Polish Journal of Microbiology
2004, Vol. 53, No 1, 41–44

Activities of Synthetic Peptides against Human Pathogenic Bacteria

KRYSTYNA BOGUCKA[1], ALEKSANDRA KRÓLICKA[2], WOJCIECH KAMYSZ[3], TADEUSZ OSSOWSKI[4], JERZY ŁUKASIAK[3] and EWA ŁOJKOWSKA[2*]

[1]Department of Bacteriology, Provincial Hospital of Gdańsk, Poland,
[2]Department of Plant Protection and Biotechnology, Intercollegiate Faculty of Biotechnology, University of Gdańsk and Medical University of Gdańsk, Poland,
[3]Department of Physical Chemistry, Faculty of Pharmacy, Medical University of Gdańsk, Poland,
[4]Department of Supramolecular Chemistry, Faculty of Chemistry, University of Gdańsk, Poland

Received 5 December 2003

Abstract

The increasing problem of antibiotic resistance among pathogenic bacteria requires development of new antimicrobial agents. Synthesis and experimental application of the hybrids peptides may be one of the interesting possibilities in antimicrobial treatment. The aim of the present investigation is to determinate *in vitro* activities of two synthetic peptide amides: cecropin-melittin hybrid peptide (CAMEL) and protegrin analogue (IB-367) against control strains and multi-resistant clinical isolates. Antimicrobial activities were measured by MIC and MBC. The tested strains were susceptible to the peptides at concentrations in the range of 1 to 32 μg ml^{-1}.

Key words: Cecropin-melittin hybrid, protegrin analague

Introduction

The introduction and increasing usage of antibiotics has initiated rapid development of antibiotic resistance in microorganisms, particularly in human pathogens (Berger-Bachi, 2002). Bacteria employ a variety of strategies to avoid the inhibitory effects of antibiotic agents and have evolved highly efficient means for the dissemination of resistance traits (Fraimow and Abrutyn, 1995). Resistance is found in numerous bacteria species, the most important of them are penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus* spp., methicillin-resistant *Staphylococcus aureus* (MRSA) and multiresistant gram-negative bacilli, including strains producing an extended – spectrum beta-lactamase (ESBL) (Fraimow and Abrutyn, 1995; Bedenic and Zagar, 1998; Özkuyumcu, 1999;). Infections with such organisms may be particularly difficult to treat (Liu, 1999, Hand, 2000). Trials to introduce new antimicrobial drugs are being carried out in response to the constantly growing bacterial resistance to antibiotics. Recently a lot of attention has been paid towards cationic antibacterial peptides (CAPs), (Ganz and Lehrer, 1999; Oh *et al*., 2000; Osusly *et al.*, 2000; Chmiel, 2001) Antibiotic peptides are a new group of antimicrobial agents with a unique mechanism of action (Gabay, 1994). Almost all antimicrobial peptides are cationic or amphiphilic and this feature determines the mode of their action (Kamysz *et al.*, 2003). A characteristic feature of most of these compounds is the presence of basic amino acid residues (Lys, Arg). Cationic parts of the peptides are capable of interacting with negatively charged structures of the microbial cell wall and finally leads to its permeabilization (Hwang and Vogel, 1998; Ganz and Lehrer, 1999; Chmiel, 2001). CAPs are ubiquitous in nature and are thought to be an important component in innate host defences against infectious agents (Hancock *et al.*, 1995). They are components of saliva, present on all surfaces exposed to the environment and are components of neutrophils as well. Often named “natural antibiotics” (produced by plants and animals), they are excellent templates for searching new antimicrobial agents. Based on the natural CAPs,

* Corresponding author: lojkowsk@biotech.univ.gda.pl

synthetic analogues with greater antimicrobial activity can be synthesised. Since many CAPs possess a strong *in vitro* activity against microorganisms which are resistant to conventional antibiotics, they provide attractive templates for designing new antimicrobials agents which could be used for specific application. Some of antimicrobial peptides are already potential candidates for clinical applications (Zasloff, 2002).

