LETTER TO THE EDITOR

Activity of antimicrobial peptides, alone or combined with conventional antibiotics, against Staphylococcus aureus isolated from the airways of cystic fibrosis patients

Katarzyna Garbacz a, Wojciech Kamysz b, and Lidia Piechowicz c

a Department of Oral Microbiology, Medical University of Gdansk, Gdansk, Poland; b Department of Inorganic Chemistry, Medical University of Gdansk, Gdansk, Poland; c Department of Medical Microbiology, Medical University of Gdansk, Gdansk, Poland

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The aim of this in vitro study was to evaluate the activity of 4 natural and 3 synthetic antimicrobial peptides (AMPs) against 215 isolates of Staphylococcus aureus from the airways of cystic fibrosis (CF) patients. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) were identified, and synergy studies between AMPs and conventional antibiotics were carried out. The growth of all investigated isolates was inhibited by AMPs at following concentrations: CA (1–7)M (2–9) (4–32 μg/mL), pexiganan (4–32 μg/mL), citropin 1.1 (16–64 μg/mL), temporin A (16–64 μg/mL), IB-367 (16–128 μg/mL), uperin 3.6, (64–128 μg/mL), aurein 1.2 (128–256 μg/mL). A synergy between IB-367, fusidic acid (FICindex = 0.395) and co-trimoxazole (FICindex = 0.409) was revealed. Our findings point the examined AMPs as promising anti-staphylococcal agents that should be subject to further research.

Cystic fibrosis (CF) is a common genetic disease of humans. It results from recessive mutation in cystic fibrosis transmembrane conductance regulator (CFTR) gene located on the long arm of chromosome 7. CFTR protein regulates membrane permeability to chloride ions. Disruption in chloride ion transport is reflected by presence of thick, sticky and hard to evacuate mucus, which aside from exocrine pancreatic insufficiency and male infertility, predisposes primarily to chronic bacterial respiratory infections. Staphylococcus aureus is an early, common pathogen of CF patients. Aside from other pathogens, also infections with S. aureus are linked to impaired function and structural damage of the lungs. Pulmonary dysfunction still constitutes the major cause of mortality among CF patients.

Frequent use of antibiotics in treatment and prevention of staphylococcal infections results in emerging antibiotic resistance of S. aureus isolates from CF patients. The fact that antimicrobial resistance may be mitigated by using non-antibiotic treatments stimulated research on agents being an alternative to conventional antibiotics. A potential candidate for such treatment seem to be antimicrobial peptides (AMPs). The interest in AMPs is not associated solely with limited toxicity of some of these compounds, low risk of resistance development and low molecular weight, but also with their established anti-inflammatory and immunomodulatory effects.

AMPs play a significant role in protection against respiratory infections. They are released from phagocytes and cells of respiratory epithelium. Together with lysozyme, lactoferrin, complement, antibodies, etc., AMPs form part of a cocktail of antimicrobial molecules, and concentrations of many of them increase in response to infection and inflammation. However, CF is associated with impaired physiological secretion of AMPs and this is postulated to be a reason behind greater susceptibility of CF patients to bacterial infections, after structural lung disease. It has been demonstrated, at least in vitro, that the airway surface liquid (ASL) secreted by cultured airway epithelial cells from CF patients has an increased salt concentration, which determines its lower antimicrobial activity. More recently, it has been suggested that the impairment of antimicrobial activity in CF lung may also depend on sequestration of AMPs by some components present in the mucus, such as DNA, F-actin and cellular debris.

AMPs raise high hopes as alternative antimicrobial treatments, among others due to their lower molecular weight as compared to conventional antibiotics. As a result, they can be administered locally, which is of vital
importance in the case of CF patients in whom inhalation is commonly used and preferred route of drug administration.13

Thus, future of AMPs as a novel treatment for staphylococcal infections seems promising. A number of research groups worldwide continue research on potential therapeutic application of AMPs, and some of these studies have already entered a clinical phase. AMPs were tested as treatments for diabetic foot syndrome,14 meningococcal meningitis,15 acne,16 oral mucosal infections,17 and local catheter-related infections.18 However, despite extensive research, still little is known on susceptibility to AMPs among common pathogens isolated from CF patients, and potential application of AMPs in the management of this condition.

