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ANTIMICROBIAL PEPTIDES AS POTENTIAL TOOL TO FIGHT BACTERIAL BIOFILM

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Abstract: Recently, the topic of biofilm has met a huge interest of researchers owing to a significant role played by this microbial life form in severe infections. These well organised three-dimensional microbial communities are characterized by a strong resistance to antimicrobials. Biofilms significantly contribute to morbidity and mortality as related infections are very difficult to treat due to their tendency to relapse after the withdrawal of antibiotics. According to the literature, antimicrobial peptides (AMPs) have a high potential as future antibiofilm agents. AMPs can influence various stages of biofilm formation and exhibit antimicrobial activity against a broad spectrum of microorganisms including multi-drug resistant strains. The purpose of the present study was to determine the activity of antimicrobial peptides against biofilms formed by a variety of bacterial strains. To do this, the following antimicrobial peptides were synthesized: Citropin 1.1, Lipopeptides Palm-KK-NH_2 and Palm-RR-NH_2, Omiganan, Pexiganan and Temporin A. Antimicrobial activity of the compounds and conventional antibiotics was determined for planktonic cells and biofilms formed by reference strains of Gram-positive (*Staphylococcus aureus*, *S. epidermidis, Streptococcus pneumoniae*, *Streptococcus pyogenes*) and Gram-negative (*Escherichia coli*, *Pseudomonas aeruginosa*, *Proteus mirabilis*) bacteria. AMPs exhibited a strong antibacterial activity against Gram-positive strains, while Gram-negative bacteria were less susceptible. Antimicrobial activity of the tested peptides against biofilms formed by Gram-positive organisms was significantly stronger as compared to that of conventional antimicrobials.

Keywords: antimicrobial peptides, lipopeptides, bacterial biofilm

Biofilm represents the basic living form of most microorganisms in natural environment and can be defined as a sessile microbial community growing on various surfaces. This highly specialized three dimensional structure is characterized by a strong resistance to antimicrobials. In extreme cases, susceptibility to antibiotic can be decreased by up to 1000 fold (1, 2). Biofilms of various species of bacteria and fungi can be formed on the surfaces of medical devices (central venous catheters, heart valves, urinary catheters, endotracheal tubes and intrauterine devices), this resulting in implant-related infections (3, 4). As one of the most common biomaterials, contact lenses constitute a suitable surface for microbial adhesion. Bacterial ocular infections are rather rare complications due to the usage of this biomaterial. However, their consequences might be severe. The ability of microbes to form biofilm on contact lenses plays a significant role in pathogenesis of several infections, such as bacterial conjunctivitis, microbial keratitis, contact lens acute red eye (CLARE) and contact lens peripheral ulcer. The etiological factors for bacterial ocular infections are: *Staphylococcus aureus, Streptococcus pneumoniae, Haemophilus influenzae, Staphylococcus epidermidis, Enterococcus spp., Moraxella spp., Escherichia coli, Streptococcus pyogenes, Serratia marcescens, Pseudomonas aeruginosa, Proteus mirabilis* and *Neisseria gonorrhoeae* (5-7). The standard treatment for bacterial eye infections is topical antibiotic therapy. Application of a broad spectrum antibiotics causes that the effectiveness of conventional antimicrobials becomes gradually suppressed. Antimicrobial peptides (AMPs) exhibit excellent activity against a number of pathogens responsible for ocular infections. AMPs are essential part of innate human immunity and their activity against broad-spectrum bacteria is well known. Owing to their unique mechanism and fast killing kinetics, the risk of the development of microbial resistance is

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significantly lower as compared to that of conventional antibiotics. There are several literature reports on successful use of AMPs in prevention of formation of biofilms as well as in elimination of the structures formed.

