform/methanol as the eluent. Spots were detected by the reversible absorption of iodine and by the UV light. Melting points were determined by Büchi B-540 apparatus and the obtained values were uncorrected. FTIR spectra were recorded on a PerkinElmer FTIR 1725 X spectrometer and elemental analyses were determined by PerkinElmer PE 2400 Series II CHNS/O Analyser. All samples were prepared by mixing FTIR-grade KBr (Sigma-Aldrich) with 1% (w/w) salt and grinding to a fine powder. Spectra were recorded over the 400–4000 cm^{-1} range without baseline corrections. ^{1}H and ^{13}C NMR spectra were recorded in DMSO-d_6 solutions with a Bruker Avance III HD 400 MHz/54 mm Ascend spectrometer (400 MHz) at room temperature. Chemical shifts are reported as δ values in ppm using TMS as an internal standard. Abbreviations for data quoted are: *s*, singlet; *d*, doublet; *t*, triplet; *m*, multiplet. Coupling constants (*J*) are given in Hz.

2.2. General procedure for synthesized compounds

An equimolar amount of the appropriate quaternization reagent (1-bromodecane, 1-bromododecane or 1-bromotetradecane) was added to solution of *N*-benzylimidazole (1.00 mmol) in dry acetone at room temperature. Due to the light sensitivity of depicted alkyl bromides the reaction mixture was kept in the dark and stirred at 70 °C for 2 to 3 days to obtain a solid product. The quaternary salts were obtained by removing the excess solvent under reduced pressure and washed several times with diethyl ether to give white crystals in yields ranging from 68.7 to 88.3%. Detailed structural characterization of the synthesized compounds is available in the Supporting information.

2.3. Microbial strains

To assess antimicrobial efficacy, **BnI-10, −12,** and **−14** QACs were tested against fourteen strains of food spoilage and emerging opportunistic pathogens, including clinical and environmental isolates stored at the Department of Biology, Faculty of Science, University of Split, Croatia, as well as the strains from the American Type Culture Collection (ATCC) (Rockville, USA).

The collection included four Gram-positive bacteria, including *Staphylococcus aureus* (ATCC 2213 and a methicillin-resistant *Staphylococcus aureus* clinical strain MRSA), *Enterococcus faecalis* ATCC 29212, and *Streptococcus pyogenes* ATCC 19615; then, six Gram-negative species: *Escherichia coli* (ATCC 25922 and an extended-spectrum-beta-lactamase (ESBL)-producing multiple-resistant clinical strain), *Klebsiella pneumoniae* (ATCC 13883 and ESBL-producing multiple-resistant clinical strain), and *Acinetobacter baumannii* (ATCC 19606 and a metallo-beta-lactamase (MBL)-producing multiple-resistant hospital strain). Clinical strains, simultaneously resistant to several classes of conventional antibiotics, were obtained from University Hospital Centre Split, Croatia. Their origin, antibiotic resistance pattern, and resistance genotype were described previously [33]. Opportunistic pathogenic yeast *Candida albicans* (ATCC 90029 and a human isolate) as well as the food isolates of the food spoilage moulds *Aspergillus niger* and *Penicillium citrinum* were tested to assess the antifungal activity.

The minimal inhibitory concentrations (MICs) of standard antibiotics were determined by Etest (AB Biodisk, Sweden) and VITEK 2 system (bioMérieux, France). The microbial strains were stored at −80 °C and subcultured on tryptic soy agar (TSA, Biolife, Italy) or Sabouraud dextrose agar (SDA, Biolife) in case of fungi before testing.

2.4. Broth microdilution assays

The antimicrobial activity was assessed using broth microdilution assay according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for testing bacteria [34] and fungi [35,36], except for Sabouraud dextrose broth (SDB; Biolife) which was used for fungal growth.

The microdilution assays were performed in 96-well microtiter plates with serial dilutions of **BnI-10, −12** and **−14** QACs ranging from 2000 μg/mL to 1.75 ng/mL. As previously described [33], 10^6 CFU/mL of mid-exponentially grown bacterial cells in Mueller-Hinton broth (MHB; Biolife) were adjusted spectrophotometrically, added to serial 2-fold dilutions of QACs in total of 100 μL per well, and incubated for 18 h at 37 °C. For fungi, an inoculum of approximately $2.5x10^5$ CFU/mL of spores/conidia were added to the serial dilutions of QACs and incubated at 35 °C for 24–48 h. The minimal inhibitory concentration (MIC) was recorded as the lowest concentration showing no visually detectable bacterial growth in the wells. For the determination of minimal bactericidal concentration (MBC), aliquots were taken from wells corresponding to the MIC, 2 MIC, and 4 MIC and plated on MHA plates. After incubation for 18 h at 37 °C, the MBC was recorded as the lowest concentration causing ~ 99.9% killing of the start inoculum.