In the present study we tested antimicrobial activity of two synthetic peptide amides: cecropin-melittin hybrid peptide (CAMEL) and protegrin analogue (IB-367) in *in vitro* conditions. These peptides are analogues of naturally occurring compounds and are known as very effective agents against human pathogens (Mosca *et al.*, 2000; Oh *et al.*, 2000). In the present study we report high activity of two synthetic peptides against chosen strains from ATCC collection and against multiresistant clinical strains isolated in Poland.

Experimental

Materials and Methods

Strains and growth conditions. The bacteria strains and their resistance to antibiotics, was determined by disc-diffusion susceptibility test (Table I). The disc-diffusion test was performed according to the guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). The disc-diffusion tests were purchased from OXOID Co. Microorganisms used as control strains were obtained from American Type of Culture Collection (ATCC). Multiresistant microorganisms were clinical isolates obtained from Laboratory of Microbiology of the Provincial Hospital in Gdańsk. *Acinetobacter baumannii, Pseudomonas aeruginosa*, *Enterococcus faecium* and *Staphylococcus aureus* MRSA were isolated from patient's wounds and *Klebsiella pneumoniae* (ESBL +) from urine tract infection.

All experiments were performed on strains grown in Mueller-Hinton II broth at 35°C for 24 h.

Antibacterial compounds. CAMEL (KWKLFKKIGAVLKVL-NH_2, cecropin – melittin hybrid peptide) and Iseganan IB-367 (RGGLCYCRGRFCVCVGR-NH_2, protegrin analogue) were synthesized manually by the solid-phase method on TentaGel S RAM resin (0.22 mmol g^{-1}; Rapp Polymere, Germany) using fluorenylmethoxycarbonyl (Fmoc) chemistry (Fields and Noble, 1990). The side-chain protecting groups of the amino acids were: t-butoxycarbonyl (Boc) for Lys and Trp, trityl for Cys, *tert*-butyl ether for Tyr, 2,2,4,6,7-pentamethyldihydro-benzofuran-5-sulfonyl (Pbf) for Arg. The peptides were synthesized using the following procedure: (i) 5 and 15 min deprotection steps using 20% piperidine in dimethylformamide (DMF) in the presence of 1% Triton; (ii) the

Table I
Characteristics of bacteria strains used in this study

Species	Gram stain	Antibiotic agents															
		PRL	TZP	CIP	CTX	CAZ	IPM	AN	OX	GN	Va	SXT	E	L	P	CN 120	AMP
Escherichia coli ATCC 25922	(–)	26 (s)	30 (s)	35 (s)	34 (s)	30 (s)	31 (s)	21 (s)	–	–	–	–	–	–	–	–	–
Pseudomonas aeruginosa ATCC 27853	(–)	26 (s)	28 (s)	26 (s)	25 (s)	26 (s)	21 (s)	23 (s)	–	–	–	–	–	–	–	–	–
Enterococcus faecalis ATCC 29212	(+)	–	–	21 (s)	–	–	–	–	–	–	17 (s)	–	–	–	–	18 (s)	25 (s)
Staphylococcus aureus ATCC 25923	(+)	–	–	22 (s)	–	–	–	–	20 (s)	22 (s)	18 (s)	26 (s)	24 (s)	24 (s)	30 (s)	–	–
Acinetobacter baumannii	(–)	6 (r)	6 (r)	6 (r)	6 (r)	12 (r)	22 (s)	15 (i)	–	–	–	–	–	–	–	–	–
Klebsiella pneumoniae	(–)	6 (r)	18 (i)	34 (s)	6 (r)	15 (i)	22 (s)	6 (r)	–	–	–	–	–	–	–	–	–
Pseudomonas aeruginosa	(–)	6 (r)	10 (r)	21 (s)	6 (r)	6 (r)	6 (r)	15 (i)	–	–	–	–	–	–	–	–	–
Enterococcus faecium	(+)	–	–	16 (i)	–	–	–	–	–	–	22 (s)	–	–	–	–	6 (r)	6 (r)
Staphylococcus aureus MRSA	(+)	–	–	6 (r)	–	–	–	–	6 (r)	6 (r)	18 (s)	19 (s)	6 (r)	6 (r)	6 (r)	–	–