In vitro tests demonstrated activity of human cathelicidin LL-37 and peptide STAMP G10KHc against biofilms formed by *Pseudomonas aeruginosa*. LL-37 also disrupted the development of biofilms formed by *Staphylococcus epidermidis* and *Pseudomonas aeruginosa* (8, 9). Dendrimeric peptides inhibited the biofilm formation and killed pregrown biofilms of *E. coli*. Short cationic lipopeptides demonstrated strong antibiofilm activity against clinical strains of *Staphylococcus aureus* (10).

Also numerous *in vivo* models have been developed to confirm the potential of AMPs as future antibiofilm agents. A chimeric peptide – DD13-RIP (composed of a dermaseptin derivative and an RNA III-inhibiting peptide) proved its ability to prevent staphylococcal infection in a rat graft infection model with methicillin-resistant *S. aureus* (MRSA) or *S. epidermidis* (MRSE) (11). The efficacy of Tachyplesin III was successfully applied in a rat model of *P. aeruginosa* urethral stent infection to prevent the biofilm formation (12). The efficacy of the treatment of central venous catheter infection caused by *S. aureus* and *E. faecalis* with linezolid was improved when the catheters were pretreated with IB-367 (13). Application of contact lenses with melamine allowed to reduce the CLARE in the *P. aeruginosa* guinea pig model (14).

AMPs can act at various stages of biofilm formation through different mechanisms of action. Therefore, the compounds have the potential as novel antimicrobials to prevent the formation of biofilm as well as to eradicate the preformed structures from the surface of contact lenses.

The aim of the present study was to evaluate a group of synthesized AMPs as potential antibiofilm agents against structures formed by some strains associated with ocular infections.

MATERIALS AND METHODS

Antimicrobial peptides

All tested peptides (Citropin 1.1, Lipopeptides Palm-KK-NH2 and Pam-RR-NH_2, Omiganan, Pexiganan and Temporin A) were synthesized manually by Fmoc chemistry on polystyrene resin modified by Rink Amide linker (15). Deprotection of the Fmoc group was carried out in 20 min using a 20% piperidine in dimethylformamide (DMF). Then, the resin was washed with DMF and DCM (dichloromethane) and a chloranil test was accomplished. All amino acids were coupled using the mixture of DMF/DCM (1 : 1, v/v) in the presence of coupling agents such as 1-hydroxybenzotriazole (HOBt) and diisopropylcarbodiimide (DIC). The degree of acylation was monitored by chloranil test. The peptides were cleaved from the resin with a mixture consisting of trifluoroacetic acid (TFA), water, triisopropylsilane (TIS) and phenol (92.5 : 2.5 : 2.5 : 2.5, v/v/v/v) as scavengers. In the next step, the peptide compounds were precipitated with cold diethyl ether and lyophilized. All crude products were purified by reversed-phase high performance liquid chromatography (RP-HPLC) in a gradient of acetonitrile – water containing 0.1% TFA. Identity of the peptides was confirmed by matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry (16).

Antimicrobial activity

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for several contact-lens involved bacterial pathogens: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* PCM 2118, *Streptococcus pneumoniae* ATCC 49619, *Streptococcus pyogenes* PCM 465, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 9027, *Proteus mirabilis* PCM 543. MIC assays for the peptides and antibiotics (bacitracin, ciprofloxacin, gentamicin, erythromycin, neomycin and polymyxin B) were performed by the broth dilution method with Mueller Hinton II broth according to the procedures recommended by CLSI (Clinical and Laboratory Standards Institute). Polypropylene 96-well plates with bacteria at initial inoculums of 5×10^5 CFU/mL exposed to tested compounds were incubated for 18 h at 37°C. MIC was taken as the lowest drug concentration at which a visible growth of microbes was inhibited. MBC was taken as the lowest concentration of compound that allowed for 99.9% reduction of the initial inoculums. Biofilms cultured on polystyrene plates for 1, 2 and 3 days were exposed to graded concentrations of peptides and antibiotics. After a 24 h incubation, resazurin was added as a cell-viability reagent and the minimum biofilm eradication concentration (MBEC) was read. The experiments were performed in triplicate.