For fungi, the aliquots taken from the wells were plated on SDA and incubated at 35 °C for 48 h. After visual inspection of the plates, MIC_{50} and MIC_{90} endpoints were recorded as the lowest concentrations of QACs that inhibited 50% and 90% of fungal growth when compared to the control. All tests were done in triplicates.

2.5. Crystal-violet (CV) biofilm assay

Exponentially grown cells ($2x10^6$ CFU/mL) of *S. aureus* ATCC 29213 were inoculated in 96-well polystyrene flat-bottomed microplates, containing the serial dilutions of **BnI-14** in a range from MIC to 1/16 MIC (200 μL/well). After 72 h of incubation, the culture suspensions were discarded and each well gently rinsed with sterile phosphate-buffered saline. The plates were then incubated for 1 h at 60 °C to fix

the formed biofilm, stained with 0.1% crystal-violet (CV) dye (Sigma, USA) for 1 h at room temperature, and finally rinsed three times with sterile distilled water. After solubilization of CV with 33% acetic acid for 30 min at room temperature, the absorbance at 600 nm was measured using a multi-well plate reader (Bio-Tek EL808). Results were obtained in two independent experiments carried out in triplicates and present the percentage of the biofilm inhibition of treated cells *vs* the untreated cells.

2.6. Atomic force and optical microscopy

E. coli ATCC 25922 cells and Petri dishes (WPI, Sarasota, USA) coated with the Cell-Tak (Corning, NY, USA) solution, were prepared as we have previously reported [37]. AFM measurements, of immobilized treated and untreated bacterial cells, were carried out by the Nanowizard IV system (JPK/Bruker, Berlin, Germany) operating in the quantitative imaging mode utilizing the MLCT-E probes (Bruker, Billerica, USA). The setpoint was kept between 0.55 and 0.65 nN while the extend/retract speed was between 60 and 150 μm/s. Each measurement was done with a resolution of 256x256 pixels. The AFM data processing was carried out with JPK data processing software.

The utilized AFM system is integrated with an inverted optical fluorescence microscope IX73 (Olympus, Tokyo, Japan). After choosing a preferred sample region using bright-field microscopy, the cells were measured by AFM, and their elongation and division were observed. Bright-field images were obtained throughout the experiment.

After confirming the growth of attached cells by AFM, the culture was washed again in fresh MHB to remove unattached cells, and the appropriate volume of benzylimidazole solution corresponding to the 16 MIC was added to the culture. All AFM measurements were performed in MHB at 37 °C. Measurement of treated cells began approximately 30 min after treatment.

Fluorescence images of treated cells were obtained immediately after the final AFM measurements. The growth medium was replaced by the physiological saline solution and the cells were stained using the green fluorescent nucleic fluorophore SYTO 9 and the red fluorescent nucleic fluorophore propidium iodide (PI) by adding 1.5 μL of each dye per mL of the culture. These dyes are included in the LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Invitrogen, Carlsbad, USA). The fluorescence images were obtained 4 h post-treatment and 20 min after adding the fluorescence dyes.

2.7. Propidium iodide (PI)-uptake assay

The propidium iodide (PI)-uptake assay was performed on *S. aureus* ATCC 29213 to assess cell membrane integrity. The bacterial culture was grown overnight in MHB at 37 °C and 210 rpm. On the following day, the culture was diluted in fresh MHB (1:30) and left under the same growth conditions until the OD_{600} value reached 0.5. The inoculum was centrifuged at 4500 × *g* for 10 min at room temperature. The bacterial pellet was washed with sterile PBS buffer and centrifuged again under the same conditions. The pellet was resuspended in sterile PBS buffer until the OD_{600} value was 1.5. The reaction mixtures were prepared in 1 mL tubes (Eppendorf). Each of them contained 500 μL of resuspended cells, 15 μM propidium iodide stain, and 500 μL **BnI-14**, resulting in final concentrations of 8 MIC, 4 MIC, MIC, and 1/2 MIC. PI-uptake was monitored in a white 96-well plate (Porvair), with each well containing 250 μL of the previously prepared sample. The PI fluorescence was immediately recorded for 540 min, at 37 °C, in the Perkin-Elmer LS55 spectrofluorometer using the excitation at 536 nm and emission at 617 nm. All measurements were performed in triplicate, the background PI fluorescence was subtracted from each measurement and Microsoft Excel was used for data analysis.

