Polish Journal of Microbiology
2014, Vol. 63, No 4, 423–431

ORIGINAL PAPER

Synthetic Amphibian Peptides and Short Amino-Acids Derivatives Against Planktonic Cells and Mature Biofilm of *Providencia stuartii* Clinical Strains

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Submitted 13 March 2014, revised 30 July 2014, accepted 15 September 2014

Abstract

Over the last decade, the growing number of multidrug resistant strains limits the use of many of the currently available chemotherapeutic agents. Furthermore, bacterial biofilm, due to its complex structure, constitutes an effective barrier to conventional antibiotics. The *in vitro* activities of naturally occurring peptide (Citropin 1.1), chemically engineered analogue (Pexiganan), newly-designed, short amino-acid derivatives (Pal-KK-NH_2, Pal-KKK-NH_2, Pal-RRR-NH_2) and six clinically used antimicrobial agents (Gatifloxacin, Ampicilin, Cefotaxime, Ceftriaxone, Cefuroxime and Cefalexin) were investigated against planktonic cells and mature biofilm of multidrug-resistant *Providencia stuartii* strains, isolated from urological catheters. The MICs, MBCs values were determined by broth microdilution technique. Inhibition of biofilm formation by antimicrobial agents as well as biofilm susceptibility assay were tested using a surrogate model based on the Crystal Violet method. The antimicrobial activity of amino-acids derivatives and synthetic peptides was compared to that of clinically used antibiotics. For planktonic cells, MICs of peptides and antibiotics ranged between 1 and 256 μg/ml and 256 and ≥ 2048 μg/ml, respectively. The MBCs values of Pexiganan, Citropin 1.1 and amino-acids derivatives were between 16 and 256 μg/ml, 64 and 256 μg/ml and 16 and 512 μg/ml, respectively. For clinically used antibiotics the MBCs values were above 2048 μg/ml. All of the tested peptides and amino-acids derivatives, showed inhibitory activity against *P. stuartii* biofilm formation, in relation to their concentrations. Pexiganan and Citropin 1.1 in concentration range 32 and 256 μg/ml caused both strong and complete suppression of biofilm formation. None of the antibiotics caused complete inhibition of biofilm formation process. The biofilm susceptibility assay verified the extremely poor antibiofilm activity of conventional antibiotics compared to synthetic peptides. The obtained results showed that synthetic peptides are generally more potent and effective than clinically used antibiotics.

Key words: antimicrobial activity, antimicrobial peptides, bacterial biofilm, urinary tract infection

Introduction

Microbial biofilms are communities of microbes that adhere to various surfaces and are engaged in a self-produced extracellular matrix. Bacterial cells being a part of biofilm are physiologically distinct from planktonic cells and what is important, are embedded within a self-produced matrix of an extracellular polymeric substance, which can increase resistance to antibiotic, drugs and chemiotherapeutics even up to 1000 times (Park *et al*., 2011). It is estimated that over 60% of bacterial infections are associated with the phenomenon of biofilm formation (Giacometti *et al.*, 2005; Hall-Stoodley and Stoodley, 2009).

This extremely unfavorable trend is particularly evident in the case of urinary tract infections (UTIs) which are the most common diagnosable chronic recurrent caused by bacterial microorganisms with an estimated annual worldwide incidence of nearly 250 million cases (Chapman and Rowland, 2014). The species composition of urinary pathogens is constantly changing, and the number of microorganisms recognized as etiological agents of UTI is steadily increasing. The use of analytical techniques such as molecular analysis of restriction fragment length polymorphism (T-RFLP), electrophoresis in denaturant gradient (DGGE), capillary electrophoresis or DNA sequencing in real time (pyrosequencing) allows a more complete description of the composition

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of pathogenic species in the urinary tract. The study of biofilms from internal urinary catheters showed the unusually frequent presence of *P. stuartii* (Ostrowska *et al.*, 2013). These organisms are opportunistic pathogens responsible for nosocomial UTI, representing nearly 50% of all bacterial nosocomial infections and in over 30% of cases, being the cause of so-called urosepsis, leading to septic shock (Kupilas, 2010).

Antibiotic therapy is still the most common treatment for UTIs. Fluoroquinolones, nitrofurans, beta-lactams, aminoglycosides, trimethoprim and sulfonamides are used predominatingly (Ostrowska *et al.*, 2013). However, biofilm, due to its complex structure, constitutes an effective barrier to the antibiotics used in the treatment of urinary tract infections. In addition, the growing number of multidrug resistant strains, limits the use of many of the currently available chemotherapeutic agents. The high resistance of *P. stuartii* clinical isolates to conventional antimicrobial agents has led us to seek alternative groups of antimicrobial compounds.

