Antimicrobial activity of Bac7 fragments against drug-resistant clinical isolates

ARTICLE *in* PEPTIDES · JANUARY 2005

Impact Factor: 2.62 · DOI: 10.1016/j.peptides.2004.08.004 · Source: PubMed

6 AUTHORS, INCLUDING:

Monica Benincasa
Università degli Studi di Trieste
50 PUBLICATIONS **1,433** CITATIONS
SEE PROFILE

Marco Scocchi
Università degli Studi di Trieste
71 PUBLICATIONS **1,705** CITATIONS
SEE PROFILE

Barbara Skerlavaj
University of Udine
39 PUBLICATIONS **1,820** CITATIONS
SEE PROFILE

Renato Gennaro
Università degli Studi di Trieste
116 PUBLICATIONS **4,980** CITATIONS
SEE PROFILE

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Peptides 25 (2004) 2055–2061
PEPTIDES
www.elsevier.com/locate/peptides

Antimicrobial activity of Bac7 fragments against drug-resistant clinical isolates

Monica Benincasa[a], Marco Scocchi[a], Elena Podda[a], Barbara Skerlavaj[b], Lucilla Dolzani[c], Renato Gennaro[a,*]

[a] *Department of Biochemistry, Biophysics and Macromolecular Chemistry, University of Trieste, Via Giorgieri, 1, I-34127 Trieste, Italy*
[b] *Department of Sciences and Medical Technologies, University of Udine, P.le Kolbe, 4, I-33100 Udine, Italy*
[c] *Department of Biomedical Sciences, University of Trieste, Via Giorgieri, 7, I-34127 Trieste, Italy*

Received 9 June 2004; received in revised form 4 August 2004; accepted 4 August 2004
Available online 17 September 2004

Abstract

Ten peptides from 13 to 35 residues in length and covering the whole sequence of the Pro-rich peptide Bac7 were synthesized to identify the domain responsible for its antimicrobial activity. At least 16 residues of the highly cationic N-terminal sequence were required to maintain the activity against Gram-negative bacteria. The fragments Bac7(1–35) and, to a lesser extent, Bac7(1–16) proved active against a panel of antibiotic-resistant clinical isolates of Gram-negative bacteria, with the notable exception of *Burkholderia cepacia*. In addition, when tested against fungi, the longer fragment was also active against collection strains and clinical isolates of *Cryptococcus neoformans*, but not towards clinical isolates of *Candida albicans*.

Keywords: Pro-rich peptide; Bactenecin 7; Cathelicidin peptide; Synthetic analog; Antimicrobial activity; Clinical isolate; Multidrug-resistance

1. Introduction

The problem of the rapid and alarming spread of antibiotic resistance among nosocomial Gram-negative strains of bacteria is worryingly increasing, with serious public health implications [23,24].

As a consequence, there is a pressing need to develop novel and effective classes of antimicrobial drugs with mechanisms of action based on cellular targets different from those of existing antibiotics.

Cationic antimicrobial peptides (AMPs) are widespread in nature and constitute an effective component of natural immunity for host defense against microbial agents [15,20]. Thanks to their capacity to rapidly inactivate bacterial, fungal or viral pathogens [33], antimicrobial peptides furnish new opportunities for the design of more effective peptidic or peptidomimetic derivatives, and thus for development into novel antibiotics.

Most AMPs kill target microorganisms by direct disruption of the integrity of their membranes [26]. However, AMPs belonging to the Pro-rich group, act without an initial damage to bacterial membranes [10,21]. Members of this group are characterized by a high content of proline residues, are mainly active against Gram-negative species, and appear to inactivate bacteria by inhibition of metabolic processes, such as protein synthesis [3] or chaperone-assisted protein folding [14].

Bac7, a cathelicidin-derived peptide from bovine neutrophils, is a member of this distinctive group that also includes the closely related Bac5 and PR-39 peptides, respectively from bovine and pig neutrophils [1,9,34], and several homologs from sheep and goat [2,27]. Bac7 has a peculiar primary structure composed of a highly cationic N-terminal region, which includes eight arginines in the first 16 residues, followed by three hydrophobic tandemly repeated sequences

* Corresponding author. Tel.: +39 040 558 3696; fax: +39 040 558 3691.
E-mail address: gennaro@bbcm.units.it (R. Gennaro).