2.8. DNA protection assay

Supercoiled pQE60 plasmid DNA (200 ng) and various concentrations of BnI-14 (0–100 mM) were incubated for 10 min at room temperature, followed by the addition of Fenton's reagent (10 μL). After a further 30 min incubation at 37 °C, a sample (10 μL) of the reaction mixture was analysed on a 1% agarose gel.

2.9. Cytotoxicity

The cytotoxicity of **BnI-14** was performed using three different human cell lines (HEK293, RPE1, HaCaT and HeLa) and was further compared to benzyldodecyldimethylammonium bromide (BAB) standard. Cells were grown in a humid environment with 5% CO_2 and at 37 °C. To determine cytotoxicity, a serial dilution of a tested compound was prepared in a 96-well plate. 5000 cells were plated in each well and the cells were grown for an additional 24 h, after which MTS reagent was added. After 3 h of incubation, absorbance was measured at 490 nm. IC_{50} values were determined by plotting compound concentration against absorbance using GraFit6 software. Measurements were performed in quadruplets and results are presented as means of at least three independent experiments with ± SD values.

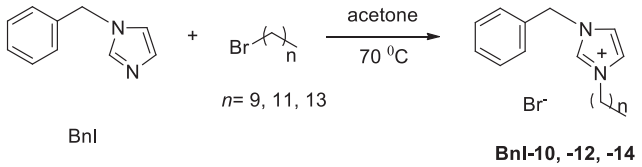
3. Results and discussion

3.1. Synthesis

In our previous work [16,19] we have shown that quaternary ammonium compounds (QACs) with less than 10 carbon atoms in an alkyl chain, result in derivatives displaying poor antimicrobial activity. Therefore, here we synthesized QACs containing *N*-benzyimidazole scaffold and long alkyl chains containing at least 10 carbon atoms (Scheme 1). All the synthesized products were of satisfactory purity and no further purification by recrystallization was necessary. The white crystals of the quaternary salts were obtained in very good yields ranging from 68.7 to 88.3%.

3.2. Antimicrobial activity

Imidazole is a well-known drug precursor and previous reports suggest that some of its derivatives have variable antibacterial activity [38]. Therefore, we aimed to test the antimicrobial activity of newly synthesized BnI QACs. The antimicrobial potential was assessed against a total of 14 strains belonging to emerging pathogenic microorganisms, including the multidrug-resistant ESKAPE pathogens *S. aureus, E. coli, K. pneumoniae,* and *A. baumannii.* As can be seen in Table 1, the compounds proved to be highly active against all pathogens tested with minimal inhibitory concentrations (MICs) ranging from 7 ng/mL to 31.25 μg/mL. Most importantly, all quaternized compounds had significantly better antimicrobial activity than the BnI precursor (*S. aureus* ATCC 29213 MIC > 200 μg/mL and *E.coli* ATCC 25922 MIC > 60 μg/mL). Particularly potent antimicrobial activity was shown by the two BnI QACs with longer alkyl chains, **BnI-12** and **BnI- 14**, providing evidence that the activity is directly dependent on the chain length as previously observed [26,38]. The MIC values for **BnI-12** against bacterial strains ranged between 0.12 and 7.8 μg/mL, while **BnI-14** was even more active, resulting in MIC values between 30 ng/mL to 1.95 μg/mL. Moreover,



Scheme 1. Synthesis of QACs from *N*-benzylimidazole and alkyl bromides.

Table 1
Antimicrobial activity of BnI QACs (μg/mL) against selected strains assessed by the microdilution assay[a].

Species	Strain origin	BnI-10 (M_r = 379.39)		BnI-12 (M_r = 407.44)		BnI-14 (M_r = 435.49)		BAB (Mr = 384.44)
		MIC		MIC		MIC		MIC
Gram-positive bacteria								
Staphylococcus aureus	ATCC 29213	1.95		0.24		0.03		1.50
Staphylococcus aureus	Clinical/MRSA	15.63		1.95		0.12		
Enterococcus faecalis	ATCC 29212	7.80		0.48		0.12		
Streptococcus pyogenes	ATCC 19615	0.98		0.12		0.03		
Gram-negative bacteria								
Escherichia coli	ATCC 25922	3.90		0.48		0.48		3.90
Escherichia coli	Clinical	15.63		3.90		0.98		
Klebsiella pneumoniae	ATCC 13883	31.25		3.90		0.48		
Klebsiella pneumoniae	Clinical	31.25		3.90		1.95		
Acinetobacter baumannii	ATCC 19606	31.25		7.80		1.95		
Acinetobacter baumannii	Clinical	31.25		3.90		1.95		
Yeasts[b]		MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	MIC_{50}	MIC_{90}	
Candida albicans	ATCC 90029	0.98	1.95	0.015	0.03	0.007	0.015	
Candida albicans	Human	15.63	31.25	1.95	3.90	0.122	0.243	
Moulds								
Aspergillus niger	Food	3.90	15.63	0.98	1.95	0.24	0.49	
Penicillium citrinum	Food	7.80	15.63	0.49	0.98	0.12	0.24	

