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In vitro and in vivo antimicrobial activity of two α-helical cathelicidin peptides and of their synthetic analogs

Monica Benincasa [a,1], Barbara Skerlavaj [b,1], Renato Gennaro [a], Antonio Pellegrini [c], Margherita Zanetti [b,d,*]

[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] Division of Applied Physiology, Institute of Veterinary Physiology, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland
[d] National Laboratory C.I.B., AREA Science Park, I-34012 Padriciano-Trieste, Italy

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Abstract

Two α-helical antimicrobial peptides (BMAP-27 and -28) and four synthetic analogs were compared for in vitro and in vivo antimicrobial efficacy. All peptides proved active in vitro at micromolar concentrations against a range of clinical isolates, including antibiotic-resistant strains. BMAP-27 and two analogs were more effective towards Gram-negative, and BMAP-28 towards Gram-positive organisms. In addition, BMAP-28 provided some protection in vitro against human herpes simplex virus type 1 (HSV-1). The parent peptides and mBMAP-28 analog protected mice from lethal i.p. infections in an acute peritonitis model at peptide doses significantly lower than those toxic to the animals, suggesting a satisfactory therapeutic index.

Keywords: Cathelicidin peptide; α-Helical antimicrobial peptide; Synthetic analog; Antimicrobial activity; Clinical isolate; In vivo protection; Infection

1. Introduction

The emergence of clinical bacterial strains exhibiting resistance against conventional antibiotics has urged the search for novel anti-infective agents. Among the compounds that are currently under investigation for their therapeutic potential are a number of cationic antimicrobial peptides of the innate immune system, and their synthetic derivatives [11,23]. Cationic antimicrobial peptides are present in all living organisms as a first-line host-defense mechanism against invading microbes. Most of these peptides rapidly inactivate bacterial, fungal or viral pathogens by a mechanism which in most cases is mediated by disruption of integrity of the microbial membranes [18]. This relatively non-specific mechanism is difficult to evade by susceptible bacteria, as indicated by the relatively rare selection of bacterial strains resistant to cationic peptides [6] and can be considered an interesting feature of these peptides in view of their development as anti-infective drugs.

In this study, we investigated the therapeutic potential of two natural peptides belonging to the mammalian cathelicidin peptide family [8,12,22]. These molecules were denoted BMAP-27 and -28 (BMAP is an acronym for 'bovine myeloid antimicrobial peptide', to indicate that both are antimicrobial components of the bovine neutrophils). The two peptides are 26 and 27 amino acid residues long, respectively, and are C-terminally amidated. They share a 28.5% sequence identity and an additional 50% similarity [19]. Prior structural studies suggested that both molecules include a 1–18 N-terminal region that undergoes an α-helical conformation in membrane mimicking environments, and a non-helical, hydrophobic C-terminal region. Both BMAPs kill Gram-negative and -positive bacteria and fungal species at micromolar concentrations, and retain strong and broad spectrum antimicrobial activity in the presence of physiologic salt concentrations [19]. In addition, in vitro antibacterial and anti-parasite efficacy against *Leptospira interrogans* serovars and against *Cryptosporidium parvum* has recently been reported for BMAP-28 [10,17]. The mechanism of action of these peptides is based on the ability to rapidly bind to and permeabilize the membranes of target microorganisms. However, BMAP-27 and -28 prove toxic to mammalian cells in a concentration range above

* Corresponding author. Tel.: +39-0432-494-390; fax: +39-0432-494-301.
E-mail address: zanetti@icgeb.trieste.it (M. Zanetti).
[1] These authors contributed equally to this work.

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10 μM, suggesting poor discrimination between prokaryotic and eukaryotic cell membranes. Structure–activity relationship (SAR) studies aimed to increase selectivity towards microbial organisms led to the design of truncated BMAP derivatives comprising the 1–18 N-terminal sequence and lacking the hydrophobic C-terminal region [19]. These compounds, denoted BMAP-27(1–18) and BMAP-28(1–18), were virtually devoid of cytotoxic and haemolytic activities and displayed antimicrobial potency comparable to, or only slightly lower than that of the parent BMAP peptides. Similar properties were also shared by an analog of BMAP-28 with modified C-terminal region, named mBMAP-28. Collectively, these SAR studies suggested that the hydrophobic C-terminal tail is a major determinant of the toxicity of BMAP molecules to mammalian cells [19].

As an important step to investigate their therapeutic potential, we extended the functional analysis of BMAPs and determined their in vitro and in vivo efficacy. Specifically, we analysed the in vitro activity of BMAP-27 and -28 and of their analogs against a wide panel of Gram-negative and -positive clinical isolates, also including many antibiotic-resistant strains, and against the human herpes simplex virus type 1 (HSV-1). Additionally, an 18-residue peptide comprising the C-terminal region and lacking the 1–9 N-terminal residues, has also been tested. Their potential to protect in vivo from infections by Gram-negative and -positive organisms was assessed using a mouse acute peritonitis model.