0196-9781/$ – see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.peptides.2004.08.004

of 14 residues [8]. This peptide exhibits strong antibacterial activity against Gram-negative microorganisms [9], neutralizes endotoxin in an experimental rat model of Gram-negative septic shock, and is not toxic to mammalian cells at concentrations well above those effective against microbes [11]. In addition, synthetic fragments of Bac7 are capable of translocation across the plasma membrane of mammalian cells without cytotoxic effects [25].

The aim of the present study was (i) to evaluate the in vitro activity of Bac7 synthetic fragments to identify shorter peptides with a potency comparable to that of the parent peptide and (ii) to characterize this activity against a wide spectrum of antibiotic-resistant nosocomial isolates.

2. Material and methods

2.1. Peptide synthesis and purification

Protected amino acids and PEG-PS resins were purchased from Advanced Biotech Italia (Milan, Italy) and Novabiochem (Merck, Darmstadt, Germany). Bac7 was extracted from bovine neutrophil granules and purified as previously reported [9]. All Bac7 fragments were synthesized by the solid phase method as previously described [29], using a Pioneer^{TM} Peptide Synthesis System and the fluorenylmethoxycarbonyl chemistry. The syntheses were performed at 48 °C by heating a jacketed column and the solvent solutions. For each coupling step, the Fmoc-protected amino acid and coupling reagents were added in a six- to eight-fold molar excess with respect to resin substitution. Couplings (60 min) were carried out with *N*-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro-borate (TBTU), except for those sequences showing a high aggregation potential, as calculated by the Peptide Companion software (CoshiSoft, Tucson, AZ, USA), for which the highly efficient acylating reagent *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was used. After Fmoc deprotection and before addition of the following residue, the resin was washed with a solution of dimethylformamide/*N*-methyl-pyrrolidone (3:1) containing 1% Triton X-100 and 2 M ethylencarbonate. Arginine side chain was 2,2,4,6,7-pentamethyl-dihydrobenzofurane-5-sulfonyl (Pbf) protected. Cleavage from the resin and deprotection of the synthesized peptides were carried out with a solution of 95% trifluoroacetic acid, 2.5% water and 2.5% triisopropylsilane. After repeated precipitation with cold ether, all the peptides were purified by reversed-phase HPLC (ÄKTA Basic, Amersham Biotech, Uppsala, Sweden) on a preparative X-Terra RP C18 column (19 mm × 300 mm; Waters, MA, USA) using appropriate 0–60% water/acetonitrile linear gradients in the presence of 0.1% trifluoroacetic acid. The molecular masses of the purified peptides were determined with an API-I electrospray mass spectrometer (PE SCIEX, Toronto, Canada). All the Bac7 fragments had a purity of at least 95% and were dissolved in double distilled water and stored at −20 °C until use. Their concentration was determined by the ultraviolet spectrophotometric method of Waddell [32] and, when possible, also by measuring the absorbance of Phe residues at 257 nm, considering an extinction coefficient of 195.1 M^{-1} cm^{-1} [4].

2.2. Microbial strains and growth conditions

The antibacterial activity of the Bac7 fragments was tested on the reference strains *Escherichia coli* ATCC 25922 and ML35 (also known as ATCC 43827), *Serratia marcescens* ATCC 8100, *Salmonella enterica* serovar Typhimurium ATCC 14028, and *Staphylococcus aureus* ATCC 25923, and on a total of 86 clinical isolates of Gram-negative (*Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *E. coli*, *S. enterica* serovars Anatum and Typhimurium, *Klebsiella pneumoniae*, and *S. marcescens)* and Gram-positive (*S. aureus*, *Enterococcus faecalis* and *faecium*) bacterial strains. These clinical isolates were collected from distinct patients with epidemiologically unrelated infections in various hospitals from Northern Italy. Half of the 20 *P. aeruginosa* isolates were collected from cystic fibrosis patients with lung disease. All the *A. baumannii* and most of the *P. aeruginosa* isolates were characterized for antibiotic resistance. Susceptibility to conventional antibiotics was tested by the disk diffusion method in accordance with the guidelines [18] and interpretative tables [19] of the National Committee for Clinical Laboratory Standards (NCCLS). Antibiotic disks were purchased from Oxoid (Oxoid SpA, Milan, Italy). All the bacterial strains were stored at −80 °C and routinely grown onto Mueller Hinton (MH, Becton Dickinson, Sparks, MD, USA) agar plates.