[a]MIC assays were carried out according to [34] protocol using 5×10^5 of exponentially grown bacterial cells/ml in 100% (v/v) Mueller Hinton Broth. [b] Determination of broth dilution MICs of BnI QACs for yeasts and conidia forming moulds were carried out by EUCAST protocols [35,36]. All assays were done in triplicates.

comparing results of **BnI-12** and **BnI-14** with commercially available benzyldodecyldimethylammonium bromide (BAB) it can be seen that both new QACs have better antimicrobial activity [16]. More specifically, **BnI-12** has 6.25 times lower MIC value against *S. aureus* and 8 times lower MIC against *E. coli* while **BnI-14** has 50 times lower MIC value against *S. aureus* and 8 times lower against *E. coli* (Table 1). Comparing their activity against Gram-positive versus Gram-negative bacteria, it can be seen that the Gram-positive strains are generally more sensitive to bactericidal effect of BnI QACs than Gram-negative strains. This is probably due to the different membrane composition of the various bacteria. The Gram-negative bacteria contain, in addition to the cell membrane, an outer membrane (OM) composed mainly of lipopolysaccharides. In contrast, Gram-positive bacteria completely lack OM but contain a thick layer of peptidoglycans made of negatively charged teichoic acid [39]. In this regard, *S. aureus* ATCC 29213 and *S. pyogenes* ATCC 19615 were the most sensitive to **BnI-12** and **BnI-14**. When these bacteria were exposed to **BnI-12,** slightly higher MICs values were observed, notably 0.12 μg/mL for *S. pyogenes* and 0.24 μg/mL for *S. aureus* ATCC 29213. However, with **BnI-14** even lower MIC values were observed with values as low as 30 ng/mL. Importantly, MRSA strain was effectively inhibited at 1.95 μg/mL of **BnI-12** and 0.12 μg/mL of **BnI-14** (Table 1).

Also, all three BnI QACs exerted very strong antifungal activity (MIC_{50} 7 ng/mL –15.625 μg/mL) against pathogenic yeast *C. albicans* and food spoilage moulds *A. niger* and *P. citrinum.* The best antifungal activity was detected towards *C. albicans* ATCC 90029, with MIC_{50} of 15 ng/mL in case of **BnI-12** and only 7 ng/mL for **BnI-14**, respectively. Two mould species were inhibited at very similar concentrations of these compounds. Notably, for *P. citrinum* MIC_{50} was 0.487 μg/mL of **BnI-12** and 0.122 μg/mL of **BnI-14**.

Overall, observed MIC values against bacteria and fungi are in a very low range, therefore **BnI-12** and **BnI-14** can be considered as good antibacterial and antifungal candidates. Thus, for further, more detailed evaluation we have chosen to analyze **BnI-14** as a candidate that exerted the best antimicrobial potential.

3.3. *Staphylococcus aureus growth kinetics and anti-biofilm activity*

The growth kinetics in the presence of BnI precursor (Fig. 1. a) and **BnI-14** (Fig. 1. b) show that both compounds inhibit *S. aureus* growth, but with the different efficacy. While BnI precursor effectively inhibits *S. aureus* at MIC, **BnI-14** was able to suppress its growth at 1/2 MIC. Both growth kinetic curves display similar shape, but different absorbance range for control and sub-MIC values. The difference of absorbance range could be explained by the different number of bacterial cells in the inoculum, or by a much higher reproduction pace in the presence of precursor as compared to **BnI-14.** A more detailed inspection of the growth kinetic data for **BnI-14** shows that 1/4 MIC effectively inhibits