Antimicrobial peptides (AMPs) are unique molecules and have been identified in all living organisms (Dean *et al.*, 2011; Żyłowska *et al.*, 2011). They are considered to be endogenous antibiotics. The sequences and structures of antimicrobial peptides are incredibly diverse, but they share some common properties, like: amphiphatic secondary structures, small size, a net positive charge at neutral pH, rapid binding to biological membranes, and what's extremely important, the ability to kill invading microorganisms within minutes (Mangoni *et al.*, 2008; Pinheiro and Machado, 2012; Meng *et al.*, 2011). It is estimated that the major targets of AMPs are intracellular molecules and cytoplasmic membrane of microorganisms (Park *et al.*, 2011). The cationic character of AMPs allows for extremely strong interaction with negatively charged phospholipids of microbial membranes whilst the hydrophobic groups facilitate the penetration into the lipid phase of the microbial cell membrane, leading to membrane disruption (Cederlund *et al.*, 2011; Baltzer and Brown, 2012). Natural antimicrobial properties of AMPs, their low propensity for the development of bacterial resistance, low toxicity, pace of action and unique mechanism of interaction with outer and inner bacterial membrane, make them a perfect alternative to conventional drugs in combating and preventing the formation of multidrug-resistant microorganisms (Baltzer and Brown, 2011; Cederlund *et al.*, 2011; Park *et al.*, 2011). Cationic peptides display antimicrobial activity against a wide range of bacteria, enveloped viruses, parasites and fungi (Baltzer and Brown, 2011; Ding *et al.*, 2014). The results presented in this paper constitute the specific comparison of antimicrobial activity presented by peptides and conventional antibiotics, against both planktonic cells and mature biofilm of *P. stuartii* clinical strains.

Experimental

Materials and Methods

Antimicrobial agents. *Antimicrobial Peptides:* Compounds included in the study (Table I) were synthesized manually by the solid-phase method using the 9-fluorenylmethoxycarbonyl chemistry (Fmoc). The completeness of each coupling reaction was monitored by the chloranil test. The peptides were cleaved from the solid support by trifluoroacetic acid in the presence of water and triisopropylsilane. The cleaved peptides were precipitated with diethyl ether and were purified by high-performance liquid chromatography (HPLC) on a Knauer K501 two-pump system with Kromasil C8 column 10 mm × 250 mm (5 µm particle diameter, 100 Å pore size) with a flow rate of 5 ml/min and gradient 20–50% A/90 min, absorbance at 226 nm. The resulting fractions of purity greater than 95–98% were tested by HPLC (Kromasil C8 column, 4.6 nm × 150 nm). Compounds were analyzed also by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Table I
Amino acid sequences of obtained antimicrobial peptides and amino-acids derivatives (Pal – palmitic acid residue)

Peptide	Amino acid sequence
Citropin 1.1	GLFDVIKKVASVIGGL-NH_2
Pexiganan	GIGKFLKKAKKFGKAFVKILKK-NH_2
Derivative 1	Pal-KK-NH_2
Derivative 2	Pal-KKK-NH_2
Derivative 3	Pal-RRR-NH_2

Amino-acids derivatives were designed to imitate the properties of AMPs. The amphipathicity was achieved by combining fatty acid with the short peptide chain. The positive net charge of compounds results from the presence of basic amino acid residues.

Stock solutions (1 mg/ml) from dry powders were prepared fresh in physiological solution on the day of experiment and the concentration range assayed was between 1 and 512 µg/ml.

Antibiotics. The following antibiotics were evaluated: amikacin, cefotaxime, cephalexin (all from Sigma-Aldrich, St. Louis, MO), cefepime and gatifloxacin (Bristol-Myers, Squibb Company, US). The Laboratory standard powders were diluted in accordance with manufacturers recommendations yielding 1 mg/ml solution. Stock solutions were prepared fresh in physiological solution on the day of experiment or stored at –80°C until the time of used, but no longer than one week. The concentration range assayed was between 1 and 2048 µg/ml.