2. Materials and methods

2.1. Materials

Fmoc-PAL-PEG-PS resin, coupling reagents for peptide synthesis and Fmoc-amino acids were purchased from Applied Biosystems (Foster City, USA), Novabiochem (Laufelfingel, Switzerland) and ChemImpex (Wood Dale, IL, USA). Peptide synthesis-grade *N*,*N*-dimethylformamide, *N*-methyl-2-pyrrolidone, dichloromethane, dimethylsulfoxide, piperidine and HPLC-grade acetonitrile were from Biosolve (Valkenswaard, The Netherlands). Trifluoroacetic acid, *N*-methylmorpholine and trifluoroethanol (TFE) were obtained from Acros Chimica (Beerse, Belgium), 1,8-diaza bicyclo (5.4.0)-undec-7-ene (DBU) and *N*-acetylimidazole from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Peptide synthesis, cleavage and purification

The peptides BMAP-27 and -28 and their analogs BMAP-27(1–18), BMAP-28(1–18), BMAP-28(10–27), and mBMAP-28 were synthesized as previously described [19] on a Milligen 9050 automated synthesizer (Applied Biosystems, Foster City, USA) using the Fmoc chemistry. Side chain protecting groups were as follows: Trityl (Trt) for His, *t*-butyl (*t*Bu) for Tyr and Ser, *t*-butyloxycarbonyl (Boc) for Lys, and Trp and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) for Arg. To obtain C-terminally amidated peptides, the Fmoc-PAL-PEG-PS resin (0.16 mEq/g) was used. The synthesis was carried out at 45 °C in *N*,*N*-dimethylformamide (DMF) with a six-fold excess of Fmoc-amino acids, activated in situ by an equivalent amount of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU), in the presence of 0.6 N *N*-methylmorpholine. To improve the yield, difficult couplings were performed with an eight-fold amino acid excess, using an equimolar amount of *O*-(7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) as coupling agent. After cleavage and deprotection with a mixture of trifluoroacetic acid/water/thioanisole/phenol/ethandithiol/triisopropylsilane (81.5:5:5:5:2.5:1, v/v) for 2 h at room temperature, the crude peptides were repeatedly extracted with methyl butyl ether and purified by reversed phase high performance liquid chromatography (RP-HPLC) on a preparative (19 mm × 300 mm) C18 Delta-Pak column (Waters, Bedford, MA, USA) using an appropriate 0–80% water/acetonitrile gradient in the presence of 0.05% trifluoroacetic acid. Molecular masses were determined by electrospray mass spectrometry (ES-MS), using an API I instrument (PE SCIEX, Toronto, Canada). Purified peptides were dissolved in 0.05% TFA at approximately 1 mM concentration, divided in small aliquots and kept frozen at −20 °C until use. The concentration of BMAP-27 and BMAP-27(1–18) was determined at 257 nm considering a molar extinction coefficient of 195.1 for each Phe residue [4], BMAP-28 peptides were measured at 280 nm, using a molar extinction coefficient of 5559 for Trp [4] and of 1280 for Tyr [7].

2.3. Bacterial strains

A total of 28 Gram-positive (10 *Staphylococcus aureus*, 10 *Enterococcus faecalis*, 5 *Enterococcus faecium*, 3 *Streptococcus agalactiae*), and 23 Gram-negative (10 *Acinetobacter baumanni*, 10 *Pseudomonas aeruginosa*, and 3 *Serratia marcescens*) bacterial strains were tested in this study. These clinical isolates were routinely characterized for antibiotic-resistance and were kindly provided by Prof. E. Tonin from the Department of Biomedical Sciences of the University of Trieste. The *Escherichia coli* O18:K1:H7 Bort, used in the acute peritonitis animal model, was a generous gift from Prof. P. Abraham from the Academic Medical Center, University of Amsterdam. All the strains were stored at −80 °C and subcultured in Luria–Bertani (LB) broth prior to use.

2.4. Antibacterial assays

The in vitro antibacterial activity of purified BMAP peptides and of their analogs was determined as the minimum inhibitory concentration (MIC) by a microdilution susceptibility test in 96-well microtiter plates, according to the

guidelines of the National Committee for Clinical Laboratory Standards (NCCLS). The activity was measured in Mueller–Hinton broth (Difco) with logarithmic-phase microorganisms as previously reported [20].

2.5. Antiviral assays

The HSV-1 and the porcine respiratory corona virus (PRCV) (kindly provided by Dr. Wunderli, Institute for Medical Virology, and Dr. Engels, Institute of Virology, Faculty of Veterinary Medicine, University of Zürich) were titrated by inoculation of Vero76 cells with HSV-1 and Swine Testicular cells with PRCV (cells kindly provided by Dr. Engels,) with 10-fold dilutions using the endpoint dilution method of Reed and Muench [16]. Cells were grown in Minimal Essential Medium (MEM) with Earle's salt and L-glutamin (Gibco BRL) and supplemented with 10% foetal calf serum (FCS, Gibco BRL), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco BRL). Swine testicular cells were cultured in Iscove's Modified Dulbecco's medium without L-glutamine, containing 25 mM HEPES, 1% non-essential amino acids and 1 mM Na-pyruvate.

