Antibacterial effects of quaternary bis-phosphonium and ammonium salts of pyridoxine on *Staphylococcus aureus* cells: A single base hitting two distinct targets?

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Abstract

We studied the effects of quaternary bis-phosphonium and bis-ammonium salts of pyridoxine on the survival and morphology of *Staphylococcus aureus* cells. We found that, while originating from the same base, they exhibit considerably different antimicrobial mechanisms. In the presence of Ca^{2+} ions the MIC and MBC values of ammonium salt increased 100-fold, suggesting that Ca^{2+} ions can successfully impede the membrane Ca^{2+} ions exchange required for ammonium salt incorporation. In contrast, in the presence of quaternary phosphonium salt, the artificial capsular-like material was formed around the cells and the filamentous and chain-like growth of the cells was observed suggesting the disruption of the cell division mechanisms. Altogether, both pyridoxine derivatives successfully inhibited the growth of gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis*, *Bacillus subtilis*) and *Escherichia coli* considerably, while demonstrated nearly no effect against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. We suggest that due to their effects on distinct and potentially complementary targets the derivatives of pyridoxine could appear perspective antibacterials with complicated adaptation and thus with lower risk of drug resistance development.

Keywords quaternary phosphonium salts, quaternary ammonium salts, pyridoxine, antibacterial activity, fluorescent microscopy

Introduction

Intensive development of antibiotics and their wide introduction into clinical practice throughout the 20th century resulted in the drastic decrease of mortality from infectious diseases. However, the uncontrolled usage of antimicrobial compounds has resulted in the emergence of antibiotic-resistant pathogenic bacteria rapidly expanding worldwide. Therefore, currently there is a high demand in the development of a new generation of antimicrobials with lower frequency of resistance development. One promising direction is the design of drugs that affect the bacterial cells using distinct and largely independent mechanisms that focus on potentially complementary targets. The appearance of strains resistant to such drugs inhibiting simultaneously two or morebiochemical pathways appears very unlikely.

In the last decade(s) quaternary phosphonium and ammonium salts were actively investigated for antibacterial activity. Among the phosphonium salts, derivatives grafted on styrene–divinylbenzene copolymers were shown to exhibit high antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Popa et al., 2003), di- and trimethyl-substituted phosphonium salts with long alkyl chaines showed bacteriostatic action against 11 widespread pathogens including methicillin-resistant *S. aureus* (MRSA) (Kanazawa et al., 1994), triphenylphosphonium-modified PPO (polyphenylene oxide) polymer were found to have antimicrobial activity on *Staphylococcus epidermidis* and *Escherichia coli* (Chang et al., 2010), quaternary phosphonium salts with reactive hydroxyl group were active against *Staphylococcus aureus* and *Escherichia coli* (Tingting et al., 2012), P- triazinylphosphonium salts exhibited bacteriostatic activity against *Staphylococcus aureus*, *Escherichia coli* and *Proteus mirabilis* (Kolesika et al., 2011). The phosphonium salts demonstrated higher antimicrobial activity against the gram-positive bacteria compared with gram-negative ones. For example, quaternary phosphonium salts based on higher alkyl halides and tertiary phosphines led to bigger growth-inhibition zones formation of *Staphylococcus* than of *Escherichia coli*,

Salmonella sp., *Klebsiella pneumonia*, *Pseudomonas aeruginosa* and *Proteus mirabilis* (Cherkasov et al., 2013). The arylmethyl triphenylphosphonium salts inhibited the growth of *Staphylococcus* and *Bacillus* and not *Escherichia coli* and *Pseudomonas aeruginosa* (Listvan et al., 2009). Currently the antibacterial properties of quaternary ammonium compounds are widely used in disinfection and antisepsis. Prominent examples include benzalkonium chloride, dequalinium chloride, benzethonium chloride and cetylpyridinium chloride that have been widely applied in clinical practice (e.g., preoperative disinfection of intact skin, treatment of mouth and vaginal bacterial infections, disinfection of noncritical surfaces etc.) (Gilbert et al., 2005).