Table II
Data of the patients from which *P. stuartii* strains were isolated

P. stuartii strain	Catheterization time [days]	Gender	Age	Microbiological examination/ UTI	Affection/ treatment
C3	21	M	79	No/No	Prostatic hypertrophy/ rinsing with 3% boric acid
C7	21	M	77	No/No	Prostatic hypertrophy/Oflodinex and rinsing with 3% boric acid
C9	14	M	73	Yes/Yes	Prostatic hypertrophy/Cipronex, Nevigramon and rinsing with 3% boric acid
C11	14	M	89	No/No	Prostatic hypertrophy/ rinsing with 3% boric acid
C12	17	M	73	No/No	Prostatic hypertrophy/ rinsing with 3% boric acid
C13	14	M	85	No/No	Prostatic hypertrophy/ rinsing with 3% boric acid
C15	14	M	82	No/No	Prostatic hypertrophy/ rinsing with 3% boric acid
C18	60	M	91	No/No	Prostatic hypertrophy/Oflodinex and rinsing with 3% boric acid
C19	21	M	80	No/No	Stroke
C24	7	M	41	No/No	Multiple sclerosis
C28	12	M	85	No/No	Spinal cord paralysis, ischemic heart disease and hypertension
C29	5	M	83	No/Yes	Prostatic hypertrophy, peptic ulcer disease/Cipronex
C31	14	M	57	No/Yes	Spinal cord injury and ventral urethral fistula paralysis/Nevigramon
C33	21	M	88	No/No	Prostatic hypertrophy, stroke, heart failure
C37	14	M	79	No/No	Prostatic hypertrophy, stroke
C44	14	M	80	No/No	Stroke
C53	10	M	87	No/No	Prostatic hypertrophy
C55	14	M	86	No/No	Prostatic hypertrophy
C56	17	M	80	No/No	Urinary retention
C85	21	M	85	No/No	Prostatic hypertrophy/ rinsing with 3% boric acid

Bacterial strains A collection of 20 clinical strains of *P. stuartii,* were isolated from urological catheters derived from long-catheterized patients, from medical facilities in Lodz (Table II). Additionally as a quality control strain *Escherichia coli* ATCC25922 was used in the study.

MIC and MBC determination MICs of synthetic antimicrobial peptides, amino-acids derivatives and antibiotics were determined with the broth microdilution technique as described by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2013). The MIC was defined as the lowest concentration of antimicrobial agent that produced complete inhibition of visible growth. The MBCs were determined at the end of the incubation process period by removing three 10-µl samples from each well in which there was no visible growth of microorganisms and plating the aliquots onto TSA plate. Bacterial colonies were counted after 24 hours incubation at 37°C. The MBC was defined as the lowest concentration of antimicrobial compound that produces 99.9% killing of the initial inoculum (Mataraci and Dosler, 2014). Concentration range of peptides used in the study was between 1 and 512 µg/ml, and from 1 and 2048 µg/ml in the case of conventional antibiotics. Experiments were performed in triplicate.

Inhibition of biofilm formation. The Wakimoto method was adapted (Wakimoto *et al.*, 2004; Chapman *et al.*, 2014). The amount of 100 µl Müeller-Hinton (MH) medium was added to each well of a 96-well, type F plate. Exponential growth phase bacteria culture (5 µl of 1×10^5 CFU/ml) was applied into each well of the plate, along with 100 µl of antibiotic or antimicrobial peptide in appropriate concentration and then incubated for 18 h at 37°C. After the incubation period, the medium was gently removed, and each well of the plates were washed four times with 150 µl of sterile dH_2O to remove unattached bacterial cells. Then 150 µl of 0.5% Crystal Violet (CV) was added to each well, incubated for 5 min. and discarded, next each well was rinsed gently with 200 µl of dH_2O and air dry. Biofilm was quantified at 595 nm. The positive control were microorganisms in MH medium without antibiotic or peptide. The Crystal Violet assay is a widely used as a screening mechanism for biofilm formation process. The inhibition of biofilm formation is possible by observing a reduction of biofilm biomass when incubated with antimicrobial agents such as antibiotics or peptides.

Testing susceptibility of *P. stuartii* biofilm at antimicrobial compounds. Bacterial strains was cultured in 5 ml of tryptic soy broth (TSB) with rotation (150 rpm), at 37°C for 24 h, and diluted in fresh MH medium to a final density of 1×10^7 CFU /ml. 100 µl of this suspension was added to each well of a 96-well polystyrene, type F microtiter plate and incubated for 24 h in 37°C.

After the incubation period, the medium was gently removed, and each well in the plates was washed four times with 150 µl of sterile dH_2O to remove unattached bacterial cells. In the next step 100 µl of a proper concentration of antimicrobial compounds was added per well and incubated for 24 h at 37°C. The concentration range of antimicrobial peptides were between 1 and 1024 µl/ml, and from 1 to 2048 µl/ml in the case of conventional antibiotics. After incubation period waste medium with antimicrobial agent were aspired and the wells of the plates were rinsed four times with 150 µl of sterile 0.85% saline solution. The wells were stained with 100 µl of 1% CV (in water) for 5 min. Excess dye was removed and each well was rinsed gently five times with 200 µl of sterile distilled water, and air dry. Next the stain was resolubilized in 100 µl of 99% ethanol with shaking for 30 sec and then the optical density was measured at 595 nm (Mataraci and Dosler, 2012). Untreated biofilms were used as positive controls were. Negative controls were only 100 µl of MH broth, with no bacteria added. The intensity of the staining corresponding to the degree of biofilm formation. It was assumed that the absorbance value was directly proportional to the number of bacterial cells adhering to the surface of the plate (Chapman *et al*., 2014). Additionally staining with (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) was performed (data not shown). Experiments were performed in triplicate.

