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SHORT COMMUNICATION

Antimicrobial Activity of 4 Novel Cyclic Peptides against a Panel of Reference and Multi-Drug Resistant Clinical Strains of Animal Origin

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ABSTRACT

In this study, antimicrobial activity of novel cyclic peptides coded as P1, P3, P5 and P8 against reference bacterial and fungal strains and clinical multidrug resistant (MDR) bacterial strains of animal origin was investigated. Good antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli, Malassetia pachydermatis* and *Candida albicans* was found, while against Gram-positive bacteria, the minimum bactericidal concentration of peptide that killed >90% of bacteria (MBC_{90}) was >100µg/mL. Excellent activity was noticed for P3 against *E. coli* MDR clinical strains (MBC_{90} 1.6-12.5µg/mL), and against *P. aeruginosa* MDR clinical strains (MBC_{90} 3.2-12.5µg/mL). The peptides readily permeabilized *P. aeruginosa* membranes as evaluated by propidium iodide dead-stain assay. The peptides showed salt dependence with hemolytic activity close to 30% at 100µg/mL. The results so far obtained indicate good and rapid antimicrobial activity of three peptides P1, P3 and P8 that prompt for further improvement in light of a potential topical therapeutic use.

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INTRODUCTION

Worldwide emergence of multi-drug resistant (MDR) bacterial strains represents a public health and a veterinary problem. As reported by the European Food Safety Authority (2013), the misuse of antimicrobials in farm, as well as companion, animals has led to the emergence of antimicrobial-resistant bacteria. European Union Member States are monitoring the occurrence of antimicrobial resistance (AMR) in zoonotic agents, as they present a potential threat to public health. A bacterial strain is defined as MDR when it is not susceptible to at least one agent of three or more different classes of antimicrobials (Magiorakos *et al.*, 2012). Given the public health relevance of the emergence of multi-resistant bacteria, the search for novel anti-infective agents has become mandatory.

Antimicrobial peptides represent an intriguing alternative to conventional antimicrobials for their low propensity to induce resistance and wide spectrum of action (McKellar, 1998). Different conformations of antimicrobial peptides (AMPs) (α-helix, β-sheet, extended and mixed conformations) along with positive charge and hydrophobicity closely relate to their broad antimicrobial activity and exert this activity by destabilizing the bacterial membrane or increasing its permeability (Zasloff, 2002).

The aim of this study was to evaluate the antimicrobial activity of four novel cyclic AMPs against reference bacterial and fungal strains and against clinical MDR bacterial strains of animal origin.

MATERIALS AND METHODS

Four novel cyclic peptides (P1, P3, P5 and P8) were designed to contain one disulfide bond by previously described screening software (Romani *et al.*, 2013). The peptides (Table 1) were synthetized by SelleckChem (Houston, Texas) with >90% purity. The secondary structures of peptides were estimated by PsiPred, while the 3D structures were built by Chimera. The structures of the 17-mer peptides were random coil cyclized by a disulphide bond (Fig. 1A). The freeze-dried peptides were dissolved in 10 mM phosphate buffer (PB) at the stock concentration of 1 mg/mL.

The antimicrobial activity of these peptides was evaluated against 9 reference strains (*Escherichia coli*

Table 1: Sequences and physicochemical properties of the tested peptides

Peptides	Peptide sequence	MW[§]	Charge	Hydrophobicity (%)	Amphipaticity	Boman index	Isoelectric point
P 1	KRLDTLCIRKKCGAKSF	1967.42	5	41.2	0.21	2.56	9.99
P 3	RNFRGKCRHKCFKKEKV	2164.62	8	29.4	0.94	4.53	10.51
P 5	KRGTCHFGRCPSHLIKG	1897.25	6	29.4	0.40	2.23	9.02
P 8	QNLRTLCVRKRCLLKSG	1746.21	5	46.7	0.26	2.30	10.75

ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Streptococcus agalactiae* ATCC 27956, *Burkholderia cepacia* ATCC 17759, *Stenotrophomonas maltophilia* ATCC 13637, *Malassetia pachydermatis* ATCC 14522 and *Candida albicans* ATCC 10231) purchased from Diagnostic International Distribution (Milano, Italy), 5 multidrug resistant swine fecal isolates of *Escherichia coli* from cases of enteritis and 5 MDR *Pseudomonas aeruginosa* strains from dog ears with otitis. All the clinical isolates were identified as MDR according to the guidelines of Magiorakos *et al.* (2012), by the Kirby-Bauer method. The antimicrobial activity was evaluated as reported elsewhere (Cabassi *et al.*, 2013), following Clinical and Laboratory Standards Institute (2008) guidelines. The minimum bactericidal concentration of each peptide that killed >90% of bacteria (MBC_{90}) was estimated in three independent experiments performed in duplicate (Romani *et al.*, 2013). The activity was also investigated on *P. aeruginosa* ATCC 27853 in the presence of four concentrations of NaCl (10, 25, 50 and 125 mM).