2.5.1. Neutral red uptake

Neutral red uptake assay was performed following a procedure based on Borenfreund and Puerner [2]. Briefly, 48 h after infection, the maintenance medium was removed and the cells were incubated for 3 h with MEM containing 50 μg/ml neutral red (Fluka) (200 μl per well). Cells were then washed and fixed with 200 μl of 4% formaldehyde and 1% $CaCl_2$. The dye taken up by the cells was extracted using 200 μl of 1% acetic acid in 50% ethanol and the absorbance was measured at 550 nm in a MR710 Dynatech Microplate®-Reader (Dynatech Laboratories Inc., Chantilly, VA, USA). Each sample was tested in triplicate and the percent protection achieved by the compounds in the infected cells was calculated as described by Pauwels et al. [15].

2.5.2. Cytopathic effect inhibition

Increasing concentrations of peptides (from 0.5 to 50 μM) were dissolved in MEM and incubated with confluent cell monolayers, infected with 10^4 $TCDI_{50}$, in 96-well tissue culture plates for 30 min at 37 °C and 5% CO_2. After removal of the medium, the cell monolayer was washed with phosphate-buffered saline (PBS) and further incubated in MEM for 48 h. The inhibition of the cytopathic effect was assessed by light microscopy and measured by the neutral red uptake assay using infected Vero cells as control. Peptide cytotoxicity was evaluated by using the same assay, incubating uninfected cells with serial dilutions of each peptide for 30 min at 37 °C and 5% CO_2.

2.5.3. Virus yield inhibition

Confluent cell monolayers were infected and treated with the peptides as described in the previous paragraph. After 24 h incubation at 37 °C and 5% CO_2, the plates were frozen and thawed three times. Ten-fold dilutions of the supernatants were used to infect confluent cell monolayers, grown in 24-well plates, to quantify HSV-1 inhibition. After 1 h at 37 °C and 5% CO_2, the inoculum was removed, cell monolayers were washed once with PBS and overlaid with MEM supplemented with 0.8% carboxymethyl cellulose, 2% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin and incubated at 37 °C and 5% CO_2 for 3 days. After removal of the medium and fixation with methanol, monolayers were stained with 0.5% crystal violet and the plaque number was counted. Results of repeated experiments performed in triplicate were expressed as percentage of plaque inhibition by comparison with untreated control cell monolayers.

2.6. In vivo toxicity

For the in vivo experiments, male Balb/c mice of approximately 20 g and 4 weeks of age were obtained from Harlan Nossan (Harlan Nossan S.r.l., Correzzana, Milan, Italy). The in vivo toxicity of the peptides was investigated by injecting mice with increasing amounts of each BMAP peptide dissolved in sterile PBS via i.p. (0.3 ml per mouse) or i.v. (0.1 ml per mouse). The controls received the vehicle alone. Animal behaviour and survival were monitored over a 7-day period and the LD_{50} was calculated according to Litchfield and Wilcoxon [13].

2.7. Murine acute peritonitis model

For these experiments, an encapsulated strain of *E. coli* O18:K1:H7 Bort, a methicillin-resistant *S. aureus* (MRSA) and a reference strain of *P. aeruginosa*, ATCC 27853, were grown in Mueller–Hinton broth. Inocula containing an amount of bacteria expected to result in 90–100% mortality were prepared by diluting log-phase cultures in PBS. Depending on the test organism, inocula ranged from 4 to 8×10^4 CFU for *E. coli* O18:K1:H7 Bort, from 4 to 7×10^7 CFU for *P. aeruginosa* ATCC 27853 and from 3 to 4×10^8 CFU per mouse for MRSA. At least 10 mice per dose level were infected and monitored for survival over a 14-day period after infection. Test peptides (0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 mg/kg of BMAP-27; 0.2, 0.4, 0.8 and 1.6 mg/kg of BMAP-28; 1 and 4 mg/kg of BMAP-27(1–18) and BMAP-28(1–18) and 0.8, 1.6 and 3.2 mg/kg of mBMAP-28) were injected i.p. immediately after bacterial challenge. Control mice were given 0.3 ml of PBS.

2.8. Statistical analysis

MIC values are presented as the mean of at least four independent experiments. Statistical significance of the differences between mortalities in the in vivo experiments was determined by Fisher's exact test. A P value ≤ 0.05 was accepted as indicating significance.