Statistical analysis and evaluation. All experiments were analyzed in three independent assays. For the susceptibility testing of *P. stuartii* biofilm to antimicrobial compounds the results are shown as means ± standard deviations of three independent experiments. A one-way analysis of variance with the Bonferroni multiple comparison test was used to compare the differences between antimicrobial-treated biofilms and untreated control. As a statistically significant a P value of < 0.001 was considered.

We define two levels of peptides suppression of biofilm formation and biomass eradication ability. A strong suppression/eradication was obtained if a peptide at a certain concentration, after adjusting for the negative control, caused ≥ 75% reduction of CV compared with the positive control. A complete suppression/eradication was obtained if a peptide at a certain concentration caused a reduction of CV ≤ negative control value (Chapman *et al*., 2014).

Results

Despite the fact that antibiotics still remain a major line of treatment for bacterial infections we have shown that antimicrobial peptides are much more effective and show a stronger bactericidal and bacteriostatic effect than standard antibiotics.

The antibiotics commonly used in the therapy of urinary tract infection: amoxicillin + clavulanic acid, cefazolin, cephalexin, cefuroxime, cefaclor, ceftriaxone, cefotaxime, cefepime, gentamicin, amikacin, ciprofloxacin, ofloxacin, norfloxacin, cotrimoxazole, doxycycline and gatifloxacin, were tested against clinical strains of *P. stuartii* (data not shown). The most promising ones: gatifloxacin, cefepime, amikacin, ceftriaxone, cephalexin and cefuroxime, were selected for further study.

Susceptibility. The *in vitro* activities of the studied antimicrobial peptides, amino-acids derivatives and clinically used antibiotics against *P. stuartii* planktonic cells are summarized in Tables III and IV. The MIC values of the antibiotics against the quality control strain *Escherichia coli* ATCC25922 were within the accuracy range described by CLSI throughout the study (CLSI, 2013). We found that all of the planktonic *P. stuartii* cells (Tab. II) were resistant to all of the examined antibiotics, with MIC values ranging between 256 and ≥ 2048 µg/ml (Tab. III).

Although we noted a major difference between both inhibitory and bactericidal concentration of the antibiotics compared to antimicrobial peptides the MICs of the peptides were in general much lower than the MICs of the antibiotics. The most effective peptides were the synthetic analogue of magainin II Pexiganan and small, highly hydrophobic Citropin 1.1, both with MIC valueings ranged between 1 and 256 µg/ml (Tab. IV). Differences in the activity of antimicrobial peptides and conventional antibiotics were also observed by comparing bactericidal concentration values. The MBC values of antimicrobial peptides were generally equal or two times greater than the MIC values. The MBC values for the most active peptides Pexiganan and Citropin 1.1 were ≤ 256 µg/m. Tangible results were also obtained for amino-acids derivatives where for Pal-KKK-NH_2 and Pal-RRR-NH_2 for 80% of tested strains MBC values were equal to 256 µg/ml, while in the case of conventionally used antibiotic MBC values were greater than the highest used concentration (> 2048 µg/ml).

Inhibition of biofilm formation. All of the tested synthetic antimicrobial peptides and amino-acids derivatives, showed inhibitory activity against *P. stuartii* biofilm formation, in relation to their concentrations. The most active were synthetic peptides. With a few exceptions (*P. stuartii* C9, C19 and C56 strains) Pexiganan and Citropin 1.1 inhibit biofilm formation by all tested strains at concentrations around the MICs. Pexiganan and Citropin 1.1 in concentration range 32 and 256 µg/ml caused both strong and complete suppression of biofilm formation (Tab. V), while with antibiotics, a concentration range of often roughly 5–10x MIC was needed to obtain a strong level of biofilm formation inhibition. None of the antibiotics caused complete inhibition of the biofilm formation process.