To assess the ability of peptides to alter the permeability of the inner membrane of bacteria, a dead-cell stain procedure employing propidium iodide (PI) was used (Williams *et al.*, 1998). The stain-dead assay was performed, using a log-phase culture of *P. aeruginosa* ATCC 27853 in the presence of 12.5µg/mL of peptides. The fluorescence emission of propidium iodide was measured after peptide addition as a function of time, up to 15 minutes. 4', 6-diamidino-2-phenylindole (DAPI) was used for counterstaining. Negative and positive controls were obtained in the absence of peptides and in the presence of 1mM EDTA and 0.5% Triton X-100, respectively. The results were analyzed by fluorescence microscopy. The hemolysis assay was performed according to the procedure described elsewhere (Romani *et al.*, 2013). The positive control (100% hemoglobin release) was obtained in the presence of 1% Tween-20.

RESULTS AND DISCUSSION

All peptides were active against *E. coli* and *P. aeruginosa* reference strains with a MBC_{90} ranging from 0.2 to 12.5µg/mL (Table 2), in particular P1 and P3 were the most active. The excellent activity of P3 was confirmed also for MDR isolates, with MBC_{90} ranging 1.6-12.5µg/mL for *E. coli* MDR isolates, and 3.2–12.5µg/mL for *P. aeruginosa* MDR isolates. A broad variability was observed for P5, showing the low activity against 3 out of 5 MDR *E. coli* isolates (MBC_{90} = 25-50 µg/mL) and against 4 out of 5 *P. aeruginosa* isolates (MBC_{90} = 12.5-100 µg/mL).

For *S. aureus* ATCC 25923, *S. agalactiae* ATCC 27956, *B. cepacia* ATCC 17759, *E. faecalis* ATCC 29212 and *S. maltophilia* ATCC 13637, the MBC_{90} was not less than 100µg/mL for all peptides. Against *M. pachydermatis* ATCC 14522, P3 exhibited an excellent activity (MBC_{90} 1.6µg/mL), while for the other peptides MBC_{90} was 6.4µg/mL. Similarly, only P3 peptide showed an excellent

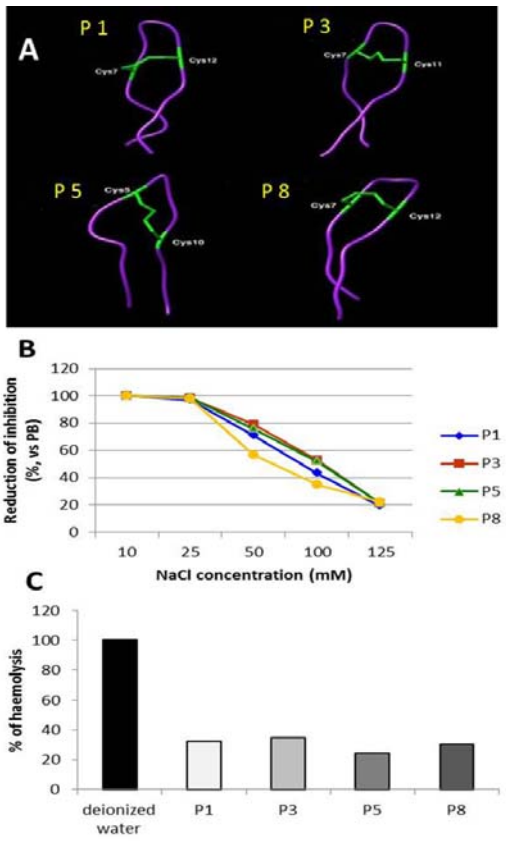

Fig. 1: Structure (A), activity at different NaCl concentrations (B) and hemolysis assay (C) of cyclic peptides (P1, P3, P5 and P8).

activity (MBC_{90} = 0.8µg/mL) against *C. albicans* ATCC 10231. The antibacterial activity of the peptides was investigated against *P. aeruginosa* ATCC 27853 in different NaCl concentrations in order to understand the influence of monovalent ions. The antimicrobial activity was fully maintained at 25mM NaCl, while it was reduced by 80% at 125mM NaCl (Fig. 1B), with no significant differences among peptides. Salt inactivation of antibacterial activity is dependent on the concentration of NaCl. The high net positive charge in P3 (Table 1), comparing to the other peptides, does not appear to have a role in maintaining antibacterial activity in the presence of NaCl. High-salt environment weakened the initial electrostatic interactions between peptides and bacterial targets, reducing bactericidal activities, as suggested by Zasloff (2002).

Due to its high sensitivity to all peptides, the mechanism of action was investigated on *P. aeruginosa* ATCC 27853, assuring comparable results. For all peptides, the stain-dead assay highlighted the fast permeabilization of the inner membrane, with the occurrence of red fluorescence within 3 min after contact with peptides (Fig. 2).