RESULTS

Activity against planktonic cells

The tested compounds have shown a diverse activity in the MIC/MBC assay performed for refer-

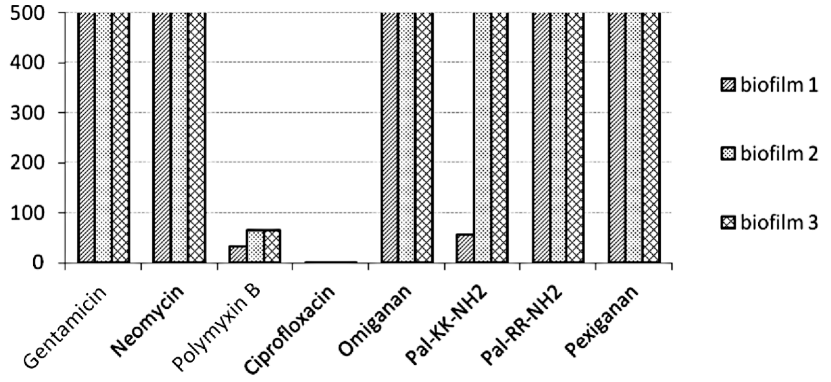

Figure 1. Activity of selected antimicrobial peptides and conventional antibiotics against biofilms formed by *Escherichia coli* ATCC 25922 on polystyrene surface for 1, 2 and 3 days

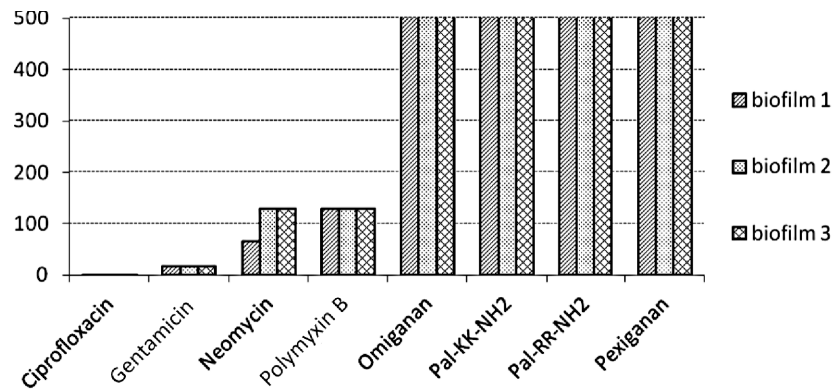

Figure 2. Activity of selected antimicrobial peptides and conventional antibiotics against biofilms formed by *Pseudomonas aeruginosa* ATCC 9027 on polystyrene surface for 1, 2 and 3 days

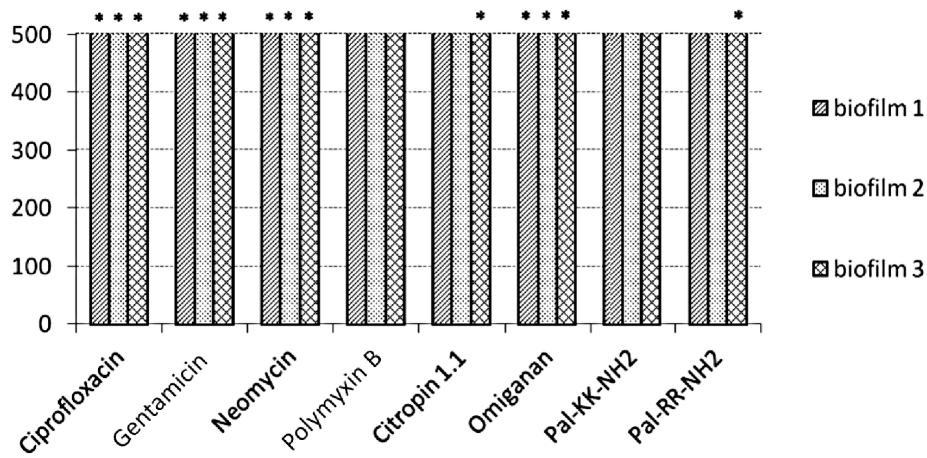

Figure 3. Activity of selected antimicrobial peptides and conventional antibiotics against biofilms formed by *Proteus mirabilis* PCM 543 on polystyrene surface for 1, 2 and 3 days (* - antimicrobial activity not observed at tested concentrations 1-512 mg/L)