3. Results

3.1. In vitro antibacterial activity

The sequences of all BMAP peptides used are shown in Table 1. The antibacterial activity of these peptides was tested by a microdilution susceptibility assay towards 53 bacterial isolates. These included a variety of clinically relevant, antibiotic-resistant species: MRSA, vancomycin-resistant *E. faecalis* (VREF) (Fig. 1) and multi-resistant strains of *P. aeruginosa* and *A. baumannii* (Fig. 2). BMAP-27 and -28 displayed a broad-spectrum activity with MIC values ranging from 1 to 8 μM in most cases. These values confirm and extend those previously obtained against a limited panel of ATCC strains [19]. Despite structural similarity, the two peptides revealed different and somewhat complementary spectra of activity, i.e. BMAP-27 was more effective against Gram-negative species (MIC values of 1–2 μM, Fig. 2 and Table 2), whereas BMAP-28 showed a better activity against Gram-positive strains (MIC values of 1–8 μM, Fig. 1 and Table 2). Importantly, similar or even lower MIC values were observed when the activity towards antibiotic-susceptible versus antibiotic-resistant strains (e.g. MSSA versus MRSA strains and VSEF versus VREF strains) was compared (Fig. 1).

Table 1
Sequences of BMAP-peptides and analogues

Peptide	Sequence
BMAP-27	GRFKRFRKKFKKLFKKLS*PVIPLLHL*-NH_2
BMAP-27(1–18)	GRFKRFRKKFKKLFKKLS-NH_2
BMAP-28	GGLRSLGRKILRAWKKYG*PIIVPIIRI*-NH_2
BMAP-28(1–18)	GGLRSLGRKILRAWKKYG-NH_2
BMAP-28(10–27)	ILRAWKKYG*PIIVPIIRI*-NH_2
mBMAP-28	GGLRSLGRKILRAWKKYG*PQATPATRQ*-NH_2

Peptides were synthesized by the solid phase method using Fmoc-chemistry. Italicized sequences correspond to the hydrophobic C-terminal region of BMAP-peptides. Modified residues in the sequence of mBMAP-28 are underlined.

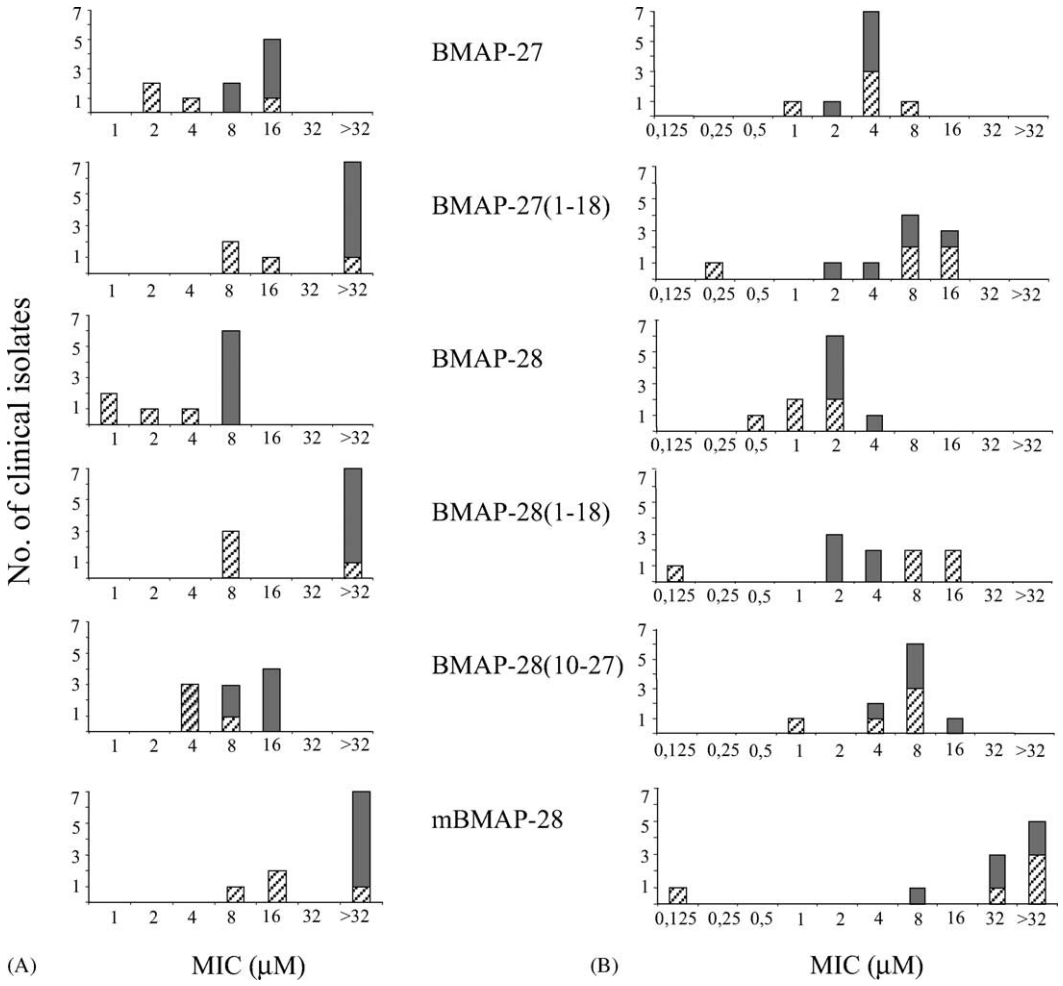

Fig. 1. Distribution of MIC values of BMAP peptides for Gram-positive clinical isolates. (A) *Enterococcus faecalis* (10 strains) and (B) *Staphylococcus aureus* (10 strains). The hatched part of bars refers to the vancomycin-resistant *E. faecalis* strains (A, four in total) or methicillin-resistant *S. aureus* strains (B, five in total).