Since it is well known that several antimicrobial peptides damage eukariotic cell membrane (Romani *et al.*, 2013), the cytotoxicity was investigated against sheep erythrocytes. After one hour of incubation in PB containing 308 mM sucrose, a negligible hemolytic activity (<1%) was

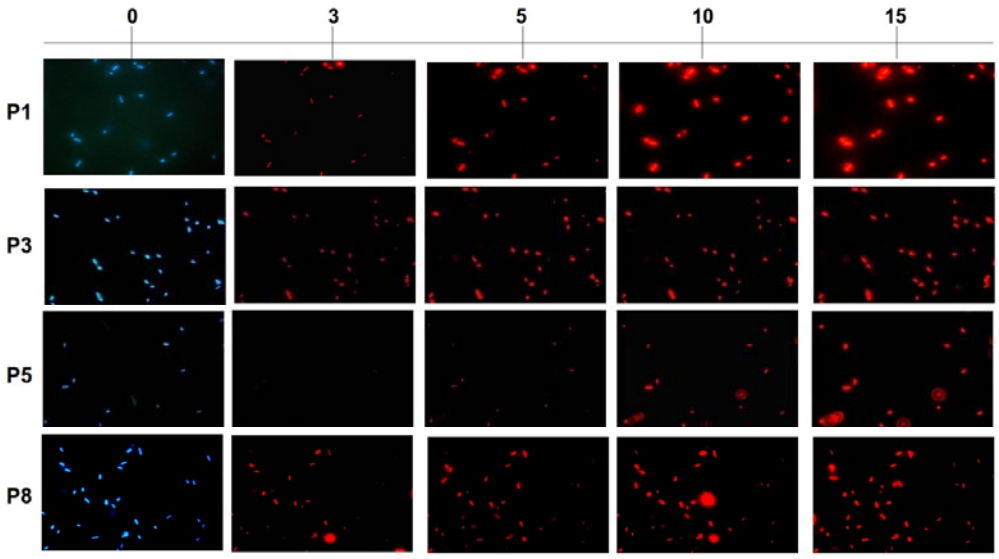

Fig. 2: Propidium iodide dead-cell stain assay: permeabilization of the inner membrane of *P. aeruginosa* ATCC 27853 as a function of contact time (min) with the peptides.

Table 2: Antimicrobial activity of cyclic peptides (P1, P3, P5 and P8)

Strains	MBC_{90} (μg/mL)			
	P1	P3	P5	P8
Gram-negative bacteria				
Burkholderia cepacia ATCC 17759	>100	>100	>100	>100
Escherichia coli ATCC 25922	1.6	1.6	12.5	12.5
Escherichia coli EC-VET 10	6.4	12.5	50	12.5
Escherichia coli EC-VET 11	1.6	3.2	1.6	0.8
Escherichia coli EC-VET 12	6.4	3.2	25	12.5
Escherichia coli EC-VET 13	6.4	3.2	6.4	12.5
Escherichia coli EC-VET 14	6.4	3.2	25	6.4
Pseudomonas aeruginosa ATCC 27853	0.2	0.8	1.6	0.4
Pseudomonas aeruginosa PA-VET 40	6.4	3.2	12.5	6.4
Pseudomonas aeruginosa PA-VET 41	6.4	12.5	100	6.4
Pseudomonas aeruginosa PA-VET 42	12.5	6.4	12.5	12.5
Pseudomonas aeruginosa PA-VET 43	3.2	6.4	12.5	6.4
Pseudomonas aeruginosa PA-VET 44	6.4	6.4	100	12.5
Stenotrophomonas maltophilia ATCC 13637	>100	>100	>100	>100
Gram-positive bacteria				
Enterococcus faecalis ATCC 29212	>100	>100	>100	>100
Staphylococcus aureus ATCC 25923	>100	>100	>100	>100
Streptococcus agalactiae ATCC 27956	>100	>100	>100	>100
Yeasts				
Candida albicans ATCC 10231	12.5	0.8	25	6.4
Malassetia pachydermatis ATCC 14522	6.4	1.6	6.4	6.4

observed in the absence of peptides, while complete hemolysis was seen in the positive control. For all the tested peptides, the percentage of hemolysis was around 30% (25-34%; Fig. 1C). It is important to stress that the information derived from *in vitro* hemolysis or cytotoxicity assays should be considered carefully in representing AMP-selective toxicity, because it may not realistically reflect the potential cytotoxicity *in vivo* (Yeaman and Yount, 2003). However, the obtained data make further development mandatory in order to reduce the cytotoxicity of the peptides. We are also aware of the fact that our *in vitro* study does not allow us to draw definitive conclusions about the *in vivo* efficacy or toxicity. However, our results represent an intriguing starting point to estimate the usefulness of these novel peptides before performing the *in vivo* experiments.

Conclusion: Taken together, these results suggest a good and fast antimicrobial activity of P1, P3 and P8 against *E. coli*, *P. aeruginosa* and yeasts, and could improve the knowledge about the structure-activity relationship for a rational development of efficient antimicrobial peptides. Moreover, the improvement of activity along with a reduction of cytotoxicity makes it possible to hypothesize a potential topical therapeutic use to counteract the infections caused by the above mentioned susceptible bacteria and yeasts.

Author's contributions: CSC and AAR conceived the study wrote and revised the manuscript; AAR carried out the design of the peptides and the molecular modelling assay; AS, DS and ST executed the experiments, analyzed the results and reviewed the manuscript; SC and MCB critically revised the manuscript. All authors have read and approved the final manuscript.

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