Very recently we have synthesized novel derivatives of quaternary phosphonium, bis-phosphonium and quaternary ammonium salts of pyridoxine and its 6-hydroxymethyl derivatives that demonstrated high antimicrobial activity mainly against gram-positive bacteria (Pugachev et al., 2013a; Pugachev et al., 2013b; Shtyrlin et al., 2015).

The antimicrobial mechanism of quaternary ammonium has been studied previously (Bruinsma et al., 2006; Crismaru et al., 2011). Low molecular weight quaternary ammonium salt compounds interact with the bacterial cell surface, followed by their integration into the cellular membrane and its disruption through creation pores in it. Such dramatic cell membrane reorganization leads to the leakage of intracellular material and thus to the cell death (Denyer, and Stewart, 1998; Marcotte et al., 2005). The antimicrobial mechanism of phosphonium salts was studied on carboxylate phosphabetaine as an example (Galkina et al., 2013). The alkylated phosphabetaines have been shown to interact with lecithin followed by pores formation, uncontrolled intracellular contents flow and ultimate cell death.

In this paper we show that both phosphonium and quaternary ammonium salts of pyridoxine affect a cellular membrane of *Staphylococcus aureus*. However, the antibacterial effect of these compounds is likely governed by different mechanisms and thus appears a promising example of a multi-target antimicrobials originating from a single base. Finally, we

discuss the specific chemical structure of these biocides and its relation with their antibacterial efficiency.

Material and methods

Chemicals and strains

The quaternary bis-phosphonium salt **1** (2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridine-5,6-diyl)bis(methylene))bis(tri-p-tolylphosphonium)dichloride) and the quaternary bis-ammonium salt 2 (N,N'-((2,2,8-trimethyl-4H-[1,3]dioxino[4,5-c]pyridine-5,6-diyl)bis(methylene))bis(N,N-dimethyloctan-1-aminium) dichloride) of pyridoxine were synthesized previously (Pugachev et al., 2013b, Shtyrlin et al., 2015), see Fig.1 for their chemical structures. Stock solutions of synthesized salts in water were sterilized by the filtration through a 0.22 µm filter.

Several strains of gram-positive (*Staphylococcus aureus* ATCC 29213, *Staphylococcus epidermidis* (clinical isolate), *Bacillus subtilis* 168) and gram-negative (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* (clinical isolate)) bacteria were chosed as test organisms. Clinical isolates were obtained from the Kazan Institute of Epidemiology and Microbiology (Kazan, Russia).

Determination of the minimal inhibitory (MIC) and bactericidal (MBC) concentration

The MICs of compounds **1** and **2** were determined by the microdilution method in Müller-Hinton broth (pH=7.3). The compounds were diluted in a 96-well microtiter plate to final concentrations ranging from 1 to 1000 µg ml^{-1} in 250-µl aliquot of the bacterial suspension ($5×10^5$ CFU/ml) followed by their incubation at 37°C for 24 h. The MIC was determined as the

lowest concentration that completely inhibited the bacterial growth. The MBC was determined as the lowest concentration that led to no CFUs in 5 μl of culture liquid in the well. Experiments were carried out in triplicate. Additionally, MICs and MBCs also were determined in the presence and absence of 0.1 M $CaCl_2$ in nutrient broth.

Time-kill curves

The 24h-cultures of *S. aureus* were diluted 1:10000 by the fresh, heated to 37°C LB broth containing different concentrations of the compounds (MIC, 1×MBC and 2×MBC) followed by their incubation with agitation at 37°C. Samples were taken each 2 h during 24 h. Viable cells were evaluated as CFUs per ml. Additionally, the *S. aureus* cells after 24 h exposition to **1** or **2** were washed six times to remove the organic components from the cell surface and analyzed by both CLSM and CEM.