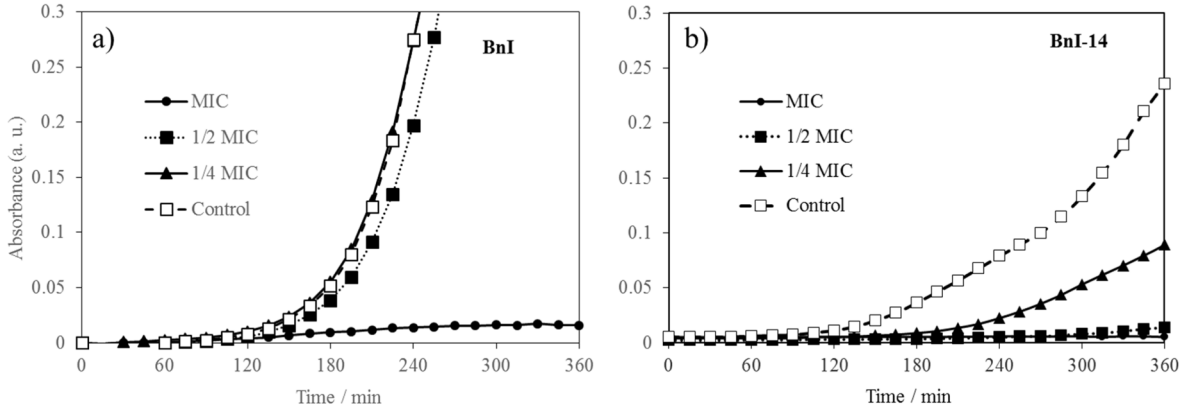


Fig. 1. The growth kinetics of *Staphylococcus aureus* ATCC 29,213 during 6 h incubation in the presence of BnI precursor (a) and **BnI-14** (b) at MIC, ½ MIC, and ¼ MIC, and with no antimicrobial agent (Control).

S. aureus during the first 3 h of incubation after which *S. aureus* continues to grow. However, in the presence of 1/2 MIC the bacterial growth is suppressed. These data along with the recorded MIC values evidence that quaternization of the precursor with long alkyl chain (14C-atoms) greatly improves the biological potential of BnI precursor.

It is generally accepted that bacteria in the form of biofilms are more resistant to antibiotics. One such biofilm-growing bacteria is *S.aureus* which is especially difficult to eradicate. After demonstrating the antimicrobial activity of new compounds, we set out to determine the antibiofilm activity of **BnI-14** toward *S. aureus* ATCC 29213. When exposed to a series of concentrations from MIC to 1/16 of MIC as presented in Fig. 2., the **BnI-14** acted variably on the formation of 3-day-old biofilm while the best activity, as anticipated, was observed at MIC value. However, the good antibiofilm potential was also evident for sub-MIC concentrations, namely 1/2 and 1/4 MIC that reduced the biofilm by 46.6% and 38.9% as compared to MIC. The inhibition rate at 1/8 and 1/16 of MIC had similar inhibition potential (13.9% and 12.4%, respectively) showing that this compound interferes with *S. aureus* growth processes even at extremely low sub-MIC doses, as much as 1.875 ng/mL (1/16 MIC). When comparing data for biofilm inhibition of **BnI-14** with BAB it can be seen that our candidate has a better inhibition rate at sub-MIC values than the commercial quaternary ammonium compound suggesting that **BnI-14** has strong antimicrobial potential.

3.4. Mode of antibacterial action

3.4.1. The effect of the *BnI-14* on the bacterial cell membrane

The AFM measurements of *E. coli* ATCC 25922 were obtained for treated and untreated cells. First, the untreated cells were measured to prove that the measurement itself does not alter the viability of the bacteria. Supplementary Figure S1 shows two consecutive AFM data sets demonstrating unperturbed cell elongation and division.

Fig. 3a shows untreated, characteristically rod-shaped bacterial cells. These AFM measurements were done just before the treatment. The cell walls appear to be preserved and relatively smooth, with no visible disruption or perturbation.

Fig. 3b shows the altered morphology of *E. coli* ATCC25922 exposed to a **BnI-14** concentration corresponding to 16 MIC obtained after 2 h of treatment. The treated cells revealed surface features that might indicate membrane blebbing and/or leakage of intracellular material. These results demonstrate the membranolytic mode of antibacterial action of **BnI-14**.

To further characterize **BnI-14** interaction with the bacterial membrane, the immobilized and treated cells were examined with optical microscopy. Fig. 4a is the brightfield image of a random sample area, while the AFM-inspected cells are indicated by the orange square.

