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DRUG BIOCHEMISTRY

SAFETY PROFILE OF ANTIMICROBIAL PEPTIDES: CAMEL, CITROPIN, PROTEGRIN, TEMPORIN A AND LIPOPEPTIDE ON HaCaT KERATINOCYTES

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Abstract: Antimicrobial peptides (AMPs) are an essential part of the innate immunity of the skin and mucosal surfaces. They have a broad spectrum of antimicrobial activity: antibacterial, antifungal, antiviral as well as antiprotozoal. Numerous studies using AMPs as potential agents against different microbes has been performed during the last two decades. Here we investigated antistaphylococcal activity and safety of following AMPs: camel, citropin, protegrin, temporin A and lipopeptide Palm-KK-NH_2. The susceptibility of the strains of *Staphylococcus aureus* isolated from the patients with erythrodermia to conventional antibiotics and AMPs was determined by the broth dilution method. The cytotoxicity assay was performed on HaCaT keratinocytes. Tested peptides turned out to be very effective against all clinical isolates, including strains resistant to conventional antibiotics. The majority of the examined peptides as well as conventional antimicrobials do not exert any toxic effect on HaCaT cells in minimal inhibitory concentration. Peptides are very promising agents for the topical treatment of staphylococcal skin infections. The most promising seem to be citropin 1.1 and temporin A, as they were toxic only in two highest concentration (50 and 100 μg/mL), with relatively low MIC values.

Keywords: *Staphylococcus aureus,* antimicrobial peptides, cytotoxicity

One of the most important innate defense mechanisms of human skin is the production of antimicrobial peptides (AMPs). They are produced mainly by keratinocytes in the stratum corneum, neutrophils or by sweat glands and are either expressed constitutively like RNase 7, psoriasin or dermcidin or after an inflammatory stimulus like β-defensin-2 (HBD-2) and -3 (HBD-3) or the cathelicidin LL-37 (1). AMPs kill bacteria by permeating their membranes, and thus the lack of a specific molecular microbial target minimizes resistance development (2). Actually, several peptides and peptide-based compounds are passing clinical trials (3). Expression levels of these natural antibiotics correlate well with susceptibility to skin infections (4).

Skin of approximately 80% of atopic dermatitis (AD) patients is colonized with *Staphylococcus aureus* (SA) (5). The pathogens' concentration (cfu/mL) on the skin of atopic dermatitis patients is significantly higher than on that of healthy population (6). Suppressed levels of ceramides, free lipoid acids, superficial polar lipids, skin natural antimicrobial peptides (IL-37, β-defensin), as well as the pH shifted to alkaline region (pH 7-8), fibronectin receptors exposure of adhesine-binding cell wall of SA and destruction of the skin barrier by substances excreted by these germs are responsible for SA skin colonization in AD (7).

Human defensins, cathelicidins, and a significant number of diverse AMPs investigated in vertebrates and invertebrates are a good template for novel antimicrobials. Manipulation of these chemical structures to create designed synthetic peptides represents a promising strategy especially for new topical medications addressed to dermatological use.

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In this study we used three peptides representing different mechanisms of action and different chemical structures. Citropin 1.1 is a basic, highly hydrophobic, 16-amino acid peptide (GLFDVIKKVASVIGGL-NH_2) produced by the green tree frog *Litoria citropa*. Citropin 1.1 is one of the simplest, wide-spectrum amphibian antimicrobial peptide reported to date (8). Protegrin 1 (PG-1), which belong to a family of five potent, naturally occurring, cationic AMPs was originally purified from porcine tissue. The arginine residues make protegrin 1 (RGGRLCTCRRRFCVCVGGR-NH_2) highly cationic molecule and this property is primarily implicated in the activity against Gram negative bacteria (9).

Temporin A is a basic, highly hydrophobic, antimicrobial peptide amide (FLPLIGRVLSGIL-NH_2) that has variable antibiotic activity against a broad spectrum of microorganisms. Like the other temporins, it is active against clinically important antibiotic resistant Gram-positive cocci. There are currently different hypotheses concerning the mechanism of action by which temporins kill organisms: insertion into the hydrophobic core of the cell membrane, interaction with anionic heads and hydrocarbon tails of bacterial phospholipids, binding to DNA or altering enzyme activities (10). Camel is a 15-residue hybrid peptide derived from the sequences of cecropin A and melittin – two insect peptide antibiotics (11). A synthetic lipopeptide (Palm-KK-NH_2) demonstrating bactericidal and fungicidal activity was used in our study. So far, different mechanisms of bactericidal activity have been identified. One of them, the most popular one, is mediated by the direct disruption of bacterial membrane electric potentials, which results in less of a likelihood for the development of cross resistance (12).