Confocal laser scanning microscopy (CLSM)

Differential fluorescent staining was used to identify cells with intact and perforated membranes (Aquino et al., 2010). The bacterial cell survival was determined after 24 h of exposure to the studied compounds and visualized by CLMS on an inverted Carl Zeiss LSM 780 confocal laser-scanning microscope (Carl Zeiss, Jena, Germany). The samples were stained for 15 min with the acridine orange (green fluorescent) and propidium iodide (red fluorescent) to differentiate between undamaged and cell surface-damaged bacteria.

Scanning electron microscopy (SEM)

For scanning electron microscopy, a bacterial suspension was fixed by 1% glutar aldehyde, then dehydrated in alcohol solutions (30, 50, 70, 80, 90, 100%) and a small drop was mounted onto a cover glass slide, air-dried and coated with Au/Pd (Quorum Q150T ES vacuum coater). Images were obtained by the scanning electron microscope Merlin (Carl Zeiss, Jena, Germany), operating at an accelerating voltage of 15 kV, SE-detector.

Results and discussion

Antibacterial activity

The MICs and MUCs characterizing the antibacterial activity of compounds **1** and **2** against Gram-positive and Gram-negative bacteria are given in Table 1. Both compounds efficiently inhibited the growth of *S.aureus* and other gram-positive bacteria while demonstrating almost no activity against gram-negative *K. pneumoniae* and *P. aeruginisa*. Notably, *E.coli* was sensitive only to bis-phosphonium salt (**1**) and not to the ammonium one. MICs and MBCs were also tested in presence of 0.1M $CaCl_2$. Interesting, in the presence of $CaCl_2$, both MICs and MBCs of pyridoxine ammonium salt (**2**) for all sensitive strains increased by two orders of magnitude, in contrast to the bis-phosphonium salt (**1**), suggesting the different mechanism of antimicrobial activity. To avoid the uncertain results related to the unknown genotype of *S. epidermidis* clinical isolate and sporulation of *B. subtilis* under sublethal concentrations of antimicrobials, these bacteria were out of further investigations.

Time-kill curves

Next for an assessment of the antimicrobial activity of the pyridoxine salts **1** and **2** against *S. aureus* ATCC 29213, the time-kill curves were obtained. For that, the *S.aureus* cells

were exposed to different concentrations of the compounds (MIC, 1×MBC and 2×MBC) in LB broth. Samples were taken each 2 h during 24 h and the number of viable cells was evaluated by the CFUs on the solid medium. A faster decrease of the CFU was observed for compound 2 compared with compound 1 already during the first hours of exposition (Fig. 2). The complete death of cells was observed after 20 h of incubation in the presence of 1×MBC of compound **1** (bis-phosphonium salt) and after 16 h in presence of its 2×MBC, whereas in presence of compound **2** (ammonium salt) at 1×MBC and 2×MBC CFUs were no longer detectable after 16 h and 12 h, respectively.

Fluorescence microscopy

This is known that quaternary salts permeabilize the cellular membrane that could be evaluated by the propidium iodide staining (Pan et al., 2011). A functional cytoplasmic membrane does not allow the accumulation of propidium into a bacterium to replace acridine orange, which readily penetrates functional cytoplasmic membranes from the DNA and RNA. To investigate the bacterial cell membrane damage by compounds **1** and **2**, the fluorescent microscopy was performed in the presence of propidium iodide as a marker of damaged membrane and acridine orange to stain all cells. Figure 3 shows the fluorescence images of *S. aureus* after 24 h cultivation with compounds **1** and **2** at different concentration.

In the absence of antibacterial compounds, all cells had a green fluorescence indicating undamaged cytoplasmic membrane (see Fig. 3A). In the presence of compound 1 at its MIC red fluorescence was observed in about 30% bacteria, whereas the addition of compound 2 (MIC) resulted in the red fluorescence of about 60% cells, indicating strong antibacterial effects. Figure 3B (compound 1, MIC) shows that a significant fraction of the bacterial cells were colored partially by both green and red that likely indicates the loss of the cell membrane integrity as a result of the treatment by quaternary bis-phosphonium salt 1. In the case of compound 2 (Fig.