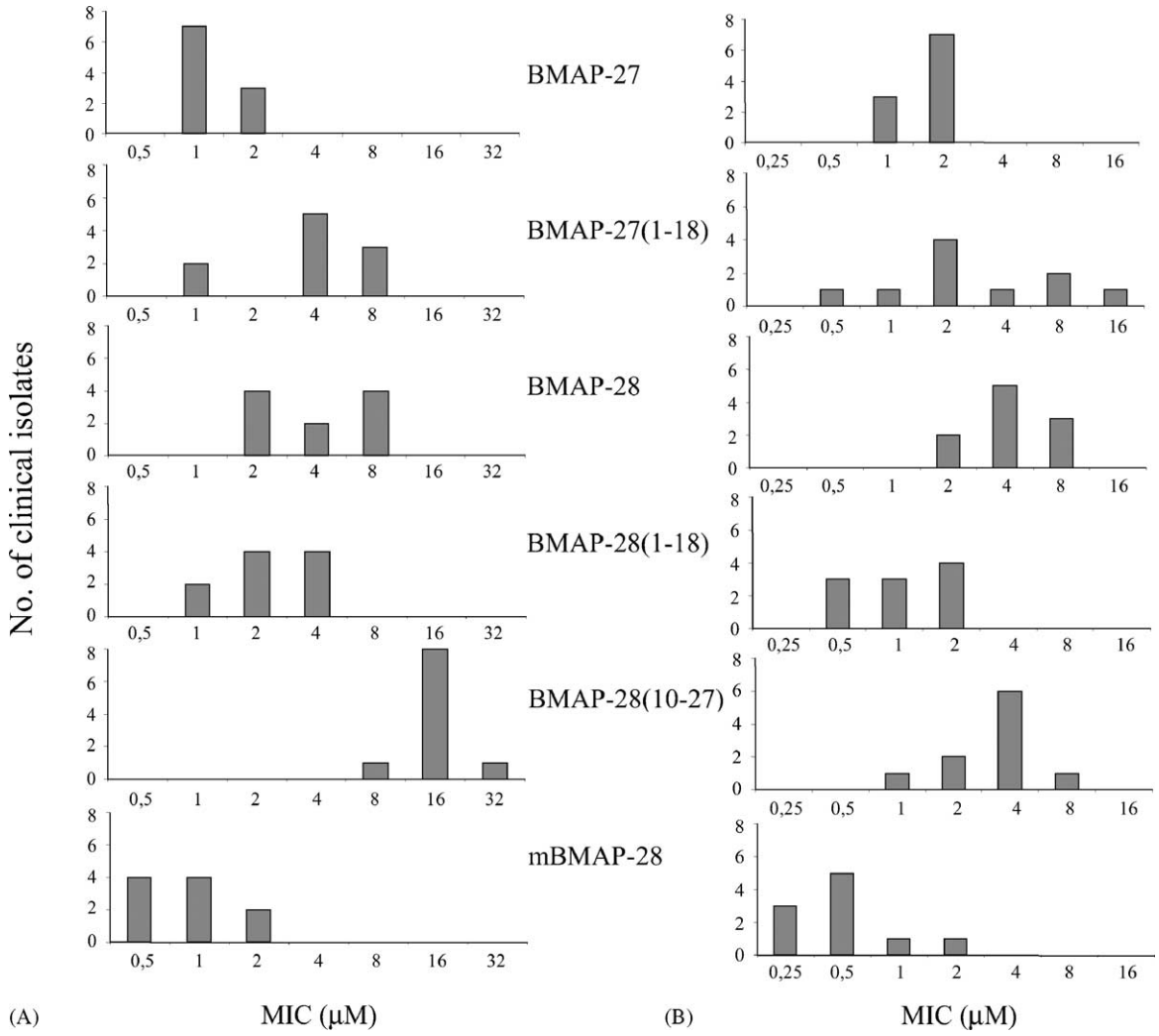

Fig. 2. Distribution of MIC values of BMAP peptides for Gram-negative clinical isolates. (A) *Pseudomonas aeruginosa* (10 strains) and (B) *Acinetobacter baumanni*.