The antifungal activity was evaluated with collection strains of *Cryptococcus neoformans* (ATCC 90112, ATCC 90113), including two acapsular mutants (ATCC 52816, ATCC 52817), and clinical isolates of *C. neoformans*, *Candida albicans* and *Pichia etchellsii*. Fungi were grown onto Sabouraud agar plates at 30 °C for 48 h; the inoculum suspensions were prepared by picking five colonies and suspending them in 5 ml of sterile PBS.

2.3. Antimicrobial assays

The minimum inhibitory concentration values (MICs) of the Bac7 fragments and of conventional antibiotics were determined by the broth microdilution susceptibility test following the guidelines of the NCCLS with mid-log phase cultures. Serial two-fold dilutions of each peptide were prepared (final volume of 50 μl) in 96-well polypropylene microtiter plates (UNIFO, Treviso, Italy) with MH broth for bacteria and RPMI-1640 for fungi. Each dilution series included control wells without peptide. A total of 50 μl of the adjusted inoculum (approximately 5×10^5 cells/ml for bacteria or 5×10^4 cells/ml for fungi, in the appropriate medium) was added to each well. To evaluate the MIC, microtiter plates

Table 1
Amino acid sequences of Bac7 and its synthetic fragments

Peptide	Sequence	
Bac7	RRIRPRPPRLPRPR	$(PRPLPFPRPGPRPI)_3$PRPL
Bac7(1–13)	RRIRPRPPRLPR	P
Bac7(1–15)	RRIRPRPPRLPRPR	P
Bac7(1–16)	RRIRPRPPRLPRPR	PR
Bac7(1–18)	RRIRPRPPRLPRPR	PRPL
Bac7(1–23)	RRIRPRPPRLPRPR	PRPLPFPRP
Bac7(5–23)	PRPPRLPRPR	PRPLPFPRP
Bac7(1–35)	RRIRPRPPRLPRPR	$(PRPLPFPRPGPRPI)_1$PRPLPFP
Bac7(5–35)	PRPPRLPRPR	$(PRPLPFPRPGPRPI)_1$PRPLPFP
Bac7(43–56)		$(PRPLPFPRPGPRPI)_1$
Bac7(29–56)		$(PRPLPFPRPGPRPI)_2$

with bacteria were incubated at 37 °C overnight, while those with fungi were incubated at 30 °C for 48 h. Aztreonam, chloramphenicol, gentamicin, tetracyclin, sulfamethoxazole and trimethoprim were purchased from Sigma (St. Louis, MI). The last two compounds were mixed in a 5:1 ratio (by weight). Ciprofloxacin was diluted from i.v. formulation from Bayer (West Haven, CT).

3. Results

3.1. Antimicrobial activity of Bac7 fragments

To identify the domain responsible for the antimicrobial activity of Bac7 and the shortest fragment still active, a total of ten N-terminal and C-terminal Bac7 fragments of different length was synthesized (sequences reported in Table 1).

The antibacterial activity of each Bac7 fragment was initially evaluated against a Gram-positive and four different Gram-negative reference ATCC strains by using the microdilution susceptibility test. The MIC values for each peptide are reported in Table 2.

Bac7 and some of its N-terminal synthetic fragments showed a potent antimicrobial activity against all the Gram-negative strains tested. In contrast, no activity was exerted against *S. aureus* (MIC ≥128 μM). Shortening of the N-terminal Bac7 fragments up to 16 residues did not result in a significant loss of activity, whereas a further shortening of the fragment of only one residue led to a dramatic loss of activity (Table 2). Among the functioning N-terminal fragments synthesized, Bac7(1–35) proved the most active, showing a potency comparable to that of the natural 60 residue peptide.

The removal of the highly cationic RRIR N-terminal sequence, as in the Bac7(5–35) fragment, resulted in a remarkable reduction of the potency of the peptide of between 32- and 128-fold, depending on the bacterial species. The absence of the same four N-terminal residues in the shorter fragment Bac7(5–23), caused an almost complete loss of activity (MIC ≥ 64 μM) with respect to the full length fragment 1–23, although the sequence was longer than that of Bac7(1–16), the shortest N-terminal fragment still active.

At variance with fragments derived from the N-terminal cationic region, those including C-terminal portions, for instance Bac7(43–56) and Bac7(29–56), which respectively include one and two copies of the tetradecamer tandem repeat, showed no antibacterial activity at all (MIC > 128 μM).