The aim of this in vitro study was to evaluate the activity of 7 AMPs, alone or combined with 11 clinically used agents, against 215 isolates of S. aureus from the airways of CF patients. We selected AMPs with an established anti-staphylococcal activity that have not been previously tested against isolates of S. aureus from CF patients, i.e. 3 synthetic compounds (CA(1–7)M(2–9), pexiganan and IB-367) and 4 natural peptides (aurein 1.2, citropin 1.1, temporin A and uperin 3.6).

All 215 investigated isolates turned out to be susceptible to AMPs used at following concentrations: CA(1–7)M(2–9) from 4 μg/mL to 32 μg/mL, pexiganan from 4 μg/mL to 32 μg/mL, citropin 1.1 from 16 μg/mL to 64 μg/mL, temporin A from 16 μg/mL to 64 μg/mL, IB-367 from 16 μg/mL to 128 μg/mL, uperin 3.6 from 64 μg/mL to 128 μg/mL and aurein 1.2 form 128 μg/mL to 256 μg/mL. MBCs for the tested AMPs were generally twice as high as their MICs. The results are summarized in Table 1. We did not observe significant differences in the susceptibility of MRSA and MSSA.

MICs for conventional antibiotics in 50 preselected susceptible strains were as follows: azithromycin (2 μg/mL), chloramphenicol (16 μg/mL), ciprofloxacin (0.5 μg/mL), clindamycin (1 μg/mL), cloxacillin (0.5 μg/mL), co-trimoxazole (2 μg/mL), fusidic acid (0.25 μg/mL), gentamicin (1 μg/mL), mupirocin (0.25 μg/mL), tetracycline (1 μg/mL), vancomycin (1 μg/mL).

Combination studies for selected strains revealed a synergy between IB-367, fusidic acid (FICindex = 0.395) and co-trimoxazole (FICindex = 0.409). The values of FICindex for other antibiotics ranged between 0.643 and 3.043 (Table 2).

Emerging antibiotic resistance of S. aureus isolates constitutes an important clinical problem in CF patients and stimulated research on novel effective treatments for this group.7 AMPs gain attention as the most promising next-generation antibiotics owing their rapid and broad spectrum antimicrobial activity.

Present study analyzed in vitro activity of AMPs, alone or combined with clinically used antibiotics, against 215 clinical isolates of S. aureus from the airways of CF patients. We selected AMPs with an established anti-staphylococcal activity that have not been previously tested against isolates of S. aureus from CF patients,19 i.e., 3 synthetic compounds (CA(1–7)M(2–9), pexiganan and IB-367) and 4 natural peptides (aurein 1.2, citropin 1.1, temporin A and uperin 3.6). Synthetic AMPs had greater antibacterial activity than the natural ones. CA(1–7)M(2–9), a 15-residue synthetic hybrid peptide derived from cecropin A and melittin, was the most effective of all analyzed AMPs. Cecropin A is a polycationic peptide with broad antimicrobial spectrum, originally isolated from hemolymph of the giant silk moth, Hyalophora cecropia.20 Melittin was originally derived from the venom of honey bee.21 CA(1–7)M(2–9) hybrid synthesized from cecropin and melittin residues is known to be more potent antibiotic than each of its components alone.21 We showed that CA(1–7)M(2–9) was highly effective against S. aureus isolates from the airways of CF patients. Although to the best of our knowledge, this AMP has not been tested against staphylococcal isolates from CF patients thus far, previous studies showed that CA(1–7)M(2–9) is effective against clinical isolates of S. aureus from patients with other infections.22,23 Therefore, CA(1–7)M(2–9) seems to be a good candidate for an anti-staphylococcal treatment for CF patients. Owing promising results of the hereby reported in vitro tests, CA(1–7)M(2–9) should be subjected to clinical trials in order to determine its pharmaco-kinetic and toxicity profiles.