Table III
The activity of conventional antibiotics used in the treatment of urinary tract infection

P. stuartii strain	Minimal Inhibitory Concentration (MIC), Minimal Bactericidal Concentration (MBC*) and values of antibiotics [μg/ml]					
	Gatifloxacin	Amikacin	Cefepime	Cefalexin	Ceftriaxone	Cefotaxime
C3	256	512	512	> 2048	256	256
C7	256	256	512	> 2048	1024	1024
C9	512	1024	> 2048	> 2048	2048	2048
C11	256	1024	2048	> 2048	1024	1024
C12	512	> 2048	> 2048	2048	> 2048	> 2048
C13	512	256	1024	> 2048	1024	1024
C15	256	512	1024	> 2048	> 2048	> 2048
C18	256	> 2048	> 2048	> 2048	> 2048	> 2048
C19	256	> 2048	> 2048	> 2048	> 2048	> 2048
C24	256	1024	512	2048	> 2048	> 2048
C28	1024	256	> 2048	2048	> 2048	> 2048
C29	512	1024	> 2048	> 2048	> 2048	> 2048
C31	1024	> 2048	> 2048	> 2048	> 2048	> 2048
C33	1024	> 2048	> 2048	> 2048	> 2048	> 2048
C37	512	> 2048	> 2048	> 2048	256	256
C44	256	> 2048	> 2048	> 2048	> 2048	> 2048
C53	512	> 2048	> 2048	> 2048	> 2048	> 2048
C55	512	> 2048	> 2048	2048	> 2048	> 2048
C56	1024	256	1024	> 2048	256	256
C85	256	512	> 2048	2048	256	256

* MBC values in all case was above 2048 μg/ml

Eradication of mature biofilm structure. Fig. 1 and Fig. 2 show the density of untreated and treated biofilm with Pexiganan in concentration 128 and 256 μg/ml. Above all tested antimicrobial agents only Pexiganan in concentration 256 μg/ml was able to cause complete and strong reduction of biofilm biomass in the case of 5% and 80% of tested strains respectively. Moreover, Pexiganan in concentration 128 μg/ml caused strong reduction of biofilm density in 60% of all tested bacterial strains (Fig. 2). Citropin 1.1 caused strong reduction of biofilm biomass in the 60% of tested strains, only when applied in concentration 256 μg/ml (Fig. 3). For Pexiganan and Citropin 1.1 in concentration 256 μg/ml treatments OD_{595} ranged between 0.091 and 0.716, and 0.193 and 1.134, respectively against a control value between 0.878 and 3.206. Statistic analysis based on

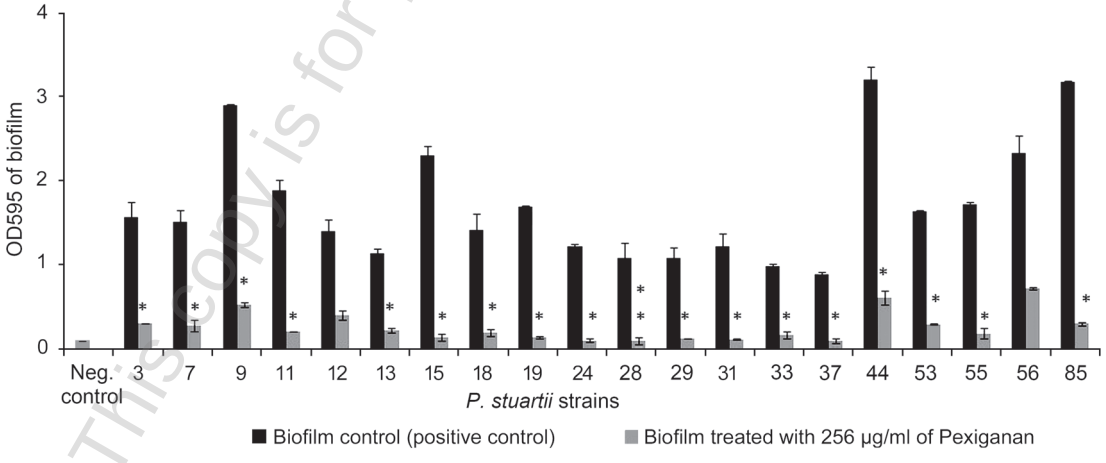

Fig. 1. Effect of 24 h treatment with 256 μg/ml of Pexiganan on 24 h biofilm of twenty *P. stuartii* clinical strains. Values are means of three experiments +SD. *Strong reduction of biofilm biomass. **Complete eradication of biofilm.