3.2. Antibacterial activity of selected Bac7 fragments against clinical isolates

Bac7(1–35) and Bac7(1–16), the longest and the shortest Bac7 fragments used in this study showing MIC values comparable to those of the parent peptide, were chosen for a further characterization of their in vitro antimicrobial activity against a panel of Gram-negative (*E. coli*, *S. enterica*, *P. aeruginosa*, *A. baumannii*, *B. cepacia* and *K. pneumoniae*) and Gram-positive (*S. aureus*, *E. faecalis*, and *E. faecium*) clinical isolates, including multi-resistant strains.

Both peptides displayed a broad-spectrum activity against most Gram-negative microorganisms (Table 3). The only Gram-negative species that was completely resistant to the

Table 2
Antibacterial activity of Bac7 and of its synthetic fragments against collection bacterial strains

Peptide	Minimum inhibitory concentration (MIC)[a] in μM				
	E. coli (ATCC 25922)	*E. coli* ML-35 (ATCC 43827)	*S. enterica* (Ser. Typhimurium) (ATCC 14028)	*S. marcescens* (ATCC8100)	*S. aureus* (ATCC 25923)
Bac7	1	2	1	1	>128
Bac7(1–35)	0.5	0.5	0.5	1	>128
Bac7(5–35)	16	32	>64	32	>128
Bac7(1–23)	1	1	2	4	>128
Bac7(5–23)	64	64	128	>128	>128
Bac7(1–18)	2	2	1	16	>128
Bac7(1–16)	2	2	1	8	>128
Bac7(1–15)	128	128	128	128	>128
Bac7(1–13)	>128	>128	>128	>128	>128
Bac7(43–56)	>128	>128	>128	>128	>128
Bac7(29–56)	>128	>128	>128	>128	>128

[a] MIC was defined as the lowest concentration of peptide that prevented bacterial visible growth after incubation for 18 h at 37 °C. Results given are mean values of at least three independent determinations.

Table 3
Antibacterial activity of Bac7(1–35) and Bac7(1–16) against clinical isolates

Organism (number of strains)	Peptide	MIC (μM)		
		Range	50%[a]	90%[a]
E. coli (10)	Bac7(1–35)	0.125–1	0.5 (2.1)[b]	1 (4.2)
	Bac7(1–16)	1 to >64	4 (8)	16 (32)
S. enterica (10)	Bac7(1–35)	0.125–0.5	0.125 (0.52)	0.25 (1)
	Bac7(1–16)	0.5–2	1 (2)	2 (4)
K. pneumoniae (13)	Bac7(1–35)	0.25–1	0.25 (1)	0.5 (2.1)
	Bac7(1–16)	1–16	8 (16)	16 (32)
A. baumannii (12)[c]	Bac7(1–35)	0.5–16	0.5 (2.1)	2 (8.4)
	Bac7(1–16)	0.5–64	8 (16)	32 (64)
P. aeruginosa (20)[d]	Bac7(1–35)	0.5–32	8 (34)	8 (34)
	Bac7(1–16)	64 to >128	>128 (>256)	>128 (>256)
B. cepacia (10)	Bac7(1–35)	>64	>64 (>268)	>64 (>268)
	Bac7(1–16)	>128	>128 (>256)	>128 (>256)

[a] MIC values at which 50% and 90% of the strains were inhibited.
[b] Data in parenthesis refer to MIC values expressed in μg/ml.
[c] 6 *A. baumannii* isolates were multi-resistant to antibacterial drugs as reported in Table 4.
[d] 8 *P. aeruginosa* isolates were resistant to at least three of the following antibacterial drugs: aztreonam, ticarcillin/clavulanic acid, ciprofloxacin, amikacin, mezlocillin and trimethoprim-sulfamethoxazole.

peptide's action was *B. cepacia*, an opportunistic pathogen of the lung in cystic fibrosis patients, known for its intrinsic resistance to many antibiotics and most AMPs [13]. In general, Bac7(1–35) was between 8- and 16-fold more active than the 1–16 fragment, with MIC values ranging in most cases from 0.125 to 8 μM for the former and from 1 to 64 μM for the latter peptide.