Table 1. Activity of peptides and conventional antimicrobials against planktonic cells of tested bacterial strains (bacteriostatic).

MIC/ MBC	*Escherichia coli*	*Proteus mirabilis*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Staphylococcus epidermidis*	*Streptococcus pneumoniae*	*Streptococcus pyogenes*
Bacitracin	512 / >512	>512 / >512	>512/>512	128 / 128	32 / 64	128 / 512	128 / 256
Ciprofloxacin	0.25 / 0.25	0.25 / 0.25	0.25 / 0.25	0.5 / 0.5	0.25 / 0.5	0.25 / 0.5	0.5 / 1
Erythromycin	64/ bs.	256 / bs.	128 / bs.	0.25 / bs.	2 / 4	0.5 / 1	4 / 16
Gentamicin	2/2	1 / 2	2 / 2	1 / 2	0.25 / 0.25	2 / 4	0.5 / 0.5
Neomycin	4/4	2 / 8	4 / 8	2 / 4	0.25 / 0.5	8 / 16	1 / 2
Polymyxin B	0.25 / 0.25	0.25 / 0.25	0.25 / 0.25	0.5 / 0.5	0.25 / 0.5	0.25 / 0.5	0.5 / 1
Citropin 1.1	32 / 64	256 / 512	128 / 256	16 / 16	8 / 8	16 / 16	16 / 32
Omiganan	16 / 64	256 / 512	32 / 128	4 / 8	1 / 2	8 / 16	8 / 16
Pal-KK-NH_2	8 / 16	256 / 512	64 / 64	8 / 16	1 / 1	8 / 16	8 / 16
Pal-RR-NH_2	16 / 32	256 / 512	64 / 256	8 / 16	1 / 1	8 / 8	4 / 8
Pexiganan	8 / 8	256 / 512	4 / 4	4 / 8	0.5 / 0.5	8 / 8	8 / 16
Temporin A	256 / >512	>512 / >512	512 / >512	8 / 8	4 / 8	4 / 8	4 / 8

ence strains of bacteria (Tab. 1). Apart from bacitracin which turned out to be the least active, conventional antimicrobials demonstrated a relatively stronger activity as compared to that of AMPs. The growth of *Escherichia coli* was inhibited by ciprofloxacin and polymyxin B at the a concentration of 0.25 mg/L whereas the most active peptides were effective at 8 mg/L. Even still larger discrepancies were observed for the remaining Gram-negative strains. These bacteria were susceptible to ciprofloxacin, gentamicin, neomycin and polymyxin B, while AMPs, bacitracin and erythromycin displayed rather a weak antibacterial activity. The tested peptides have shown a markedly stronger activity against Gram-positive bacteria. The most susceptible strain was *Staphylococcus epidermidis* (MIC value of lipopeptides, pexiganan and omiganan were 0.5-1 mg/L). Erythromycin and bacitracin displayed a stronger activity towards Gram-positive strains as well. The remaining conventional antibiotics showed approximately equal antimicrobial activity against all tested bacteria. In general, the tested compounds presented bactericidal mode of action as their MBCs were equal or twice higher in comparison with their MICs. Only erythromycin showed bacteriostatic action against *S. aureus, P. aeruginosa* and *P. mirabilis*.