3E) cells were stained evenly by either red or green. The 1× and 2× the MBCs of compounds 1 caused the loss of the membrane integrity for 93 and 99 % cells, respectively (Fig. 3C,D). The 1× and 2×MBCs of quaternary ammonium salt (2) caused the loss of membrane integrity for 92 and 97 % of all bacteria, respectively (Fig. 3F,G).

SEM microscopy

Additionally, the morphological changes of cellular membranes of *S. aureus* exposed to compounds **1** and **2** for 24 h were analyzed by scanning electron microscopy. The untreated cells had intact, smooth and spherical morphology (see Figs. 4 and 5A, E). The bacterial cells exposed to 1 μg/ml (MIC) of compound **1** (see Fig. 4B, F) or to 8 μg/ml (MIC) of compound **2** (see Fig. 5B, F) did not show significant changes, despite of a moderate increase in the number of lysed cells that could be observed.

The exposure to higher concentrations of bis-phosphonium salt **1** (32 and 64 μg/ml, 1×MBC and at 2×MBC respectively) led to the roughened bacterial surface with boss-like protuberances and unusual protrusions (see Fig. 4C,D,G,F) that were not observed in control cells (see Fig. 4A,E). Additionally these cells exhibited drastically altered surfaces and prominent cleavage sites (see Fig. 4C, G). Numerous lysed cells accompanied by cellular debris and release of intracellular components could be observed.

S. aureus cells were drastically altered by the exposure to lethal concentrations of quaternary ammonium salt **2** (1×MBC and 2×MBC) (see Figs. 5 C, D, G, H). Antimicrobial-induced modifications included filamentous growth, ghosts, antler-like protrusions, mini-cells, elongated linear cells, blebs, and deep web-like fissures. Additionally, some cells had deep craters on the cell walls confirming an idea of the membrane damage leading to the loss of the cell integrity as a result of the treatment by compound **2** (see Figs. 5 G, H).

Discussion

Understanding the mechanisms governing the antimicrobial effects of the newly synthetized biocides is essential to reveal their potential area of practical application. Both compounds tested in our study exhibited high activity only against gram-positive microorganisms (with the exception of *E.coli*). This suggests that their antimicrobial target could be related to the cell wall of these bacteria. We also like to note that the selectivity of phosphonium salts against gram-positive microorganisms was shown previously (Kanazava et al., 1994).

The investigation of antimicrobial activity of bis-phosphonium and ammonium salts of pyridoxine revealed differences in the destruction of gram-positive bacterial cell (see Table 1). We suggest that the ammonium salt of pyridoxine damage the cellular membrane permeability, since the propidium iodid penetrated into the *Staphylococcus* cells exposed to the sublethal concentration (MIC) of the ammonium salt **2** (see Fig. 3). Scanning electron microscopy demonstrated that lethal concentrations of compound **2** disrupted the cellular integrity of *S. aureus* and formed unusual and bizarre morphological forms (see Fig. 5). As reported earlier, the membrane-integrand biocides force the removal of Ca2+ ions from the membrane (Das et al., 2014). Since Ca^{2+} plays an important role in the membrane charge stability that is essential for the integrity of the outer lipopolysaccharide layer and cell walls of bacterial cells, its elimination can lead to the loss of the cell integrity. The addition of Ca^{2+} ions yielded longer survival of intact *Staphylococcus* and *Bacillus* cells in presence of compound **2**, suggesting that positively charged Ca^{2+} ions can impede the exchange of membrane Ca^{2+} ions by ammonium salt of pyridoxine. Accordingly the antimicrobial mechanism of compound **2** is likely similar to other quaternary ammonium salts (Crismaru et al., 2011). We believe that the antibacterial action of the ammonium salt of pyridoxine is largely determined by the liphophilic fragments of $-C_8H_{17}$ that are able to integrate into the lipid layers of the bacterial cell membrane and damage it. This mechanism is typical for many alkylated compounds that form pores and this way disrupt the

membrane functions (Togashi et al., 2007; Galkina et al., 2013). Nevertheless, revealing the particular metabolic pathway that is affected requires further investigation.