All of the 10 isolates of *Salmonella* tested were highly susceptible to both fragments, with Bac7(1–35) and Bac7(1–16) respectively showing MIC values of 0.25 and 2 μM for 90% of the strains. Nosocomial isolates of *K. pneumoniae* (13 strains), *E. coli* (10 strains) and *A. baumannii* (12 strains) were definitely more susceptible to Bac7(1–35) than to the shorter 1–16 fragment (Table 3). Interestingly, the *A. baumannii* strains also included multidrug-resistant isolates that were not susceptible to amikacin, ceftazidime, piperacillin, ciprofloxacin, trimethoprim-sulfamethoxazole, aztreonam, gentamicin, chloramphenicol and tetracyclin. Comparative tests run in parallel indicate that Bac7(1–35) has a potent activity against these multi-resistant clinical isolates, with MICs that are in most cases equal to or lower than 8 μg/ml (Table 4). In addition, similar MIC values were observed when the activity towards multi-resistant versus antibiotic-susceptible strains was compared, suggesting that Bac7 and its fragments act with a mechanism that is different from those of the above mentioned drugs. A variable susceptibility of *P. aeruginosa* strains to Bac7(1–35) was observed with MIC values ranging from 0.5 to 32 μM. In contrast, Bac7(1–16) was virtually ineffective against all the isolates of this species. Eight of the *P. aeruginosa* isolates tested were resistant to at least three of the following antibacterial drugs: aztreonam, ticarcillin/clavulanic acid, ciprofloxacin, amikacin, mezlocillin and trimethoprim-sulfamethoxazole. As for *A. baumannii*, also in this case there was no significant difference in their susceptibility to Bac7(1–35) with respect to antibiotic-sensitive isolates. In contrast to Gram-

Table 4
Antibacterial activity of Bac7(1–35), Bac7(1–16) and conventional antibiotics against multidrug-resistant clinical isolates of *A. baumannii*

Isolate	MIC[a] (μg/ml)							
	Bac7(1–35)	Bac7(1–16)	CIP	GEN	AZT	CHL	TMP-SMX[b]	TET
A. baumannii 1B	2	16	>64[c]	>128	128	256	64	>128
A. baumannii 2B	8	64	32	>128	256	256	128	>128
A. baumannii 23B	4	32	>64	16	256	256	64	128
A. baumannii 24B	16	128	8	>128	256	256	64	>128
A. baumannii 176A	2	16	32	2	256	128	128	64
A. baumannii 179A	8	64	8	>128	64	256	64	>128

Assays were run in duplicate in Muller-Hinton broth according to the NCCLS guidelines. CIP, ciprofloxacin; GEN, gentamicin; AZT, aztreonam; CHL, chloramphenicol; TMP-SMX, trimethoprim-sulfamethoxazole; TET, tetracyclin.
[a] MIC was defined as the lowest concentration of antibacterial compound that prevented bacterial visible growth after incubation for 18 h at 37 °C.
[b] Trimethoprim and sulfamethoxazole were mixed in a 5:1 ratio (by weight).
[c] The *A. baumannii* isolates are considered resistant to antibiotics when the MIC values were ≥4 μg/ml for ciprofloxacin, ≥16 μg/ml for gentamicin, ≥32 μg/ml for aztreonam; ≥32 μg/ml for chloramphenicol; 8 μg/ml for trimethoprim-sulfamethoxazole, ≥16 μg/ml for tetracyclin (Ref. [19]).

Table 5
Antifungal activity of Bac7(1–35) against collection strains and clinical isolates

Organism (number of strains)	MIC (μM) or range of MIC[a]
C. neoformans ATCC 90112	32
C. neoformans ATCC 52817[b]	8
C. neoformans ATCC 52816[b]	8
C. neoformans ATCC 90113	4
C. neoformans (9)	1–32
P. etchellsii (2)	>32
C. albicans (6)	>64

[a] MIC was defined as the lowest concentration of peptide that prevented fungal visible growth after incubation for 48 h at 30 °C. Results given are mean values of at least three independent determinations.
[b] Acapsular mutant.

negative bacteria, both Bac7 fragments were ineffective against the Gram-positive isolates tested (*Staphylococcus* and *Enterococcus*), showing MIC values >64 μM (data not shown).