Activity against biofilm

The tested antimicrobials exhibited a very diverse activity against biofilms depending on the tested strain and the maturity of structure. For instance, *E. coli* formed biofilm susceptible to ciprofloxacin applied at a concentration of 1 mg/L and to polymyxin B at concentrations of 32-64 mg/L. Gentamicin, neomycin and antimicrobial peptides eliminated preformed biofilms at the highest concentration applied (Fig. 1). Biofilms formed by *P. aeruginosa* were susceptible to ciprofloxacin, and gentamicin (MBEC obtained for 3 days old structures = 4 and 16 mg/L respectively). A lower activity was exhibited by neomycin and polymyxin B, while AMPs turned out to be the weakest antibiofilm agents (MBEC = 512 mg/L) (Fig. 2). *P. mirabilis* formed biofilms resistant to almost all the tested compounds. Only polymyxin B and lipopeptide Palm-KK-NH_2 were active at the concentration of 512 mg/L. Citropin 1.1 was effective at the same concentration against biofilm grown for 1 and 2 days, while a 3-day structure was unsusceptible. The remaining compounds did not show antibiofilm activity up to a concentration of 512 mg/L (Fig. 3).

Antimicrobial peptides were very effective against biofilm formed by Gram-positive bacteria. *Staphylococcus aureus* formed biofilms resistant to the conventional antibiotics, while AMPs acted on all the formed structures at concentrations of 32-64 mg/L (Fig. 4). Also *S. epidermidis* biofilms were much more senstitive to AMPs in comparison to conventional compounds. A remarkable dependence of the maturity of the structure on the activity of the antibiotics was observed. Namely, biofilms grown for 1 day were sensitive to all compounds, while after 3 days bacteria were resistant to gentamicin, neomycin and ciprofloxacin (Fig. 5). All the living forms of *Streptococcus pyogenes* were very susceptible to ciprofloxacin, while neomycin and antimicrobial peptides were active at higher concentrations. Again, erythromycin and gentamicin turned out to be ineffective at the tested concentrations (Fig. 6). Similar results were obtained for *S. pneumoniae*. However, the activity of ciprofloxacin against biofilm after 3 days was significantly lower (Fig. 7).

DISCUSSION AND CONCLUSION

Extended wear of contact lenses significantly contributes to the development of corneal infections (17). Several side effects related to wearing of con-

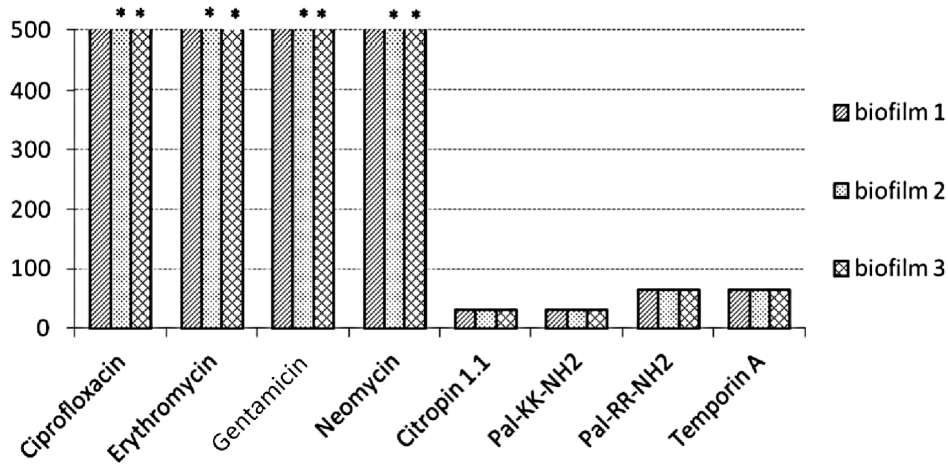

Figure 4. Activity of selected antimicrobial peptides and conventional antibiotics against biofilms formed by *Staphylococcus aureus* ATCC 25923 on polystyrene surface for 1, 2 and 3 days (* - antimicrobial activity not observed at tested concentrations 1-512 mg/L)