Table IV
The activity of antimicrobial peptides and amino-acids derivatives against clinical strains of *P. stuartii*

P. stuartii strain	Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) values of antimicrobial peptides [μg/ml]				
	Pexiganan	Citropin 1.1	Pal-KK-NH_2	Pal-KKK-NH_2	Pal-RRR-NH_2
C3	8/32	128/128	256/256	256/256	128/215
C7	8/16	128/128	256/512	128/256	128/256
C9	128/256	256/256	128/256	128/512	128/256
C11	1/16	1/64	1/16	1/16	2/16
C12	256/256	256/256	128/256	128/256	128/256
C13	8/128	64/128	128/256	128/256	128/256
C15	32/256	64/256	128/128	128/128	128/256
C18	4/16	64/256	128/256	128/256	128/256
C19	128/256	256/256	256/256	256/256	128/256
C24	32/256	128/128	256/256	256/256	256/256
C28	256/256	256/256	256/512	256/256	256/512
C29	256/256	256/256	256/256	256/512	256/256
C31	64/256	256/256	256/256	128/512	256/256
C33	32/128	128/128	256/512	256/256	256/256
C37	128/256	256/256	256/256	256/256	256/256
C44	64/256	256/256	256/256	256/512	256/512
C53	16/128	128/256	256/512	128/256	64/512
C55	16/256	64/256	256/256	128/256	64/512
C56	128/256	256/256	256/256	128/512	256/512
C85	128/128	128/256	128/512	256/256	256/256

one-way analysis of variance with the Bonferroni test (P value < 0.001) indicated that only Pexiganan at concentration 256 μg/ml was able to significantly eradicate 16/20 biofilms of *P. stuartii*. Analogous analysis for amino-acids derivatives showed no significantly abilities of these compounds in the eradication of biofilm structure. The biofilm susceptibility assay verified the extremely poor antibiofilm activity of conventional antibiotics compared to synthetic peptides.

Discussion

Nowadays the antibiofilm activities of antimicrobial agents are becoming an important part in treating biofilm-related infections, such as urinary tract infections or catheter associated infections caused by *P. stuartii*. In this study, we investigated the *in vitro* activities of clinically available antibiotic and synthetic antimicrobial peptides and amino-acids derivatives against *P. stuartii*

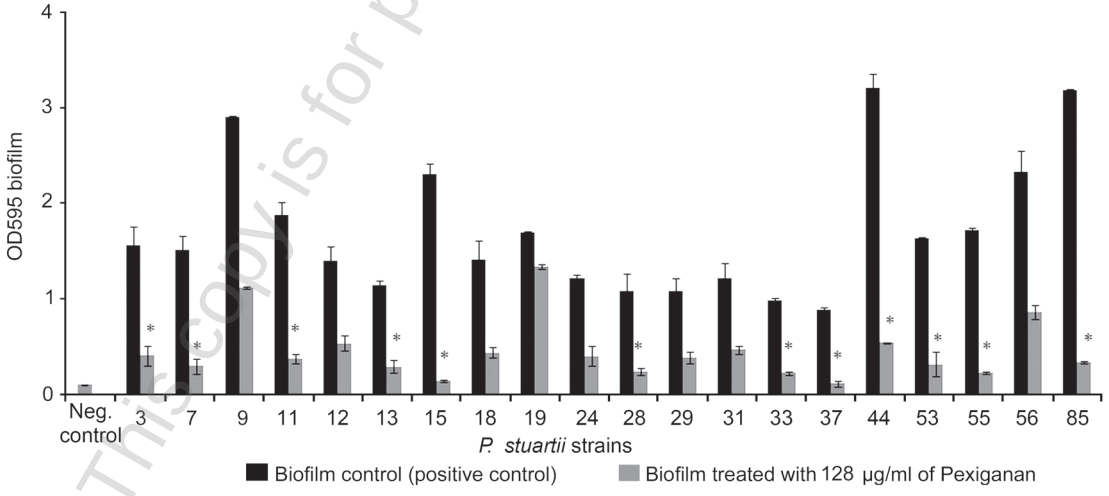

Fig. 2. Effect of 24 h treatment with 128 μg/ml of Pexiganan on 24 h biofilm of twenty *P. stuartii* clinical strains. Values are means of three experiments +SD. *Strong reduction of biofilm biomass. **Complete eradication of biofilm.

Table V
Type of inhibition of biofilm formation process caused by peptides and amino-acids derivatives in their most active concentration range