3.3. Activity of Bac7 fragments against fungal isolates

The antifungal activity of Bac7(1–35) was tested against collection strains and clinical isolates of *C. neoformans*, as well as against clinical isolates of *C. albicans* and *P. etchellsii*. The peptide was active against all the strains of *C. neoformans* tested, with MIC values ranging from 1 to 32 μM (Table 5). The antifungal activity was not significantly different between encapsulated and acapsular mutant strains of *Cryptococcus*, indicating that the structure of the capsule is not a barrier for the peptide. By contrast, *C. albicans* and *P. etchellsii* were completely resistant to Bac7(1–35) (MIC > 64 μM).

4. Discussion

In this study we investigated the in vitro antimicrobial activity of synthetic fragments of the Pro-rich peptide Bac7. The peculiar sequence of this peptide prompted us to try to identify which region between the highly cationic N-terminus and the following tandem tetradecamer repeats, was required for antimicrobial activity. With this aim, ten peptides of different length (from 13 to 35 residues) and covering the whole sequence of Bac7 were chemically synthesized. Their activity was tested against a panel of reference and clinical isolates of bacteria and fungi, including multi-drug resistant isolates.

The results showed that the minimum requirements to maintain antimicrobial activity are an intact cationic N-terminal sequence and a length of at least 16 residues. In fact, removal of the C-terminal Arg from Bac7(1–16), to give Bac7(1–15), virtually abolished the activity of the peptide. Similarly, deletion of the four N-terminal residues (RRIR) in peptides Bac7(5–23) and Bac7(5–35) greatly diminished or virtually abolished the antimicrobial activity, albeit a sufficient length was present. By this study, we also determined that Bac7(1–35) displayed an activity identical or, in some cases, slightly higher than that of the 60-residue Bac7, thereby making this fragment a model for the whole natural peptide.

The observation that the N-terminal region is the active domain of Bac7 is in agreement with previous structure/activity relationship studies carried out on Bac5 and PR-39, two other mammalian Pro-rich peptides. Although showing a low degree of sequence identity, these peptides share a similar arrangement of the molecule. They all show a highly cationic N-terminal region followed by more hydrophobic, proline-rich repeats, and arginine rather than lysine is the cationic residue. Furthermore, in all cases it was found that their antimicrobial activity was dependent on the N-terminal region and could be maintained by fragments of 15–18 residues [6,28,30].

The present study also adds to the notion that the Pro-rich peptides are mainly active against Gram-negative species and are completely ineffective against *Staphylococcus* and *Enterococcus* strains [10]. In addition, it shows that Bac7(1–35) exerts a considerable activity against collection strains and clinical isolates of *C. neoformans*, irrespective of the presence or absence of a polysaccharide capsule. In contrast, none of the peptides was active against *C. albicans* or *P. etchellsii*. Overall, these results are partially in contrast with those of Sadler et al. [25], reporting that Bac7 and some of its fragments were also able to kill *S. aureus* and *C. albicans* at micromolar concentrations. In addition, they observed that some C-terminal fragments of Bac7 were active against these microorganisms, although at a higher concentration than the N-terminal fragments [25]. These contrasting results find an explanation in the different antimicrobial assays used, that in the case Sadler et al. [25] include low concentrations of surfactants (0.02% Tween 20) that are known to greatly increase the susceptibility of bacteria to peptides' action, by altering the membrane permeability properties [16].

Following the initial SAR studies with collection strains, Bac7(1–35) and Bac7(1–16) were assayed against a panel of nosocomial bacterial isolates. Both of them showed a considerable antimicrobial activity against Gram-negative strains with species-specific differences in their potency. A high activity was in general displayed towards the *Enterobacteriaceae*. In particular, all the *E. coli* and *K. pneumoniae* strains were found to be highly susceptible to the action of Bac7(1–35) and, to a minor extent, of Bac7(1–16). The remarkable activity against the above species is particularly important when considering that *E. coli* and *K. pneumoniae* account for about 95% of urinary tract infections. These are the most common infections in the hospital setting, and rank second among the most common infections in the general population [31]. In addition to the antibacterial activity against *E. coli* and *K. pneumoniae* strains, both fragments were also highly effective against all the ten isolates of *S. enterica* tested (Table 3). This result is noteworthy when considering that

S. enterica resistance to several critical antimicrobials used to treat invasive salmonellosis, such as extended spectrum cephalosporins and quinolones, is increasing at a worrying rate [22].