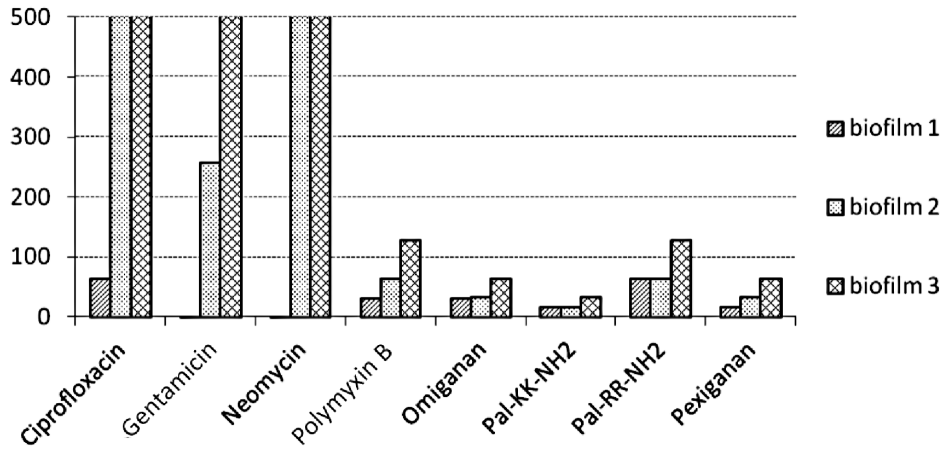

Figure 5. Activity of selected antimicrobial peptides and conventional antibiotics against biofilms formed by *Staphylococcus epidermidis* PCM 2118 on polystyrene surface for 1, 2 and 3 days

tact lenses occur due to the bacterial adhesion to their surface. In order to reduce the bacterially driven adverse responses, novel materials and contact lens liquids that contain safe antimicrobials should be considered.

The eye possesses an array of native defense components protecting the ocular structures against microbial infections. The tear film is one of the crucial elements of this protection. It contains antimicrobials and is responsible for the purification, nutrition and moisturizing of the eye (18). The tear film contains a plenty of proteins and peptides which include lysozyme, mucins and endogenous antimicrobial peptides. These molecules together with complement compounds constitute the main elements of the antimicrobial defense of human tears (19). Defensins seem to be interesting candidates as therapeutic agents for ocular infections. These compounds belong to AMPs found in human eye and display antimicrobial activity against a broad spectrum of microorganisms (20). Owing to the mechanism based on altering of the permeability properties of the microbial cell plasma membrane (21), the risk to develop bacterial resistance to AMPs is relatively low. This hypothesis was confirmed by several *in vitro* studies. The resistance of *Pseudomonas aeruginosa* to peptides was increased only by two to fourfold after 30 passages below their MIC (22), while