In contrast to the ammonium salt, the bis-phosphonium salt of pyridoxine apparently affects the cell in a different way, since its MIC or MBC values did not change in presence of Ca^{2+} ions. Both CLSM and SEM data indicate the absence of the membrane damage and leakage of the intracellular substances (see Figs. 3 and 4, respectively). The pronounced anomalies in the *S. aureus* cell structure treated by lethal concentrations of bis-phosphonium salt suggest the production of defective cell-wall material that likely led to the formation of aberrant septation.

Therefore we suggest that the bis-phosphonium salt of pyridoxine affects the cell wall metabolism or membrane components. Similar results were obtained for some lipopeptide antibiotics such as daptomycin (Greenwood and O'Grady, 1969; Wale et al., 1989; Conrad et al., 1998). It exhibited a similar high specificity for Gram-positive bacteria, did not react to the presence of Ca^{2+}, and also led to the incrustation protrusions on cell surface and affected the staphylococcal cell division and resulted of chains formation.

Conclusion

To summarize, our investigations revealed that, while originating from the same base, quaternary bis-phosphonium and bis-ammonium salts of pyridoxine exhibit considerably different antimicrobial mechanisms. While the ammonium salt forces the removal of the Ca^{2+} ions from the membrane causes the disruption of the cell integrity, the quaternary phosphonium salt leads to the formation of the artificial capsular-like material around the cells likely affecting the cell division mechanisms. Altogether, both pyridoxine derivatives successfully inhibited the growth of gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis*, *Bacillus subtilis*) and *Escherichia coli* considerably, while demonstrated nearly no effect against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. We suggest that due to their effects on

distinct and potentially complementary targets the derivatives of pyridoxine could appear perspective antibacterials with complicated adaptation and thus with lower risk of drug resistance development.

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Fig.1. The chemical structures of the bis-phosphonium salt (compound 1) and ammonium salt (compound 2) of pyridoxine

Fig.2 Time-kill curves of *S. aureus* ATCC 29213 exposed to compound **1** (A) and compound **2** (B) in various concentrations (MIC, 1× MBC and 2×MBC)

Fig. 3 Fluorescent confocal microscopy of *S. aureus* ATCC 29213 after 24 hours of exposition to the compounds **1** and **2** in nutrient broth and stained with propidium iodide and acridine orange. (A) No compounds; (B) compound **1** MIC; (C) compound **1** 1×MBC; and (D) compound **1** 2×MBC, (E) compound **2**MIC; (F) compound **2** 1×MBC; and (G) compound **2** 2×MBC. The bar corresponds to 2 µm.

Fig. 4 Scanning electron microscopy (SEM) of *S. aureus* ATCC 29213 after 24 hours of exposition to the compound **1** in nutrient broth: (A, E) No compounds; (B, F) MIC; (C, G) 1×MBC; and (D, H) 1 2×MBC. The magnification of A-D is 10000, magnification of E-H is 20000 or 30000

Fig. 5 Scanning electron microscopy (SEM) of *S. aureus* ATCC 29213 after 24 hours of exposition to the compound **2** in nutrient broth. (A, E) No compounds; (B, F) MIC; (C, G) 1×MBC; (D, H) 2×MBC. The magnification of A-D is 10000, magnification of E-H is 30000 or 50000

Table 1 Antibacterial activity of the bis-phosphonium salt (compound **1**) and ammonium salt (compound **2**) of pyridoxine (MICs and MBCs, µg/ml)

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