Inhibition zone diameter (mm) in disc-diffusion susceptibility test ; s – sensitive, i – intermediate, r – resistance
PRL – Piperacillin, TZP – Piperacillin/Tazobactam, CIP – Ciprofloxacin, CTX – Cefotaxime, CAZ – Ceftazidime, IPM – Imipenem, AN – Amikacin, OX – Oxacilin, GN – Gentamicin, Va – Vancomycin, SXT – Sulphamethoxazole/Trimethoprim, E – Erythromycin, L – Lincomycin, P – Pennicilin G, CN 120 – Gentamicin, AMP – Ampicillin

coupling reactions carried out with the protected amino acid diluted in a mixture of dimethylformamide and N-methyl-2-pyrrolidone (DMF/NMP), (1:1, v/v) in the presence of 1% Triton using N,N'-diisopropylcarbodiimide (DIC) as the coupling reagent in the presence of 1-hydroxybenzotriazole (HOBt); (Fmoc-AA:DIC:HOBt:1:1:1) for 1.5 h. The completeness of each coupling reaction was monitored with the chloranil test (Christensen, 1979). The peptides were cleaved from the solid support with trifluoroacetic acid (TFA) in the presence of water (2.5%), ethanedithiol (2,5%) and triisopropylsilane (2.5%) as scavengers. The cleaved peptides were precipitated with diethyl ether. IB-367 was cyclized by air oxidation as described (Chen *et al.,* 2000). The peptides were purified by the solid-phase extraction (SPE) on a sorbent Kromasil C8 (5 μm particle size, 100 Å) using the protocol described previously (Kamysz *et al*., 2002). The resulting fractions with purity greater than 95–98% were tested by High performance Liquid Chromatography (HPLC). The peptides were analyzed with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF).

Test with a commercial antibiotic: ciprofloxacin – fluoroquinolone (KRKA, d.d., Novo Mesto, Slovenia) was included for comparative purposes.

MIC and MBC determinations. Solutions of CAPs freshly prepared on the day of the assay were applied to determine the lowest concentration inhibiting bacterial growth. The concentration range assayed for CAMEL, IB-367 and ciprofloxacin was from 0.06 to 256 μg ml^{-1}. The minimal inhibitory concentration (MIC) of each compound was determined using the broth macrodilution method with Mueller-Hinton II broth and an initial inoculum of 5×10^5 cfu ml^{-1} (Thornsberry, 1991). The incubation was performed for 18 h at 35°C and followed by the determination of MIC. In order to establish the minimal bactericidal concentrations (MBC) 100 μl of the contents of the wells showing no visible growth of bacteria on Mueller-Hinton was plated out on agar plates, distributed evenly with sterile bent glass rods and incubated for 18 h at 35°C. The MBC was defined as the lowest concentration of the substrate that reduced the inoculum by 99.9% within 24 h (Thornsberry, 1991). All experiments were performed in triplicate.

Results and Discussion

A large number of diverse natural antimicrobial peptides have been discovered the last two decades (Nicholas and Mor, 1995; Hancocock *et al*., 1995) These natural products vary greatly in their biological activity spectrum, killing bacteria at concentrations from 0.25 to 4 μg ml^{-1}. Present study was performed to estimate the influence of two synthetic cationic peptides (CAMEL and Iseganan) on growth and multiplication of chosen ATCC strains and several multiresistant clinical isolates. MIC and MBC tests were used to compare antibacterial activity of CAMEL and Iseganan and commercial antibiotic – ciprofloxacin.