MATERIALS AND METHODS

Antimicrobial peptides

Peptides (camel, citropin 1.1, protegrin 1, temporin A, lipopeptide) included in the study were synthesized manually by the solid-phase method using the 9-fluorenylmethoxycarbonyl chemistry (Fmoc). The peptides were purified by high-performance liquid chromatography (HPLC). The resulting fractions of purity greater than 95-98% were tested by HPLC. The peptides were analyzed also by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS).

Bacterial isolates

Fifty patients with erythrodermia, hospitalized at the Department of Dermatology, Venereology and Allergology from January 2007 to December 2010, were enrolled in the study. From each patient, skin swabs were taken. All samples were inoculated onto selective Staphylococcus 110 medium and incubated for 24 h at 37°C. Colonies were subcultured on the Columbia agar plates and incubated at 37°C for 24 h, yellow colonies of *S. aureus* were observed. Gram staining confirmed the presence of Gram-positive bacteria. Isolated strains were treated with hydrogen peroxide in order to detect the enzyme catalase. The presence of protein A as well as the clumping factor were confirmed by the Slidex Staph Plus test (bioMerieux, France).

Antibiotics and AMPs susceptibility assay

Minimum inhibitory concentration (MIC) for peptides and conventional antimicrobials (chloramphenicol, erythromycin, fusidic acid, mupirocin) was determined using a broth dilution method as

Table 1. The activity of antimicrobial peptides and conventional antibiotics against *S. aureus* clinical isolates.

	MIC [µg/mL]			ATCC	MBC [µg/mL]			ATCC
	range	50%	90%	25923	range	50%	90%	25923
Camel	4 - 16	4	8	16	4 - 32	4	8	16
Citropin 1.1	8 - 32	8	32	32	8 - 64	16	32	32
Lipopeptide	2 - 16	4	8	4	4 - 16	8	16	8
Protegrin 1	2 - 16	4	8	4	2 - 32	8	16	8
Temporin A	8 - 32	8	8	8	8 - 64	8	16	8
Chloramphenicol	4 - 128	8	128	16	-	-	-	-
Erythromycin	0.25 - > 512	0.5	> 512	0.25	-	-	-	-
Fusidic acid	0.25 - 2	0.25	0.25	0.25	0.25 - 4	0.25	1	0.25
Mupirocin	0.25 - > 512	0.25	2	0.25	0.25 - > 512	1	2	0.5

recommended by CLSI (Clinical Laboratory Standards Institute) guidelines. Polypropylene 96-well plates with tested compounds serially diluted in the Mueller Hinton II broth and initial SA inoculums of 5×10^5 cfu/mL were incubated for 18 h at 37°C. MIC was taken as the lowest drug concentration at which a noticeable growth was inhibited. Minimum bactericidal concentration was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction of the initial inoculums. The experiments were performed in triplicate.

Culture of HaCaT cell line

Human HaCaT keratinocytes were grown in Dulbecco's modified Eagle's medium (DMEM), with 4500 mg/L glucose, 584 mg/L, L-glutamine, sodium pyruvate, and sodium bicarbonate. Medium contained 10% FCS was supplemented with 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were cultured in humidified atmosphere with 5% CO_2 at 37°C. Cells were seeded at a density described below and grown for 24 h in the media with FCS. Thereafter, media were changed to serum-free before adding peptides at the concentrations listed in the figures.

Cell proliferation assay

Keratinocytes were seeded at a density of 5000 cells per well into 96-well plates in medium supplemented with serum (10%). After 24 h, media were changed to serum-free DMEM medium containing graded concentrations of peptides. The cells were incubated with peptides for 48 h incubation, thereafter, 20 µL MTT (5 mg/mL in PBS) was added and the plates were incubated at 37°C for 4 h in the presence of 5% CO_2. At the end of the incubation period, media were discarded and 100 µL of acid (0.1 mol/L hydrochloric acid) isopropanol was added before measuring optical density at 570 nm with a plate reader (13).