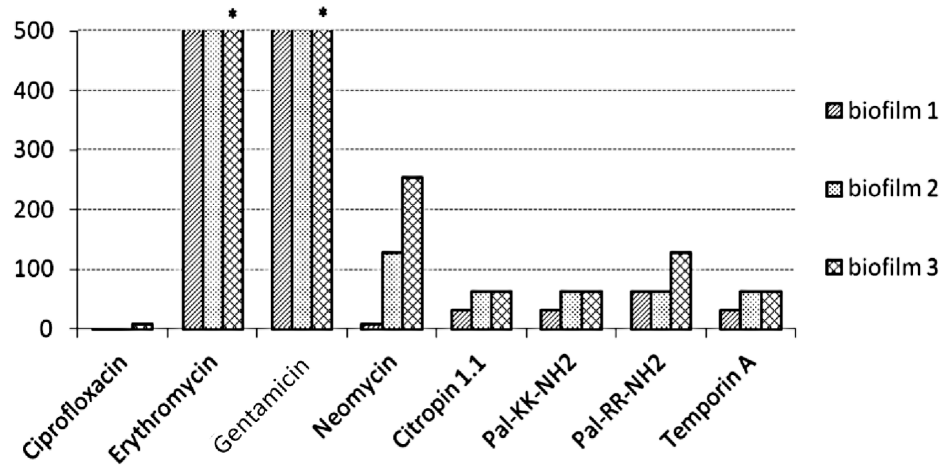

Figure 6. Activity of selected antimicrobial peptides and conventional antibiotics against biofilms formed by *Streptococcus pyogenes* PCM 465 on polystyrene surface for 1, 2 and 3 days (* - antimicrobial activity not observed at tested concentrations 1-512 mg/L)

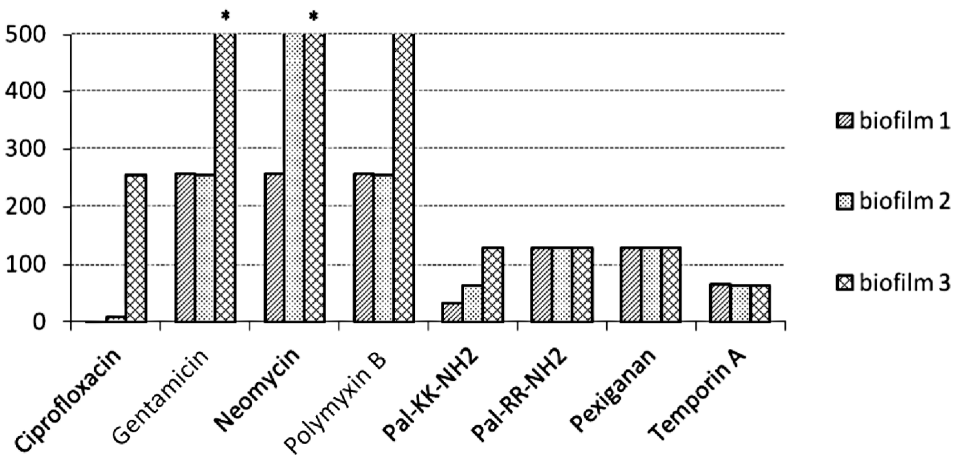

Figure 7. Activity of selected antimicrobial peptides and conventional antibiotics against biofilms formed by *Streptococcus pneumoniae* ATCC 49619 on polystyrene surface for 1, 2 and 3 days (* - antimicrobial activity not observed at tested concentrations 1-512 mg/L)

the application of lipopeptide Laur-KK-NH_2 proved to reduce the ability of strains of *Enterococcus faec*alis to daptomycin (23).

The above mentioned phenomena together with the activity of AMPs against multi-drug resistant microorganisms and their fast killing kinetics (24, 25) in comparison to the majority of the conventional antibiotics represent suitable features for novel antimicrobials. It is worth noting that antimicrobial peptides also demonstrate strong antimicrobial activity against biofilm structure. Microbial cells in such communities are very difficult to eliminate with conventional antibiotics, while the same cells are sensitive in their planktonic state (1). Bacteria and fungi colonizing the surface of contact lenses are very likely to grow in this living form.

Although antimicrobial peptides clearly represent a great potential for the management of ocular infections, their practical usage remains at a very early stage of development (26). Ophthalmic pharmaceutical companies have not focused on AMPs as yet, but several *in vitro* and *in vivo* studies have been performed worldwide by scientific groups.

In vitro activity of AMPs against numerous pathogens that could be linked with ocular infections, has been confirmed. Human β-defensins HB43, HB55 and HBPM4 demonstrated activity towards meticillin-resistant *S. aureus* (MRSA), while strains *P. aeruginosa* strain was susceptible to HBD-1, HBD-2 HBD-3, HBCM2, HBCM3 and HB14 (27). Human β-defensin 3 was also an effective agent against *S. aureus* in the biofilm form (28). Defensins isolated from rabbits (NP-1, NP-5) showed an *in vitro* activity against pathogens isolated from human and horses suffering ocular infections (29).