The presented results indicated that both peptides exhibited antibacterial activity (Tab. II). CAMEL showed insignificantly lower activity inhibiting the growth of tested strains in higher concentrations (MIC range 2–32 μg ml^{-1}, MBC range 4–32 μg ml^{-1}) than Iseganan (MIC range 1–16 μg ml^{-1}, MBC range 2–32 μg ml^{-1}) (Tab. II). Both peptides appeared less active against resistant clinical isolate *P. areuginosa* (MIC = 16 μg ml^{-1}, MBC = 32 μg ml^{-1}) than ciprofloxacin. However, in case of *A. baumannii* and *S. aureus* MRSA (clinical isolates resistant to ciprofloxacin), CAMEL and Iseganan demonstrated good activities inhibiting their growth in concentrations from 2 to 4 μg ml^{-1}, respectively. The level of the resistance to tested CAPs of the control strains and the multiresistant clinical isolates was not significantly different when tested *in vitro*. Antimicrobial activity of the tested peptides was similar in case of both gram-positive and gram-negative bacteria.

Table II
Comparison of minimal inhibitory concentrations (MIC) and minimal bactericidal concentrations (MBC) of synthetic peptides and ciprofloxacin

Species	CAMEL (μg/ml)		Iseganan IB-367 (μg/ml)		Ciprofloxacin (μg/ml)	
	MIC	MBC	MIC	MBC	MIC	MBC
Escherichia coli ATCC 25922	4	4	8	8	< 0.06	< 0.06
Pseudomonas aeruginosa ATCC 27853	8	16	4	32	1	2
Enterococcus faecalis ATCC 29212	32	32	4	8	0.5	2
Staphylococcus aureus ATCC 25923	4	8	2	2	0.5	8
Acinetobacter baumannii	2	2	2	4	>256	>256
Klebsiella pneumoniae	8	8	8	16	<0.06	<0.06
Pseudomonas aeruginosa	16	32	16	32	0.5	1
Enterococcus faecium	4	8	1	2	2	4
Staphylococcus aureus MRSA	4	8	2	8	16	> 256

Andreu *et al.*, 1992, Oh *et al.,* 2000 and Giles *et al.*, 2002 demonstrated that CAMEL and Iseganan exhibit activities against other aerobic bacteria species as well as against a wide range of anaerobic gram-positive and gram-negative bacteria when tested *in vitro*. Among others endogenic CAPs which are exhibit antimicrobial properties some are worth mentioning. For example – MSI-78, Pexiganan, and buforin II which are already known as a broad spectrum bactericidal agent active *in vitro* against both anaerobic and aerobic bacteria (Fuchs *et al.*, 1998; Ge *et al.*, 1999; Park *et al.*, 2000).

The present study indicated that CAMEL, Iseganan are effective antimicrobial agents however a particular study of their antibacterial toxicity and *in vivo* efficacy are necessary.

Literature

Andreu D., J. Ubach, A. Boman, B. Wahlin, D. Wade, R.B. Merrifield and H.G. Boman. 1992. Shortened cecropin A-melittin hybrids. Significant size reduction retains potent antibiotic activity. *FEBS Lett*. **296**: 190–194.

Bedenic B. and Z. Zagar. 1998. Extended-spectrum beta-lactamases in clinical isolates of *Klebsiella pneumoniae* from Zagreb, Croatia. *J. Chemother*. **10**: 449–459.

Berger-Bachi B. 2002. Resistance mechanisms of gram-positive bacteria. *Int. J. Med. Microbiol.* **292**: 27–35.

Chen J., T.J. Falla, H. Liu, M.A. Hurst, C.A. Fujii, D.A. Mosca, J.R. Embree, D.J. Loury, P.A. Radel, C. Cheng Chang, L. Gu and J.C. Fiddes. 2000. Development of protegrins for the treatment and prevention of oral mucositis: structure-activity relationships of synthetic protegrin analogues. *Biopolymers* **55**: 88–98.

Chmiel D. 2001. Mode of action of antimicrobial peptides. *Adv. Cell Biol*. **16**: 261–273.

Christensen T. 1979. Qualitative test for monitoring coupling completeness in solid phase peptide synthesis using chloranil. *Acta Chem. Scand. B,* **33**: 763–776.

Fields G.B. and R.L. Noble. 1990. Solid phase peptide synthesis utilizing 9-fluorenylmethoxycarbonyl amino acids. *Int. J. Peptide Protein Res*. **35**: 161–214.