Pexiganan, a synthetic 22-residue analog of magainin II, was the second most potent AMP in our study.24 Magainins are a class of cationic α-helical peptides isolated from the skin of African frog, Xenopus laevis. Pexiganan was previously shown to be a more potent antibiotic than magainin II,25 and our study demonstrated that it is also

| Peptide       | MIC (μg/mL) | MBC (μg/mL) |
|---------------|-------------|-------------|
|               | range       | 50%¹ | 90%² | range       | 50%³ | 90%⁴ |
| CA(1–7)M(2–9) | 4–32        | 8    | 16   | 4–32        | 16   | 32   |
| pexiganan     | 4–32        | 16   | 32   | 16–64       | 32   | 64   |
| citropin 1.1  | 16–64       | 32   | 16–128 | 64   | 64   |
| temporin A    | 16–64       | 16   | 32   | 16–64       | 32   | 64   |
| IB-367        | 16–128      | 32   | 64   | 32–128      | 64   | 64   |
| uperin 3.6    | 128–256     | 128  | 128  | 128–256     | 128  | 128  |
| aurein 1.2    | 128–256     | 128  | 128  | 128–256     | 128  | 128  |

Note. ¹Inhibitory concentration for ≥50% of the tested isolates; ²inhibitory concentration for ≥90% of the tested isolates; ³bactericidal concentration for ≥50% of the tested isolates; ⁴bactericidal concentration for ≥90% of the tested isolates.
active against S. aureus. Pexiganan deserves attention not only because of its anti-staphylococcal activity, but also in view of the fact that its efficiency was already confirmed in clinical trials. An in vivo study of patients with mildly infected diabetic foot ulcers demonstrated that topical pexiganan is as efficacious as oral oxolinic in terms of clinical and microbiological improvement and wound healing rates. Another unquestioned advantage of pexiganan is its recently documented ability to inhibit the synthesis of biofilm, which may further potentiate its antibacterial efficacy in CF patients.

Natural peptides being a subject of this study were originally isolated from the frog skin, a rich source of AMPs. Natural AMPs are synthesized and accumulated in specialized structures of the skin, referred to as granular glands. AMPs were found in the secretions from approximately 20 species of Australian tailless amphibians, including Litoria spp. and Uperoleia spp. Two AMPs analyzed in our study, citropin 1.1 and aurein 1.2, are naturally found in the Australian green tree frog, Litoria citropa, and golden bell frog, Litoria aurea. Uperin 3.6 was isolated from the Australian toadlet, Uperoleia mjobergii, and temporin A from skin of the European red frog, Rana temporaria.

We demonstrated that natural AMPs are less potent than synthetic CA(1–7)M(2–9) and pexiganan. Citropin 1.1 and temporin A turned out to be the most efficient anti-staphylococcal agents among natural AMPs tested in our study. However, their MICs/MBCs were several times higher than previously documented in the case of S. aureus isolates from patients with other infections. This was even more evident in the case of uperin 3.6 and aurein 1.2, whose MICs/MBCs were up to 16-fold higher than reported previously. However, MICs/MBCs of uperin 3.6 and aurein 1.2 documented in a study of clinical isolates from skin infections were similar to those recorded in our series, which implies that the activity of AMPs against S. aureus may be infection-specific. Lower susceptibility to AMPs among staphylococcal isolates being a subject of our study might also result from their adaptation to the specific microenvironment of CF airways.

We demonstrated a synergy between IB-367, fusidic acid and co-trimoxazole. IB-367 (also referred to as iseganan) is a 17-amino acid synthetic analog of protegrin. Protegrins, originally isolated from porcine neutrophils, belong to the cathelicidin family showing activity against a broad spectrum of bacteria. IB-367 demonstrates stronger bactericidal activity than native protegrins. Moreover, previous studies showed that some peptides and hydrophobic antibiotics, such as rifampicin, macrolides, fusidic acid and novobiocin, may act synergistically. The exact mechanism behind this positive interaction is not understood, and perhaps may be complex. Presumably, IB-367 is absorbed to the membrane surface, being incorporated in the hydrophobic core in a transmembrane orientation and forming peptide dimers. The latter oligomerize to form water-filled transmembrane pores, which is reflected by unrestricted transport of ions. The enhanced permeability of cytoplasmic membrane facilitates influx of antibiotics. Perhaps, the synergy may also result from disintegration of bacterial membranes by IB-367 and resultant better access of fusidic acid and co-trimoxazole to their intracellular targets, i.e. such biochemical pathways as synthesis of protein or dihydrofolic acid.