In addition, we found that the activity of the two fragments is not limited to enteric bacteria but also includes non-fermenting Gram-negative species, such as *A. baumannii* and *P. aeruginosa*, and fungi, such as *C. neoformans*, all known pathogens causing opportunistic infections in critically ill patients and nosocomial outbreak situations [7,17]. Susceptibility of *A. baumannii* and *P. aeruginosa* to Bac7(1–35) is worthy of note, since they are generally characterized by high rates of intrinsic resistance to antibiotics [13] and represent a problem for the management of hospitalized patients [5].

Understanding the molecular basis of the differences in the effectiveness of the various fragments here discussed will require deeper investigations on the mechanism of action of Bac7. The fact that this peptide and its active fragments are effective against Gram-negative bacteria suggests that the outer membrane of these microorganisms is not a barrier. Bac7 is known to interact with LPS [11,27], and translocation through this membrane may occur via the "self-promoted uptake" mechanism [12]. As killing activity requires the presence of the cationic N-terminal region and a minimum length of 16 residues, based on the proposed mechanism of action of Pro-rich peptides, it can be hypothesized that either Bac7(1–15) is too short to cross the bacterial membranes or that it has lost the capacity to interact with a putative intracellular target. The former hypothesis is less likely as Sadler et al. [25] observed that the 10-residue peptide Bac7(15–24) maintains a relevant cell permeant activity in eukaryotic cells in spite of a complete loss of antibacterial activity [25]. The fact that Gram-positive microorganisms are resistant to Bac7 suggests that either the internal target is missing, or that translocation across the membrane does not occur. This could derive from the different membrane composition if translocation is self-promoted, or from the absence of the appropriate translocation machinery, if it is assisted. The indication that Bac7 is active on *S. aureus* if the membrane is rendered more permeable with detergent, suggests that this resistance is likely translocation-dependent.

The above data indicate that truncated variants of Bac7 retain antibacterial activity when an intact N-terminal domain and a sufficient length are present, thereby providing fragments of Bac7 that may constitute a reasonable starting point for the design of peptides or peptidomimetic molecules to be tested as novel lead compounds for the treatment of infections caused by multidrug-resistant Gram-negative species.

Acknowledgments

We thank Dr. Alex Tossi for critically reading the manuscript. This work was supported by grants from MIUR (PRIN 2002) and from Regione Friuli Venezia Giulia (research grants LR n. 3/1998).

References

[1] Agerberth B, Lee JY, Bergman T, Carlquist M, Boman HG, Mutt V, et al. Amino acid sequence of PR-39. Isolation from pig intestine of a new member of the family of proline-arginine-rich antibacterial peptides. Eur J Biochem 1991;202:849–54.
[2] Anderson RC, Hancock RE, Yu PL. Antimicrobial activity and bacterial-membrane interaction of ovine-derived cathelicidins. Antimicrob Agents Chemother 2004;48:673–6.
[3] Boman HG, Agerberth B, Boman A. Mechanisms of action on *Escherichia coli* of cecropin P1 and PR-39, two antibacterial peptides from pig intestine. Infect Immun 1993;61:2978–84.
[4] Buck MA, Olah TA, Weitzmann CJ, Cooperman BS. Protein estimation by the product of integrated peak area and flow rate. Anal Biochem 1989;182:295–9.
[5] Canton R, Coque TM, Baquero F. Multi-resistant Gram-negative bacilli: from epidemics to endemics. Curr Opin Infect Dis 2003;16:315–25.
[6] Chan YR, Zanetti M, Gennaro R, Gallo RL. Anti-microbial activity and cell binding are controlled by sequence determinants in the antimicrobial peptide PR-39. J Invest Dermatol 2001;116:230–5.
[7] Clark NM, Patterson J, Lynch 3rd JP. Antimicrobial resistance among gram-negative organisms in the intensive care unit. Curr Opin Crit Care 2003;9:413–23.
[8] Frank RW, Gennaro R, Schneider K, Przybylski M, Romeo D. Amino acid sequences of two proline-rich bactenecins. Antimicrobial peptides of bovine neutrophils. J Biol Chem 1990;265:18871–4.
[9] Gennaro R, Skerlavaj B, Romeo D. Purification, composition, and activity of two bactenecins, antibacterial peptides of bovine neutrophils. Infect Immun 1989;57:3142–6.
[10] Gennaro R, Zanetti M, Benincasa M, Podda E, Miani M. Pro-rich antimicrobial peptides from animals: structure, biological functions and mechanism of action. Curr Pharm Des 2002;8:763–78.
[11] Ghiselli R, Giacometti A, Cirioni O, Circo R, Mocchegiani F, Skerlavaj B, et al. Neutralization of endotoxin in vitro and in vivo by Bac7(1–35), a proline-rich antibacterial peptide. Shock 2003;19:577–81.
[12] Hancock RE. Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect Dis 2001;1:156–64.
[13] Hancock RE. Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. Clin Infect Dis 1998;27(Suppl 1):S93–9.
[14] Kragol G, Lovas S, Varadi G, Condie BA, Hoffmann R, Otvos Jr L. The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry 2001;40:3016–26.
[15] Lehrer RI, Ganz T. Antimicrobial peptides in mammalian and insect host defence. Curr Opin Immunol 1999;11:23–7.
[16] Li J, Chikindas ML, Ludescher RD, Montville TJ. Temperature- and surfactant-induced membrane modifications that alter Listeria monocytogenes nisin sensitivity by different mechanisms. Appl Environ Microbiol 2002;68:5904–10.
[17] Lozano-Chiu M, Paetznick VL, Ghannoum MA, Rex JH. Detection of resistance to amphotericin B among *Cryptococcus neoformans* clinical isolates: performances of three different media assessed by using E-test and National Committee for Clinical Laboratory Standards M27-A methodologies. J Clin Microbiol 1998;36:2817–22.
[18] NCCLS, Performance standards for antimicrobial disk susceptibility tests; Approved standard, Seventh edition (M2-A7). Wayne, PA: National Committee for Clinical Laboratory Standards.
[19] NCCLS, Performance standards for antimicrobial susceptibility testing; Eleventh informational supplement (M100-S11). Wayne, PA: National Committee for Clinical Laboratory Standards.
[20] Oppenheim JJ, Biragyn A, Kwak LW, Yang D. Roles of antimicrobial peptides such as defensins in innate and adaptive immunity. Ann Rheum Dis 2003;62(Suppl 2):17–21.