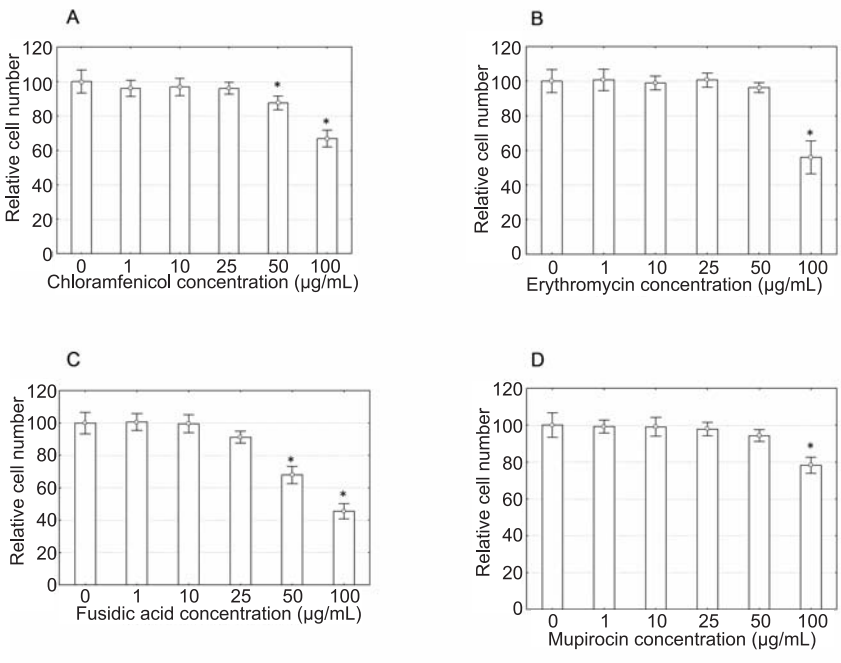

Figure 1. Effect of chloramphenicol (**A**), erythromycin (**B**), fusidic acid (**C**) and mupirocin (**D**) on the proliferation of HaCaT keratinocytes. Cells were plated at a density of 5000 cells per well in 96-well plates. After 48 h of incubation with antimicrobial agent at graded concentrations, the growth of cells was estimated by MTT method as described in "Materials and Methods". Results are presented as the mean ± standard deviation (SD) of quadruplicate wells. *$p < 0.05$ for cell growth with *versus* without antimicrobials

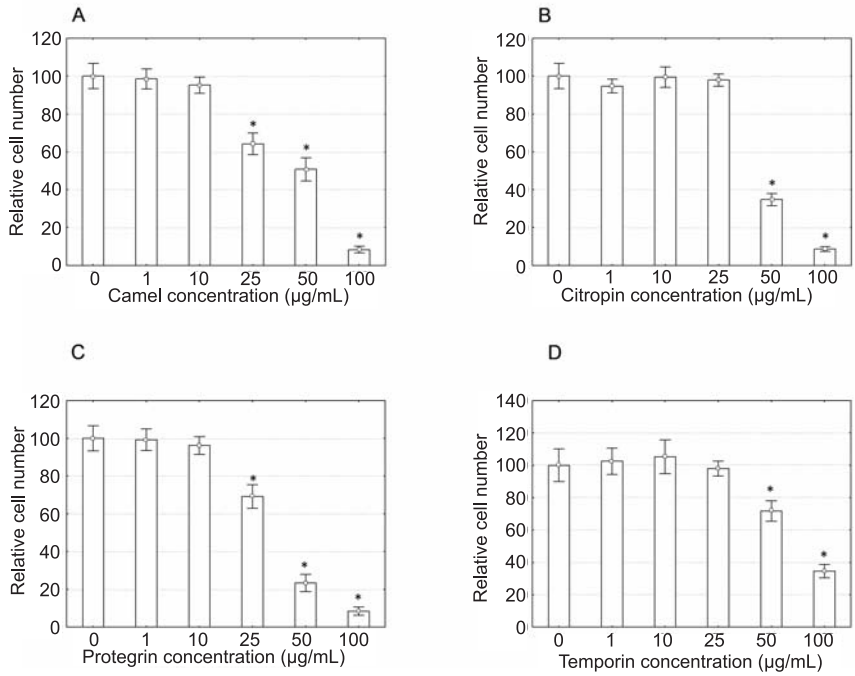

Figure 2. Effect of camel (**A**), citropin 1.1 (**B**), protegrin 1 (**C**) and temporin A (**D**) on the proliferation of HaCaT keratinocytes. Cells were plated at a density of 5000 cells per well in 96-well plates. After 48 h of incubation with peptide at graded concentrations, the growth of cells was estimated by MTT method as described in "Materials and Methods". Results are presented as the mean ± standard deviation (SD) of quadruplicate wells. *$p < 0.05$ for cell growth with *versus* without peptide