IB-367 was already a subject of clinical trials as a preventive measure of ventilator-associated pneumonia. Unsatisfactory outcomes of these trials might result from an inappropriate pharmaceutical formulation of IB-367. According to some authors, a mucosa-adhesive paste should be used in future trials, rather than the previously tested aqueous solution of IB-367. Consequently, further studies are needed to verify if the synergistic effect of IB-367, fusidic acid and co-trimoxazole documented in our in vitro experiments can be also achieved in vivo, for IB-367 used in an appropriate pharmaceutical formulation. This question should be addressed in an adequately-designed clinical trial.

Noticeably, both fusidic acid and co-trimoxazole are used in the treatment of CF-related infections.
Recently, fusidic acid proved to be effective not only in the treatment of staphylococcal infections but also in the decolonization of airways in CF patients.\textsuperscript{45,46} Moreover, it was demonstrated to act synergistically with trimethoprim, a component of co-trimoxazole.\textsuperscript{45}

Due to a large proportion of strains being susceptible to these agents, fusidic acid and co-trimoxazole are commonly used in the treatment of MRSA infections. Our study showed that both MSSA and MRSA showed similar degree of susceptibility to the analyzed AMPs. This with no doubt represents an advantage of AMPs, since antibiotic therapy of MRSA infections is usually more challenging than those caused by MSSA. Our findings are consistent with the results of previous studies in which the risk of developing bacterial resistance to AMPs was relatively low.\textsuperscript{8}

In conclusion, we showed that CA(1–7)M(2–9), a synthetic AMP, is a potent antibiotic active against \emph{S. aureus} isolates from the airways of CF patients. Moreover, we demonstrated a synergy between IB-367, fusidic acid and co-trimoxazole, i.e., antibiotic used routinely in the treatment of CF-related infections. Finally, AMPs were shown to be equally efficient against methicillin-resistant and susceptible staphylococcal isolates from CF patients. Our findings seem to be particularly interesting from a clinical perspective, as they point to AMPs as promising anti-staphylococcal agents that should be subject to further research, and due to the hereby documented synergistic effect may be also potentially used as a component of combined antimicrobial therapy.

The study included material from 107 CF patients aged 1 month to 47 years, treated at the Outpatient Cystic Fibrosis Clinic of “Polanki” Children Hospital in Gdańsk between 2012 and 2014. A total of 215 \emph{S. aureus} isolates from throat swabs, sputum and bronchoalveolar lavage were studied. Isolates belonged to multiple spa-types (to be published in detail elsewhere). Throat swabs were obtained after provoking a cough (deep swabs). The material was subcultured onto Columbia blood agar and incubated at 35°C for 24 hours. Suspected staphylococcal isolates were identified on the basis of colony characteristics, pigment production, Gram-stained appearance, hemolysis and Staphyslide agglutination test (BioMerieux, France). In addition, all strains identified as \emph{S. aureus} were analyzed for the presence of species-specific thermostable nuclease gene (\textit{nucSA}) as described by Baron et al.\textsuperscript{47}

The isolates were screened for their resistance to oxacillin on the basis of growth of blue colonies in the selective medium (Oxacillin Resistance Screening Agar Base; ORSAB, Oxoid, Poland). Suspected methicillin-resistant \emph{S. aureus} (MRSA) isolates were further examined for the presence of \emph{S. aureus} mecA gene, as described elsewhere.\textsuperscript{48} \emph{S. aureus} ATCC 25923 (methicillin-susceptible and \emph{S. aureus} ATCC 43300 (methicillin-resistant) strains were used as reference strains. MRSA represented 12 out of the 215 (5.6%) examined isolates.

The isolates were stored at –70°C in Tripticase Soy Broth (Oxoid, England) supplemented with 15% glycerol.