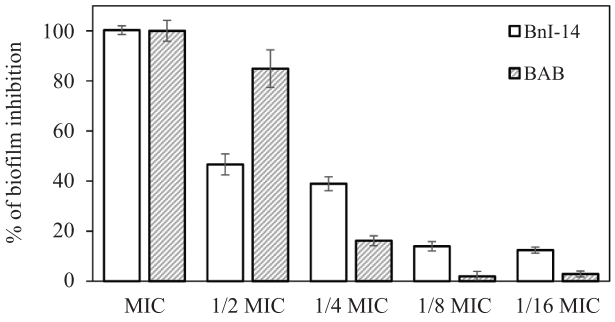


Fig. 2. The effect of **BnI-14** and benzyldodecyldimethylammonium bromide (BAB) on *Staphylococcus aureus* ATCC 29213 biofilm formation. The biofilm formation was assessed across a series of concentrations of antimicrobial agent ranging from 1/16 MIC to MIC. Control represents *S. aureus* ATCC 29213 biofilm formation with no antimicrobial agent present in suspension. Two independent experiments were carried in triplicates, and the results are given as the mean ± SD.

Fig. 4b and c show the corresponding SYTO 9 and PI fluorescence signals.

The PI passes through the bacterial membrane barrier only when the membrane is disrupted and subsequently binds to DNA. On the other hand, SYTO 9 penetrates live and dead bacterial cells [40].

Fig. 4 reveals that all inspected cells are permeabilized after 4 h of **BnI-14** treatment at 16 MIC, i.e. the treatment resulted in membrane disruption.

The PI-uptake experiment further supported the optical microscopy and AFM observations. When *E. coli* ATCC25922 cells were treated with a range of super-MIC and sub-MIC **BnI-14** concentrations, the increase in the PI intensity was found to be time and concentration dependent (Fig. 5). The 8 MIC concentration shifted the PI fluorescence toward its maximum values almost immediately, whereas the 4 MIC fluorescence increased steadily with time. These results, together with the microscopy data, indicate that the bacterial membrane becomes more damaged and consequently more permeable to PI upon prolonged exposure to **BnI-14**.

3.4.2. The DNA protection assay

This DNA protection assay evaluates the ability of the tested sample to prevent linearization of plasmid DNA upon its exposure to pro-oxidative agents such as those present in the Fenton's reagent (e.g., hydroxyl radicals,·OH). Free radicals from the Fenton's reagent react with DNA to form base and sugar radicals resulting in breakage of the sugar-phosphate backbone and linearization of plasmid DNA [41]. When the tested sample reacts with DNA, the DNA nicking and linearization is omitted and the original plasmid forms are preserved.

Therefore, to further investigate whether **BnI-14** binds to DNA and protects it from the free radicals in Fenton's reagent, we set out to perform the DNA protection assay.

Fig. 6. shows DNA footprints corresponding to different plasmid forms and degree of DNA damage. Lane 1, free plasmid DNA, gives two bands in an agarose gel corresponding to supercoiled and linear plasmid forms. The fully linear form of DNA is seen in lane 2, where the DNA sample was exposed only to the Fenton's reagent, resulting in plasmid damage and linearization. On the other hand, when the plasmid DNA was co-incubated with different concentrations of **BnI-14**, it can be seen that despite exposure to Fenton's reagent, the original form was maintained as in lane 1 (lanes 6–12).

This leads us to conclusion that **BnI-14** interacts with DNA, as evidenced by its ability to shield DNA from the Fe^{3+}-dependent nicking, and that this activity is concentration-dependent. This observation could be explained by the structure of **BnI-14** whereby the positive nitrogen atom electrostatically interacts with the DNA phosphate backbone. We hypothesise that this interaction is similar to the interaction of ethidium bromide (EtBr) and DNA, since EtBr is also a QAC. We and others have shown that the mode of antibacterial action of QACs, apart from membrane disruption, is even more complex and could involve interaction of QACs with intracellular proteins and nucleic acids [42,43]. This interaction in turn could cause interference with the bacterial growth and/or division process, leading to bacterial cell death in addition to membrane disruption.