The synthetic BMAP-27(1–18), BMAP-28(1–18) and BMAP-28(10–27) fragments retained antimicrobial activity comparable to that of the parent peptides, with some differences. The BMAP-27(1–18) fragment was slightly less effective than BMAP-27, with 2–4-fold higher MIC values against both Gram-negative and -positive bacteria (Figs. 1 and 2 and Table 2). Conversely, BMAP-28(1–18) was slightly less effective than BMAP-28 against Gram-positive strains but showed significantly improved activity against Gram-negative strains. Thus, based on these in vitro data, removal of the C-terminal hydrophobic sequence not only results in improved selectivity towards bacterial versus mammalian cells, as already reported [19], but also towards Gram-negative versus Gram-positive microorganisms. The BMAP-28(10–27) fragment, which lacks part of the N-terminal α-helix and maintains the C-terminal hydrophobic region of BMAP-28, showed comparable activity against both Gram-positive and -negative microorganisms, and was only slightly less potent than BMAP-28 against *P. aeruginosa* (MIC values increased from 2- to 4-fold, Fig. 2). The modified mBMAP-28 analog, characterized by a more hydrophilic C-terminal region than BMAP-28, was highly potent against Gram-negative strains, with MIC values significantly lower than those of BMAP-28 (MICs of 0.25–1 μM) towards *A. baumannii* and *P. aeruginosa* (Fig. 2). By contrast, this analog showed a marked decrease in the activity against Gram-positive strains (most MICs are >32 μM), with the remarkable exception of three highly susceptible *S. agalactiae* strains.

4. Antiviral activity in vitro

The ability of BMAP-27 and -28 and of their synthetic 1–18 fragments to inhibit the cytopathic effect of HSV-1 was determined with the neutral red uptake assay, using a broad range of peptide concentrations. Vero76 cell monolayers

Table 2
Antibacterial activity of BMAP-peptides against *E. faecium*, *S. agalactiae* and *S. marcescens*

Organism (no. of strains)	Peptide	Range of MIC[a] (μM)
E. faecium (5)	BMAP-27	2–16
	BMAP-27(1–18)	8–>32
	BMAP-28	1–8
	BMAP-28(1–18)	8–>32
	BMAP-28(10–27)	4–16
	mBMAP-28	16–>32
S. agalactiae (3)	BMAP-27	2–4
	BMAP-27(1–18)	1–4
	BMAP-28	2–4
	BMAP-28(1–18)	2–8
	BMAP-28(10–27)	4
	mBMAP-28	1–2
S. marcescens (3)	BMAP-27	1–8
	BMAP-27(1–18)	4–>32
	BMAP-28	4–16
	BMAP-28(1–18)	4–>32
	BMAP-28(10–27)	8–>32
	mBMAP-28	0.5–>32

[a] MIC was defined as the lowest concentration of peptide preventing visible bacterial growth after incubation for 18 h at 37 °C. All the strains were grown in Mueller–Hinton broth. Results were determined with 1–2 × 10^5 CFU/ml and are the mean of at least four independent experiments.

were incubated with 10^4 $TCDI_{50}$ of HSV-1 particles and 0.5–50 μM peptide for 30 min, then washed and incubated in the absence of the peptide for 48 h to observe cell growth. BMAP-28 was poorly cytotoxic at 5 μM, with only 17% of the cells taking up the neutral red dye. At this concentration, the peptide provided 72% protection from HSV-1 and inhibited viral replication to the same extent, as assessed by the virus yield inhibition assay (Table 3). BMAP-28(1–18) was also not toxic to Vero cells at 5 μM, but unlike BMAP-28, this peptide did not exert any protection from viral infection at any concentration tested (not shown). Despite structural resemblance to BMAP-28, BMAP-27 was cytotoxic even at the lowest peptide concentration tested (not shown), thereby precluding the evaluation of its antiviral activity. Both peptides were also tested against porcine respiratory corona virus using Swine Testicular (ST) cells. However, none of the peptides proved active against this virus at 0.5–50 μM (not shown), further supporting poor antiviral activity of these peptides.

Table 3
Antiviral activity of BMAP-28 against human herpes simplex virus type 1

Concentration (μM)	Cell survival[a] (%)	Cell protection[b] (%)	Virus yield inhibition[c] (%)
0.5	100	0	10
5	83	72	72
50	5	0	Not tested

[a] Confluent Vero76 cells were incubated with BMAP-28 for 30 min at 37 °C, then washed and resuspended in growth medium. Cell survival was determined by measuring the uptake of neutral red after incubation for 48 h at 37 °C.

[b] Cells were incubated with peptide and viral particles (10^4 $TCDI_{50}$) for 30 min at 37 °C, then washed and resuspended in growth medium. Cell protection was determined by measuring the uptake of neutral red after 48 h incubation at 37 °C.

[c] Quantification of HSV-1 inhibition was performed by virus yield inhibition assay, as described in Section 2. The results are the mean of two independent experiments performed in triplicate.