Fraimow H.S. and E. Abrutyn. 1995. Pathogens resistant to antimicrobial agents. Epidemiology, molecular mechanisms, and clinical management. *Infect. Dis. Clin. North. Am.* **9**: 497–530.

Fuchs P.C., A.L. Barry and S.D. Brown. 1998. *In vitro* antimicrobial activity of MSI-78, a magainin analog. *Antimicrob. Agents. Chemother*. **35**: 45–53.

Gabay J.E. 1994. Ubiquitous natural antibiotics. *Science* **264**: 373–374.

Ganz T. and R.I. Lehrer. 1999. Antibiotic peptides from higher eucaryotes and applications. *Mol. Med. Today* **5**: 292–297.

Ge Y., D.L. MacDonalds, K.J. Holroyd, C. Thornsberry, H. Wexler and M. Zasloff. 1999. In vitro antibacterial properties of Pexiganan, an analog of magainin. *Antimicrob. Agents. Chemother*. **43**: 782–788.

Giles F.J, R. Redman, S. Yazji and L. Bellm. 2002. Iseganan HCl: a novel antimicrobial agent. *Expert Opin*. *Investig*. *Drugs* **11**: 1–8.

Hancock R.E.W., T.J. Falla and M. Brown. 1995. Cationic antimicrobial peptides. *Adv. Microb. Physiol*. **37**: 136–175.

Hand W.L. 2000. Current challenges in antibiotic resistance. *Adolesc*. *Med*. **11**: 427–428.

Hwang P.M. and H.J. Vogel. 1998. Structure-function relationships of antimicrobial peptides. *Biochem. Cell Biol*. **76**: 235–246.

Kamysz W., M. Okrój and J. Łukasiak. 2003. Novel properties of antimicrobial peptides. *Acta Bioch*. *Pol*. **50**: 461–469.

Kamysz W., B. Kochańska, A. Kędzia, J. Ochocińska, Z. Maćkiewicz and G. Kupryszewski. 2002. Statherin SV2 and its analogue. Synthesis and evaluation of antimicrobial activity. *Pol. J. Chem*. **76**: 801–806.

Liu H.H. 1999. Antibiotic resistance in bacteria. A current and future problem. *Adv*. *Exp*. *Med*. *Biol*. **455**: 387–396.

Mosca D.A., M.A. Hurst, W. So, B.S.C. Viajar, C.A. Fujii and T.J. Falla. 2000. IB-367, a protegrin peptide with *in vitro* and *in vivo* activities against the microflora associated with oral mucositis. *Antimicrob*. *Agents*. *Chemother*. **44**: 1803–1808.

Nicholas P. and A. Mor. 1995. Peptides as weapons against microorganisms in the chemical defence system of vertebrates. *Annu*. *Rev*. *Microbiol*. **49**: 277–304.

Oh H., M. Hedberg, D. Wade and C. Edlund. 2000. Activities of synthetic hybrid peptides against anaerobic bacteria: aspects of methodology and stability. *Antimicrob. Agents. Chemother*. **44**: 68–72.

Osusly, M. Zhou, G. Osuska, L. Hancock, R.E. Kay, W.W. and S. Mistra. 2000. Transgenic plants expressing cationic peptide chimeras exhibit broad-spectrum resistance to phytopathogens. *Nat. Biotechnol*. **18**:1162–1166.

Özkuyumcu C. 1999. Resistant enterococci: prevalence and factors associated with colonization in a Turkish university hospital. *Acta Microbiol*. *Pol*. **48**: 203–207.

Park C.B., K.S. Yi, K. Matsuzaki, M.S. Kim and S.C. Kim. 2000. Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. *Proc*. *Natl*. *Acad*. *Sci*. *USA* **97**: 8245–8250.

Thornsberry C. 1991. Antimicrobial susceptibility testing: general considerations, p. 1059–1201. In: Manual of clinical microbiology, 5th ed, Balows A., W.J. Hausler, K.L. Herrmann, H.D. Isenberg and H.J. Shadomy (eds) American Society for Microbiology, Washington D.C.

Zasloff M. 2002. Antimicrobial peptides of multicellular organisms. *Nature* **415**: 389–395.