RESULTS

Staphylococcus aureus isolation

In the group of the total 50 of the patients with erythrodermia the presence of *Staphylococcus aureus* (SA) was confirmed in 37 cases. Negative cultures for SA were noticed in 13 patients: 8 with psoriasis and 5 with generalized drug eruption.

Antibiotics and AMPs susceptibility determination

All among 37 tested strains were susceptible to antimicrobial peptides in following concentrations: camel (4-16 µg/mL), citropin (8-32 µg/mL), lipopeptide (2-16 µg/mL), protegrin 1 (1-4 µg/mL) and temporin A (8-32 µg/mL). No strains with reduced susceptibility to peptide antibiotics were found. Tested peptides have presented bactericidal activity as their MBC values were equal with their MICs. Conventional antimicrobials have shown significant diversity in activity depending on tested strains. While fusidic acid acted constanlty similarly to AMPs (Table 1), for remaining compounds (chloramphenicol, erythromycin and mupirocin) significant differences in effectivness were noticed.

Effect of AMPs on HaCAT keratinocytes

The cytotoxicity activity of antimicrobial peptides as well as conventional antimicrobials was evaluated using MTT test. HaCaT keratinocytes were exposed for 48 h to peptides at doses ranging from 0.1 to 100 µg/mL. It was found that HaCaT proliferation was inhibited by lipopeptide in the concentrations of 1.0, 10.0 and 100.0 µg/mL, as reflected by the lower absorbance in the MTT test (Fig. 3; $p < 0.05$). Lipopeptide at 0.1 µg/mL was the only concentration that did not show a significant decrease in viability of keratinocytes at 48 h. Camel, fusidic acid and protegrin 1 inhibited the proliferation of HaCaT keratinocytes in the concentration 25.0 µg/mL (Fig. 1C, 2A and 2C; $p < 0.05$), while the remaining peptide antibiotics and chloramphenicol were proven to be cytotoxic in two highest concentrations of 50.0 and 100.0 µg/mL. (Fig. 1A, 2B

and 2D; $p < 0.05$). Erythromycin and mupirocin did not show cytotoxic activity up to concentration 100.0 µg/mL.

DISCUSSION AND CONCLUSION

We found that from the skin of 37 out of 50 erythrodermic patients staphylococci were isolated. Several staphylococcal strains were resistant to chloramphenicol, erythromycin and mupirocin. The purpose of the study was to investigate antistaphylococcal activity of antimicrobial peptides and estimate their safety profile due to HaCAT cell lines.

Several studies on the effect of antimicrobial treatment on the colonization with SA and the severity of inflammation, gave conflicting results. In several open or double-blind placebo-controlled trials, topical or systemic antibiotics were able to reduce colonization density and led to a partial improvement of skin lesions (14, 15). On the other hand, treatment with oral antibiotics did not lead to a significant improvement of AD in two double-blind placebo controlled studies (16, 17). No matter what kind of the treatment has been adopted, recolonization occurred after 4-8 weeks (18).

Our results have shown constant antistaphylococcal activity of tested peptides against all clinical isolates, including strains resistant to conventional antimicrobials. From a clinical point of view, our study has several implications. Considering that erythrodermic patients are frequently treated with various antibiotics, the question may be raised whether excessive use of antibiotics and induction of resistance is associated with cross-resistance to AMPs. We found no evidence for the development of the AMP resistance in relation to antibiotic susceptibility, likely reflecting the fact that the mode of action of the antibiotics investigated herein is not shared with AMPs. An interesting finding of the high efficacy of AMPs, against clinical strains of SA makes them attractive candidates for therapeutic application.