Percentage of *P. stuartii* strains [%]	Antimicrobial agent							
	Strong suppression of biofilm formation (≥75%)				Complete suppression of biofilm formation (≥ neg. control 0.091)			
Concentration [μg/ml]	256	128	64	32	256	128	64	32
Pexiganan	50	50	60	65	40	20	10	10
Citropin 1.1	30	75	75	65	60	10	5	5
Derivative 1	20	15	10	10	10	5	5	5
Derivative 2	35	45	45	45	20	5	5	5
Derivative 3	35	40	45	40	35	10	5	5

planktonic cells and mature biofilm. We found that planktonic cells of all tested *P. stuartii* clinical strains were highly resistant to conventional antibiotics used in the treatment of urinary tract infections caused by these microorganisms and demonstrating MIC values between 256 and ≥2048 μg/ml. The most effective antibiotics were fourth-generation fluoroquinolones (gatifloksacin) and aminoglycosides (amikacin). Also, considering the ability of antibiotics to inhibit biofilm formation process we noted that the concentration range of these compounds required to cause strong inhibition of biofilm formation, was roughly 5–10x MIC. None of the antibiotics caused complete inhibition of the biofilm formation process. When we considered the antibiofilm activities of the tested antibiotics, concentration values were at least 10 fold times higher compared to MIC. The concentration ranges required by all the tested antibiotics to disrupt the biofilm formation process were too high to be used in therapy.

Antimicrobial peptides have the desirable properties to become excellent antimicrobial agents, and is this regard, they are considered one of the most promising antimicrobial substances for future use as medications for health-threatening and chronic infections (Mataraci and Dosler, 2012). The antimicrobial and antibiofilm activities presented by synthetic peptides and amino-acids derivatives used in our study were much stronger compared to antibiotics and demonstrated MIC values between 1 and ≥512 μg/ml. The most active of the tested peptides against all tested *P. stuartii* strains were Pexiganan and Citropin 1.1 with MIC values ranging between 1 and 256 μg/ml. Although, the MIC values of the synthetic peptides and amino-acids derivatives were not as low as we expected, it was notable that the peptides demonstrated greater antimicrobial activity than conventional antibiotics against *P. stuartii* clinical strains and that there were no very high MIC ratios, as seen with the antibiotics.

All of the tested synthetic antimicrobial peptides and amino-acids derivatives, showed inhibitory activity against *P. stuartii* biofilm formation, in relation to their concentrations. The most active were the synthetic peptides. With a few exceptions (*P. stuartii* C9, C19 and C56 strains) Pexiganan and Citropin 1.1 inhibited biofilm formation of all tested strains at concentrations around their MICs. Pexiganan and Citropin 1.1 in concentration range 32 and 256 μg/ml caused both strong and complete suppression of biofilm formation.

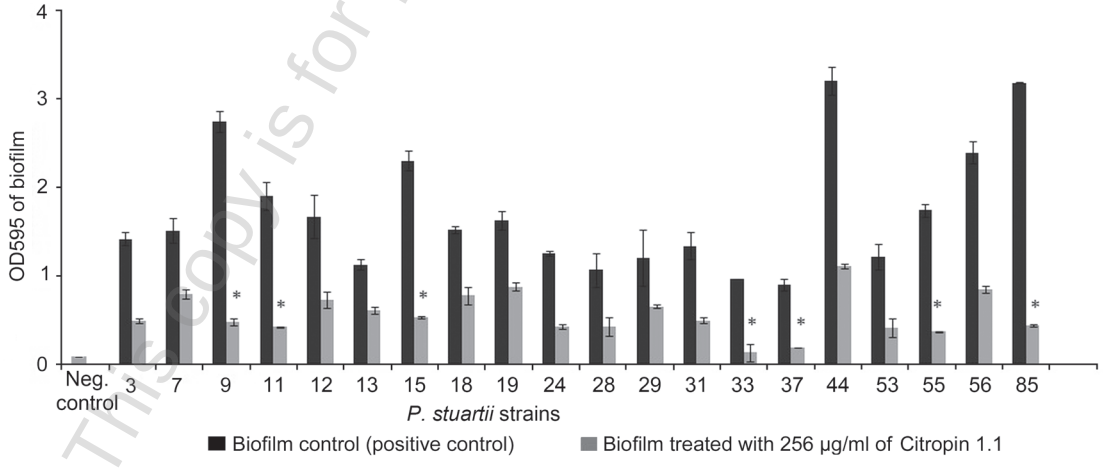

Fig. 3. Effect of 24 h treatment with 256 μg/ml of Citropin 1.1 on 24 h biofilm of twenty *P. stuartii* clinical strains. Values are means of three experiments +SD. *Strong reduction of biofilm biomass. **Complete eradication of biofilm.

All of the tested synthetic antimicrobial peptides showed the ability to eradicate biofilm structure, but only Pexiganan in concentration 256 μg/ml was able to cause both, complete (5% of strains) and strong (80% of strains) reduction of biofilm biomass. Moreover, Pexiganan in concentration 128 μg/ml caused a strong reduction of biofilm density in 60% of all the tested bacterial strains. Citropin 1.1 caused a strong reduction of biofilm biomass in the 60% of tested strains, only at concentration 256 μg/ml.