[21] Otvos Jr L. The short proline-rich antibacterial peptide family. Cell Mol Life Sci 2002;59:1138–50.
[22] Parry CM. Antimicrobial drug resistance in *Salmonella enterica*. Curr Opin Infect Dis 2003;16:467–72.
[23] Poole K. Multidrug resistance in Gram-negative bacteria. Curr Opin Microbiol 2001;4:500–8.
[24] Poole K. Overcoming multidrug resistance in gram-negative bacteria. Curr Opin Investig Drugs 2003;4:128–39.
[25] Sadler K, Eom KD, Yang JL, Dimitrova Y, Tam JP. Translocating proline-rich peptides from the antimicrobial peptide bactenecin 7. Biochemistry 2002;41:14150–7.
[26] Shai Y. Mode of action of membrane active antimicrobial peptides. Biopolymers 2002;66:236–48.
[27] Shamova O, Brogden KA, Zhao C, Nguyen T, Kokryakov VN, Lehrer RI. Purification and properties of proline-rich antimicrobial peptides from sheep and goat leukocytes. Infect Immun 1999;67:4106–11.
[28] Shi J, Ross CR, Chengappa MM, Sylte MJ, McVey DS, Blecha F. Antibacterial activity of a synthetic peptide (PR-26) derived from PR-39, a proline-arginine-rich neutrophil antimicrobial peptide. Antimicrob Agents Chemother 1996;40:115–21.
[29] Skerlavaj B, Benincasa M, Risso A, Zanetti M, Gennaro R. SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes. FEBS Lett 1999;463:58–62.
[30] Tokunaga Y, Niidome T, Hatakeyama T, Aoyagi H. Antibacterial activity of bactenecin 5 fragments and their interaction with phospholipid membranes. J Pept Sci 2001;7:297–304.
[31] Valiquette L. Urinary tract infections in women. Can J Urol 2001;8:6–12.
[32] Waddell WJA. Simple ultraviolet spectrophotometric method for the determination of protein. J Lab Clin Med 1956;48:311–4.
[33] Yasin B, Pang M, Turner JS, Cho Y, Dinh N-N, Waring AJ, et al. Evaluation of the inactivation of infectious herpes simplex virus by host-defense peptides. Eur J Clin Microbiol Infect Dis 2000;19:187–94.
[34] Zanetti M, Gennaro R, Romeo D. Cathelicidins: a novel protein family with a common proregion and a variable C-terminal antimicrobial domain. FEBS Lett 1995;374:1–5.