3.5. Cytotoxic activity of *BnI-14*

Given the broad spectrum of antimicrobial activity and the potential application of **BnI-14** as a new antimicrobial agent, we set out to investigate the toxicity of **BnI-14** to human cells. The cytotoxicity was studied on several healthy human cell lines, HEK293, RPE1, and HaCaT, as well as on the tumor cell line, HeLa and the data obtained were compared to commercial quaternary ammonium compound BAB. Firstly, we tested toxicity of BnI precursor and results showed that BnI is not toxic to HEK293, HaCaT and HeLa cell lines up to the concentration of 8 mg/mL. As can be seen in Fig. 7, the toxicity of **BnI-14** proved to be comparable to BAB, but BAB generally exerts lower toxicity on all cell

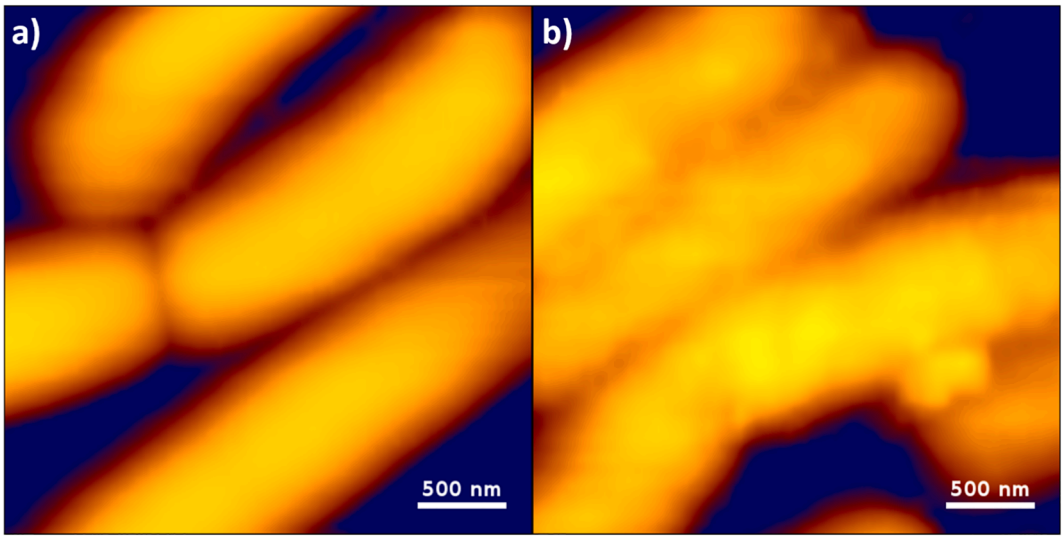


Fig 3. Atomic force microscopy images of immobilized **a**) untreated and **b**) **BnI-14**-treated *Escherichia coli* ATCC25922 cells.

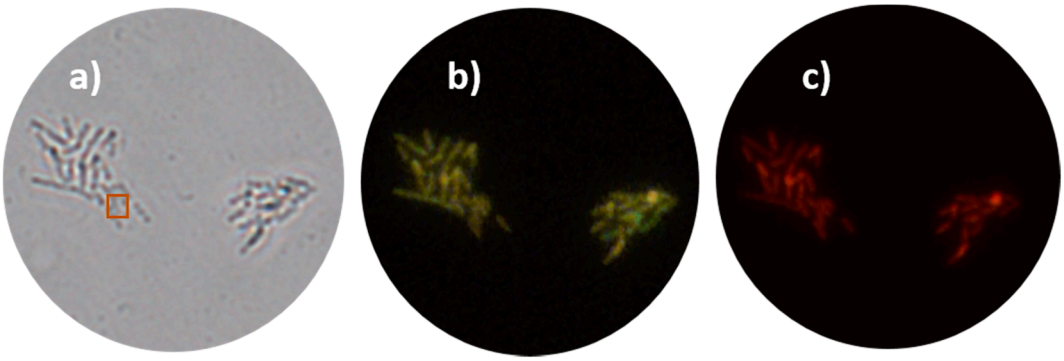


Fig 4. Bright-field image of:**a)** immobilized treated *Escherichia coli* ATCC25922 cells; **b)** same cells stained with SYTO9 and **c)** same cells stained with Propidium iodide (PI).

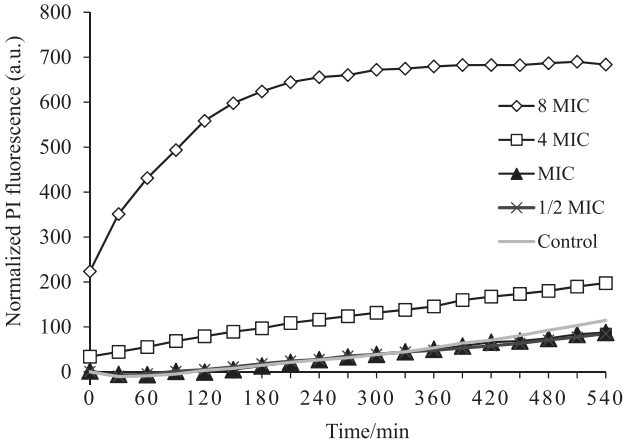


Fig 5. The propidium iodide (PI)-uptake of the *Escherichia coli* ATCC25922 without an antimicrobial agent (control) and exposed to 8 MIC, 4 MIC, MIC, and 1/2 MIC of **BnI-14** for 540 min.

lines tested, except HaCaT.