Therefore, it is essential to assay cytotoxicity of peptide antibiotics on human cells, with special regard to keratinocytes, which form main cellular component of the epidermis. In our study, we decided to use HaCaT cells as an experimental model since they closely resemble normal primary keratinocytes. HaCaT keratinocytes are immortalized cells derived from normal epidermal keratinocytes, that represent an attractive *in vitro* testing model [13]. They maintain most of the normal keratinocytes functions including differentiation potential and response to different stimuli. Moreover, they are characterized by a very high homogeneity and lack of the donor-to-donor variability (19, 20).

In this paper, we describe the analysis of cytotoxicity of 5 peptides and 4 conventional antibiotics.

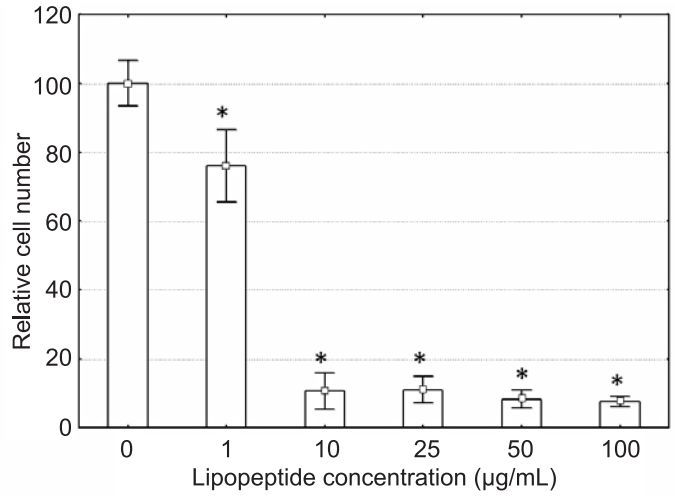

Figure 3. Effect of lipopeptide on the proliferation of HaCaT keratinocytes. Cells were plated at a density of 5000 cells per well in 96-well plates. After 48 h of incubation with peptide at graded concentrations, the growth of cells was estimated by MTT method as described in "Materials and Methods". Results are presented as the mean ± standard deviation (SD) of quadruplicate wells. *$p < 0.05$ for cell growth with *versus* without peptide

The method used to assess cytotoxic properties of the above mentioned compounds was performed using colorimetric MTT test. In our experiments, cell were stimulated with the examined compounds in medium without serum for 48 h. Lack of the serum in the culture media, resulting in deprivation of protective effect of the serum components (albumin, growth factors, protease inhibitors), leads to cells sensitization to any of possible toxic stimulus coming from the vicinity. Therefore, our experimental model based on the serum-free medium and high reproducibility due to cell line – HaCaT seems to be fully reliable.

The obtained results show that the majority of the examined peptides (apart from lipopeptide) do not exert any toxic effect on HaCaT cells at their minimal inhibitory concentration (MIC). The most promising peptide seems to be citropin 1.1 and temporin A, as it was toxic only in two highest concentrations (50 and 100 μg/mL), with relatively low MIC values (8.0 μg/mL). Therefore, the examined peptides may be dedicated to treatment of skin infections, both intact skin with preserved epidermal barrier, as well as wounded skin. Additionally, all tested conventional antibiotics proved non-toxic to cells in concentrations commonly used in dermatology (ointments 1-4% corresponding concentrations of 10-40 μg/mL). Only the fusidic acid in concentration 25 μg/mL proved to be toxic (in dermatology ointments 10 and 20 μg/mL). Mupirocin and erythromycin occurred to be the safest for the cells, as their cytotoxicity was observed only in the concentration 100 μg/mL. Even though, MIC values for these antibiotics were lower than the values of cytotoxic concentrations, bacteria, especially staphylococci show increasing resistance to the drugs. Hence, arises the need for using other antibacterial compounds, such as AMPs, inducing minimal drug resistance in various bacterial strains (21–23).

The balance between antibacterial activity and cellular toxicity is especially important since antibiotics may inhibit natural wound-healing processes (24). It is because topical antibacterial agents usually are cytotoxic to the skin cells, including the cells of the dermis and epidermis (25). Our data suggest that antimicrobial peptides may be applied not only on the non-injured skin but also directly on living layers of epidermis and skin. It is especially important before and after grafting of skin substitute and cultured keratinocytes since there is a high possibility of bacterial infections.

In conclusion, our work presents an effective and safe potential strategy for the treatment of bacterial skin infections.

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