Statistic analysis showed that only Pexiganan in concentration 128 and 256 μg/ml was able to significantly eradicate biofilm structure in the case of 2/20 and 16/20 *P. stuartii* strains, respectively.

A major advantage of antimicrobial peptides is based on the principle of microbial cell membrane permeabilization. Peptides by binding to the components of the bacterial membrane, destabilize the structure of the lipid bilayer and inhibit the synthesis of membrane proteins (Cederlund *et al*., 2011; Mangoni *et al*., 2008; Gottler and Ramamoorthy, 2009; Lohner and Blondelle, 2005; Nicolas, 2009). Although the mechanisms inducing antibiotic-resistance are also diverse, the cellular action of antimicrobial peptides is separated from this mechanism. For that reason, antimicrobial peptides have the potential for use in a unique antibiotic drug for combating or preventing the formation of multidrug-resistant bacteria (Park *et al*., 2011; Flemming *et al*., 2009). Moreover, indisputable advantage of the endogenous peptide antibiotics in comparison to conventional antibiotics is the relatively low risk of the development of microbial resistance to these substances, as confirmed by *in vitro* studies (Zhang *et al*., 2005). Among these, is the composed of 22 amino acids Pexiganan also known as MSI – 78, an analogue of naturally occurring magainin II, extracted from the skin of the *Xenopus laevis*. Our results showed good *in vitro* activity of Pexiganan against both planktonic cell and mature biofilm of *P. stuartii* clinical strains. It is one of the AMPs with extremely high level of effectiveness and broad spectrum of activity. Pexiganan exerts its great antimicrobial activity to its ability to form toroidal pores in the bacterial membrane. The mechanism of cell membrane disruption by Pexiganan was confirmed by monitoring the leakage or uptake of fluorescent molecules from either *E. coli* or lipid vesicles. Pexiganan effectively induced the leakage of carboxyfluorescence from a model system for microbial cell membranes (POPC/POPG vesicles) as well as induced the uptake of ANS into *E. coli* cell membranes (Gottler and Ramamoorthy, 2009).

Due to its complex structure a bacterial biofilm constitutes an effective barrier to conventional antibiotics and also enhances antimicrobial resistance. An essential element that distinguishes planktonic cells from biofilm cells, and one of the key factors that gives biofilm significant resistance to antimicrobial agents are extracellular polymers forming a matrix surrounding the cells in a biofilm.

Microorganisms of the *Providencia* genus are opportunistic pathogens responsible for nosocomial urinary tract infections (UTI), representing nearly 50% of all bacterial nosocomial infections and in over 30% of cases, being the cause of the so-called urosepsis, leading to septic shock. *P. stuartii* has the capacity to colonize the surface of both tissues and implanted medical devices such as urological catheters made from such polymers as propylene, polystyrene, silicone, polyvinyl chloride or silicone latex. Obstruction of the urinary flow through the catheter may cause incontinence due to the leakage of urine around the catheter or painful distention of the bladder. Those microorganisms are the cause of many types of infections ranging from relatively minor inflammation of the bladder to the acute pyelonephritis, uremia and urological catheters stenosis, renal failure, or the formation of infectious urinary stones (Jiang *et al*., 2012). Pathological states caused by these microorganisms are recurrent and their therapy is extremely long and difficult.

However, treatment of infections caused by Gram-negative *P. stuartii* is additionally extremely difficult because of the presence of lipopolisaccharide (LPS) in the cell wall of these bacteria (Gruendheid and Moual, 2011; Gottler and Ramamoorthy, 2009). LPS consists the major structural component of the outer membrane in the case of Gram-negative bacteria and protects them from multiplicity factors, including AMPs. A huge number of conventional, clinically used antibiotics, lead to the release of LPS from the bacterial cell wall, which results in the secretion of pro-inflamatory cytokines (*e.g.* IL-6, TNFα or IL-6). In extreme cases this can lead to a harmful host response known as septic shock (Mangoni *et al.*, 2008; Mukhopadhyay *et al.*, 2004; Gee *et al.*, 2003). However, AMPs are believed to have a potential as antibiofilm agents, due to their different mechanisms, which include functional inhibition of proteins, membrane-disrupting action, binding with DNA and most importantly detoxification of lipoteichoic acid and LPS (Park *et al*., 2011; Mangoni *et al*., 2008; Cohen, 2002).

Therefore, understanding the potential application of antimicrobial peptide in the treatment of bacterial infections is extremely important, especially in the case of Gram-negative microorganisms of the family *Enterobacteriaceae*, causing the most common infections affecting man during his life.

Acknowledgements

This work was funded by the University of Łódź research grant (No. 545/789) and supported by Operational Programme of Innovative Economy WND-POIG.01.04.00-22-052/11.

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