More importantly, the IC_{50} values for **BnI-14** against the most sensitive cell line, HEK293 (0.3 μg/mL) are 10 times higher than the MIC against *S. aureus* (0.03 μg/mL). These values are even much higher for RPE1 and HaCaT, 117- and 85-fold, respectively.

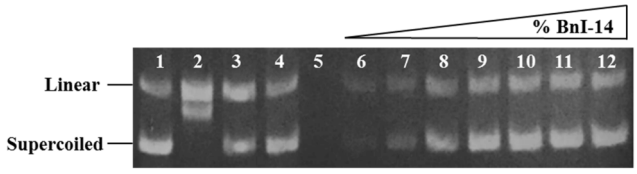


Fig 6. The pQE60 plasmid (DNA) in the presence and absence of the DNA nicking reagent (Fenton's reagent). Lane 1. DNA; lane 2: DNA incubated with Fenton's reagent; lane 3–4: DNA, Fenton's reagent and 2.5 and 5.0 U of catalase, and lanes 6–12: DNA, Fenton's reagent and 1.565, 3.125, 6.25, 12.5, 25, 50 and 100% BnI-14.

Taken together, these data suggest that **BnI-14** has a lower toxicity to human cells discriminating well enough mammalian over bacterial cell types. Since the HaCaT cell line (human keratinocytes) is a good model for potential topical application of the antimicrobial candidate, our results represent a good starting point for the development of new disinfectants and/or antiseptics. This is particularly interesting in times of pandemics and growing bacterial resistance, when antibacterial and antiviral agents are increasingly used.

4. Conclusion

In this study, we have synthesized quaternary *N*-benzylimidazole salts with alkyl chains of different lengths to investigate their

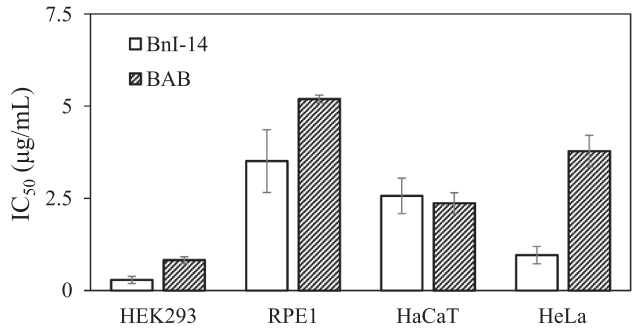


Fig. 7. Cytotoxicity of **BnI-14** and benzyldodecyldimethylammonium bromide (BAB) against HEK293, RPE1, HaCaT, and HeLa cell lines. The presented IC_{50} values represent an average of at least three independent experiments performed in quadruplets with standard deviation presented with error bars.

antimicrobial potentials and mechanisms of action. Overall, the MICs values observed against bacteria and fungi are in a very low range (as low as 7 ng/mL). Antibacterial growth kinetics and antibiofilm data for **BnI-14** showed better antibacterial activity than the commercial quaternary ammonium compound BAB, even at sub-MIC values. The AFM and optical microscopy data suggest that the bacterial membrane becomes more damaged and consequently more permeabile to PI with prolonged exposure to **BnI-14**. However, we have shown that **BnI-14** not only attacks the bacterial membrane, but also interacts with DNA which may indicate a more complex mode of action mechanism than previously observed. This interaction, in turn, could cause interference with the bacterial growth and/or division process. In addition, **BnI-14** showed lower toxicity against the HaCaT cell line, indicating good discrimination of mammalian over bacterial cells.

The results of the present study provide a new perspective on the antibacterial mode of action of QACs. As we have shown, QACs target the bacterial membrane but are also bound by other cellular macromolecules. In the future, the study of other possible intracellular interactors of QACs, such as proteins involved in the bacterial division process, may be an interesting direction in the field. In addition, our results represent a promising starting point for the structural optimization of ammonium salts based on the benzylimidazole scaffold. This is particularly interesting in times of pandemics and growing bacterial resistance, when antibacterial and antiviral agents are more widely used.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioorg.2021.104938.

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