4.1. In vivo toxicity

As a first step to evaluate the therapeutic potential of BMAP peptides, the in vivo toxicity of the parent peptides and of their 1–18 fragments was determined in mice injected intraperitoneally and/or intravenously with increasing single peptide doses. No toxicity was observed when any of the peptides were administered i.p. up to 32 mg/kg, whereas doses of 46 mg/kg resulted in 80–100% mortality within 3 days from injection, and the LD_{50} values were determined to be in the range 38–44 mg/kg. The toxicity of BMAP-27 and -28 after intravenous administration proved much higher, with LD_{50} values of 10 and 15 mg/kg, respectively. These results indicate that the compounds are not safe via i.v., and therefore, their therapeutic potential is restricted to topical applications.

4.2. In vivo efficacy in an acute peritonitis model

The potential of BMAPs to protect mice from a bacterial challenge was tested in a model of acute peritonitis induced by i.p. injection of a lethal dose of *P. aeruginosa* ATCC 27853 (4–7 × 10^7 CFU/ml), *E. coli* O18:K1:H7 Bort (4–8 × 10^4 CFU/ml), an encapsulated strain isolated from a neonatal meningitis patient, or MRSA (3–4 × 10^8 CFU/ml). All these strains were susceptible to BMAPs in vitro in the micromolar concentration range, as shown in Table 4. Adult

Table 4
In vitro susceptibility of the bacterial strains used in peritonitis animal model to BMAP peptides

Bacterial strain	MIC (μM)					
	BMAP-27	BMAP-27(1–18)	BMAP-28	BMAP-28(1–18)	BMAP-28(10–27)	mBMAP-28
E. coli O18:K1:H7 Bort	0.5	8	2	2	2	2
P. aeruginosa ATCC 27853	1	2	2	2	8	2
S. aureus MRSA	4	4–8	2	4–8	8	>32

Table 5
Protective effect of BMAP-27 and -28 against *E. coli*, *P. aeruginosa* and *S. aureus* in an acute peritonitis mouse model

Mouse group receiving	*E. coli* O18:K1:H7		*P. aeruginosa* ATCC 27853		*S. aureus* (MRSA)	
	Survival[a]	*P*[b]	Survival[a]	*P*[b]	Survival[a]	*P*[c]
No infection	10/10		10/10		10/10	
No peptide	0/10		0/10		0/10	
BMAP-27 (mg/kg)						
0.2[c]	10/10	<0.0001	–		–	
0.4	10/10	<0.0001	8/10	0.0007	–	
0.8	–		10/10	<0.0001	–	
1.6	–		–		3/10	ns[d]
3.2	–		–		2/10	ns
6.4	–		–		2/10	ns
BMAP-28 (mg/kg)						
0.2	–		–		5/10	0.0325
0.4	2/10	ns	1/10	ns	8/10	0.0007
0.8	8/10	0.0007	3/10	ns	10/10	<0.0001
1.6	10/10	<0.0001	8/10	0.0007	–	

[a] Number of mice surviving/total number.
[b] By Fisher's exact test.
[c] Dose of peptide administered i.p. in a single injection after intraperitoneal bacterial challenge.
[d] Not significant.

male Balb/c mice were infected i.p. with a lethal dose of the test microorganism, immediately followed by a single dose of peptide. The number of survivors was monitored for 2 weeks and compared to that of control mice that only received the lethal bacterial challenge or the vehicle. The results of these experiments are summarized in Table 5.

As little as 0.2 mg/kg of BMAP-27 resulted in full protection of *E. coli*-infected mice. A higher dose (0.8 mg/kg) of this peptide was required to obtain 100% protection from *P. aeruginosa*. Conversely, BMAP-27 was poorly effective against *S. aureus*, even at 6.4 mg/kg peptide concentration. BMAP-28 provided a 100% protection against *S. aureus* and *E. coli*-infected mice, respectively, at 0.8 and 1.6 mg/kg. This compound was somewhat less effective against *P. aeruginosa*, with 80% protection at a dose of 1.6 mg/kg. The in vivo results thus reflect the in vitro behaviour of the two peptides, BMAP-27 being more effective against Gram-negative, and BMAP-28 against Gram-positive bacterial strains.

The effects of the 1–18 fragments were tested in mice i.p. infected with *P. aeruginosa* and MRSA. The compounds were administered i.p. at 1 or 4 mg/kg immediately after bacterial challenge. Only BMAP-27(1–18) showed 100% protection of *P. aeruginosa*-infected mice at 4 mg/kg, and neither peptide was effective against *S. aureus* challenge (data not shown). Finally, the protective effect of mBMAP-28 against a lethal dose of i.p. injected *P. aeruginosa* was tested at 0.8, 1.6 and 3.2 mg/kg. The former dose was ineffective, while the latter two doses protected 60 and 100% of the infected mice, respectively (data not shown). The mBMAP-28 analog was not tested in the MRSA-induced peritonitis model, since it proved inactive in vitro against Gram-positive microorganisms.