LL-37, a human cathelicidin was also found to be expressed in human corneal and conjunctival epithelia (30). The compound disrupted the formation of biofilm as well as eradicated the pre-grown biofilms of *Pseudomonas aeruginosa*. LL-37 also inhibited the attachment and development of biofilms of *Staphylococcus epidermidis* (31).

Lactoferrin (LF), a human antimicrobial protein present in saliva, milk and tears prevented the formation of biofilm by *Streptococcus gordonii* and *S. mutans* in oral cavity (32, 33). LF also showed a synergic action with lysozyme and vancomycin against *S. epidermidis* biofilms (34). Another promising candidate to fight biofilm is lactoferricin B – a product of enzymatic digestion of LF. Application of the compound in combination with conventional antifungals allowed to reduce their dosage significantly. The combinations were active towards common keratitis-associated fungal pathogens (*Aspergillus fumigatus*, *Fusarium solani*, *Candida albicans*) in both living forms: planktonic cells and biofilms (35).

A number of sequences isolated from other organisms and their analogues have also been tested. Mannis et al. have tested peptides CCI A, B, C and COL-1 on human ocular isolates and in a rabbit model of *Pseudomonas keratitis*. The compounds were effective *in vitro* but not *in vivo* (26). Derivatives of cecropin demonstrated activity against strains isolated from patients suffering ocular infections (36). Cecropin-mellitin hybrid peptides were effective in the management of *P. aeruginosa* ocular infection developed in rabbits (37), while contact lenses with another hybrid peptide – melamine (mellitin-protamine), reduced the CLARE in the *P. aeruginosa* guinea pig model (14). In contrast to mellitin, the hybrids proved non toxic to the eukaryotic cells. We have synthesized an analogue of indolicidin-peptide primarily isolated from borvine neutrofils – omiganan. The compound eradicated staphylococcal biofilm at a considerably low concentration and exhibited some activity against structures formed by *Escherichia coli*.

An amphibian peptide citropin 1.1 demonstrated synergic action with rifampin and minocycline against a *S. aureus* biofilm (38). In our study, the compound alone was very effective against biofim formed by *S. aureus* and *S. pyogenes*. The determined MBEC were significantly lower in comparison to those obtained for conventional antimicrobials. Moreover, its activity did not depend on the time of biofilm cultivation. Another amphibian peptide temporin A was also active against biofilm formed by Gram-positive strains. The tested amphibian peptides demonstrated a minor antibacterial activity against Gram-negative strains. Similar results were obtained for pexiganan - an analogue of another amphibian peptide – magainin II. A peptide obtained from hemocytes of the horseshoe crab, tachyplesin III prevented the formation of *Pseudomonas aeruginosa* biofilm in a rat model of ureteral stent infection (12). Another study reported iseganan as a potential adjunctive agent to linezolid in the treatment of central venous catheter infection (13).

In our study, Palm-KK-NH_2 and Palm-RR-NH_2 proved to be potent agents towards Gram-positive biofilm displaying some activity towards both living forms of Gram-negative strains, while in the previous work both compounds were the most active lipopeptides tested against staphylococcal biofilms produced by dermatological isolates of *S. aureus* (10).

Based on the literature data and the obtained results we can expect that AMPs could be applied for the management as well as prevention of ocular infections. However, before the application of the peptides in ophthalmology several issues need to be considered, such as their toxicity, immunogenicity, stability and route of administration.

In general, AMPs have shown a considerably stronger antimicrobial activity against biofilm formed by Gram-positive bacteria than conventional antibiotics. A significantly lower activity of AMPs was observed in the case of Gram-negative strains. As the Gram-positive organisms are the most common isolates among ocular infections (39), we can assume that antimicrobial peptides possess a potential for development as therapeutic antimicrobials for the ocular infections. Moreover, immobilization of AMP onto the surface of biomaterials such as contact lenses might result in reduction of biofilm formation.

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