AMPs (aurein 1.2, CA(1–7)M(2–9), citropin 1.1, IB-367, pexiganan, temporin A, uperin 3.6; Table 3) were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) solid-phase method, and purified by high-pressure liquid chromatography (HPLC) on a Knauer K501 2-pump system with a Kromasil C8 column (4.6 mm × 150 mm). The peptides were analyzed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOP).\textsuperscript{49}

Minimal inhibitory concentrations (MICs) for tested peptides and conventional antibiotics (azithromycin, chloramphenicol, ciprofloxacin, clindamycin, cloxacillin, co-trimoxazole, fusidic acid, gentamicin, mupirocin, tetracycline, vancomycin; all provided by Sigma-Aldrich) were determined using a broth dilution method as recommended by Clinical Laboratory Standards Institute (CLSI) guidelines.\textsuperscript{50} Polypropylene 96-well plates with investigated compounds serially diluted in Mueller Hinton Broth 2 (Sigma-Aldrich) and initial inoculum 5 × 10^8 cfu/mL were incubated at 37°C for 18 h. MIC was taken as the lowest drug concentration at which an observable growth was inhibited. Minimal bactericidal concentration (MBC) was determined in a sample taken from each test tube in which no growth was observed in the MIC assay. The loopful (10 µl) of the tested sample was transferred to Triptic Soy Broth (TSB, BD Difco) and incubated at 37°C for 48 h. MBC was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction of initial inoculum. Solutions of drugs were made fresh on the day of the assay or sored at −80°C in the dark up to 2 weeks. All experiments were performed in triplicate.

Synergy studies included 50 selected strains from various patients, with previously established (different)

| Peptide   | Amino acid sequence                   |
|-----------|---------------------------------------|
| aurein 1.2| GLFDIILKKAESF-NH₂                    |
| CA(1–7)M(2–9)| KWIILFKKAGLYKL-NH₂            |
| citropin 1.1| GLFDVKKVAYSVGL-NH₂                |
| IB-367    | RGLYCRGGRGVCOVGR-NH₂               |
| pexiganan | GIGKFKKAKKFHKAFVKILKK-NH2          |
| temporin A | FPLGLGRLSGL-NH₂                   |
| uperin 3.6 | GVIDACKVNVKLKFL-NH₂                |

\textbf{Table 3.} Amino acid sequences of tested antimicrobial peptides.
antibiotic susceptibility profiles and with different spa-types (not shown). All these strains proved susceptible to the tested antibiotics. The tested dilutions amounted to 0.5–256 μg/mL for antimicrobial peptides and to 0.125–256 μg/mL for conventional antibiotics. Fractional inhibitory concentration (FIC) index for combinations of 2 antimicrobials was calculated according to the equation: 

\[
FIC_{index} = FIC_A + FIC_B = A/MIC_A + B/MIC_B,
\]

where A and B are the MICs of drug A and B in the combination, \(MIC_A\) and \(MIC_B\) are the MICs of drug A and drug B alone, and \(FIC_A\) and \(FIC_B\) are the FICs of drug A and drug B. The values of FIC index were interpreted as follows: ≤0.5 – synergy, >0.5 to 1.0 – addition, >1.0 to <4.0 – indifference, and ≥4.0 – antagonism.\(^{51}\)

### Abbreviations

- AMP: antimicrobial peptide
- ASL: airway surface liquid
- ATCC: American Type Culture Collection
- CF: cystic fibrosis
- CFTR: cystic fibrosis transmembrane conductance regulator
- CLSI: Clinical Laboratory Standards Institute
- Fmoc: 9-fluorenylmethoxycarbonyl
- FIC: fractional inhibitory concentration
- HPLC: high-pressure liquid chromatography
- MALDI-TOF: matrix-assisted laser desorption ionization
- MBC: minimal bactericidal concentration (MBC)
- MIC: minimal inhibitory concentration
- MRSA: methicillin-resistant S. aureus
- MSSA: methicillin-susceptible S. aureus
- nucSA: thermostable nuclease gene
- SCV: small-colony variant

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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