5. Discussion

In this study, we investigated the in vitro and in vivo activity of two cathelicidin peptides, BMAP-27 and -28, and of fragments and analogs derived from them. The in vitro activity was tested against a wide panel of clinical isolates including MRSA and VREF strains, as well as *A. baumannii* and *P. aeruginosa* multi-resistant strains. The results indicate a broad spectrum activity for the parent BMAP-27 and -28, which are comparably effective against antibiotic-resistant and antibiotic-susceptible isolates. This further confirms that the mechanism of action of these peptides, based on their ability to permeabilize bacterial membranes [19], is different from that of the antibiotics to which the strains tested are resistant.

The truncated and substitution analogs, designed to probe the importance of the hydrophobic C-terminal region, showed significant differences in activity when compared with the parent BMAPs. The lack of the C-terminal region, or replacement of hydrophobic residues in this region with more hydrophilic ones, had no effect towards Gram-negative microorganisms and in some cases led to improved activity, as specially observed with mBMAP-28. Conversely, these modifications resulted in decreased activity against Gram-positive strains, with the notable exception of *S. agalactiae*, which remained highly susceptible. It is interesting to note that the decreased activity against Gram-positive bacteria parallels the decreased cytotoxicity towards mammalian cells observed upon removal or modification of the C-terminal tail [19]. Understanding of these differences in activity will require deeper investigations of bacterial surface components and of their interaction with the peptides.

While indicating potent and wide spectrum in vitro antibacterial activity, the results of this study do not support antiviral efficacy of BMAP peptides. In fact, only BMAP-28 provides some protection in vitro against HSV-1, whereas all the other peptides are ineffective at non-cytotoxic concentrations.

The results obtained with in vitro assays prompted us to exploit the in vivo potential of these peptides against lethal bacterial challenge in animals. The use of an acute peritonitis model in mice was suggested by the results of in vivo toxicity tests, that indicated a much better LD_{50} for i.p. rather than i.v. administration of these peptides. The in vivo results using the parent BMAPs are encouraging and parallel those obtained in vitro. BMAP-27 was highly protective against *E. coli* and *P. aeruginosa*, with 100% protection at respectively 0.2 and 0.8 mg/kg, and was considerably less effective against i.p. challenge with *S. aureus* (Table 5). Conversely, administration of BMAP-28 at 0.8 mg/kg resulted in 100% protection against i.p. injection of an MRSA strain and was less effective against *E. coli* and *P. aeruginosa*. Importantly, protection is achieved at peptide doses that are significantly lower than those which are toxic to animals (i.e. no i.p. toxicity up to 36 mg/kg), suggesting a satisfactory therapeutic index for these compounds. In keeping with the in vitro results, mBMAP-28 also protects mice from lethal i.p. dose of *P. aeruginosa*, whereas the 1–18 fragments are more effective in vitro than in vivo. These peptides are poorly or no protective in the mouse model used, suggesting that the hydrophobic C-terminal region is important for the in vivo activity.

Despite a wealth of published in vitro activity studies of antimicrobial peptides against antibiotic-resistant clinical isolates, there are only a few published reports of their in vivo activity. PG-1, a 18-residue cathelicidin peptide with a β-sheet structure, was tested in a mouse model of acute peritonitis induced by *S. aureus* and *P. aeruginosa* [21]. The effective doses of PG-1 compare well with those of BMAP-27 and -28. A highly potent PG-1 analog proved effective in reducing oral microflora in humans, suggesting that antimicrobial peptides may be useful for treatment of oral infections [14]. Other in vivo studies examined the effect exerted by the sheep cathelicidin peptide SMAP-29 in an ovine model of acute pulmonary infection. This peptide is highly similar to BMAP-28 (63% identity) and proved effective after pulmonary deposition in a lamb pneumonia model induced by *Mannheimia haemolytica*. SMAP-29 reduced the bacterial concentration in bronchoalveolar fluid and in consolidated pulmonary tissues, as well as the severity of the lesions in the lungs, suggesting it may find an application in the treatment of respiratory tract infections [3]. Further in vivo studies were performed with indolicidin, a bovine cathelicidin peptide, using an animal model of systemic fungal infection caused by intravenous injection of *Aspergillus fumigatus* spores [1]. Free indolicidin was highly toxic to mice when injected i.v. (all treated animals died at 12 mg/kg peptide), whereas administration of the peptide encapsulated in liposomes significantly reduced toxicity. Liposomal entrapped indolicidin at 40 mg/kg considerably increased animal survival, suggesting that liposome carriers may improve the therapeutic index of toxic peptides [1]. Finally, indolicidin and other antimicrobial peptides were investigated in three rat models of septic shock. Treatment with all the peptides used in this study resulted in a significant reduction in plasma endotoxin and TNF-α concentrations and reduced the rates of animal deaths as compared to controls [9].

In addition to the above reported in vivo studies, several clinical trials have been performed or are currently underway on analogs derived from natural antimicrobial peptides. The results so far obtained indicate that, despite failure in a few cases, these peptides still have therapeutic potential, particularly for topical applications [5,23].

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