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SMAP-29: a potent antibacterial and antifungal peptide from sheep leukocytes

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Abstract SMAP-29 is a cathelicidin-derived peptide deduced from sheep myeloid mRNA. The C-terminally amidated form of this peptide was chemically synthesized and shown to exert a potent antimicrobial activity. Antibiotic-resistant clinical isolates highly susceptible to this peptide include MRSA and VREF isolates, that are a major worldwide problem, and mucoid *Pseudomonas aeruginosa* associated with chronic respiratory inflammation in CF patients. In addition, SMAP-29 is also active against fungi, including *Cryptococcus neoformans* isolated from immunocompromised patients. SMAP-29 causes significant morphological alterations of the bacterial surfaces, as shown by scanning electron microscopy, and is also hemolytic against human, but not sheep erythrocytes. Its potent antimicrobial activity suggests that this peptide is an excellent candidate as a lead compound for the development of novel antiinfective agents.

Key words: Antimicrobial peptide; Cathelicidin; Amphipathic helix; Lytic peptide

1. Introduction

Gene-encoded antimicrobial peptides are a widespread host-defense mechanism. A great number has been characterized in the last 15 years in animals, plants and bacteria [1–3]. Most of these peptides display a good selectivity for microbial vs. host membranes. This is thought to result from differences in membrane composition, e.g. a high content of anionic phospholipids on the surface of the bacterial cytoplasmic membrane, presence of LPS in the outer membrane of Gram-negative microorganisms, and lack of cholesterol in bacterial membranes [4].

The protective function of antimicrobial peptides in host-defense has been convincingly demonstrated in *Drosophila*, where their reduced expression dramatically decreases survival after microbial challenge [5,6]. In mammals, this function is suggested by defective bacterial killing in the lung of cystic fibrosis (CF) patients and in the small intestine of MAT$^{-/-}$ mice. In CF patients this deficit is attributed to an abnormally high salt concentration in the airway surface fluid that inhibits the activity of β-defensin-1, an antimicrobial peptide expressed in human airway epithelial cells [7]. In MAT$^{-/-}$ mice the defect in bacterial killing depends on lack of matrilysin, a metalloproteinase of the Paneth cells that cleaves inactive procryptdins to active cryptdins, antimicrobial peptides that are released in small intestine [8].

The antimicrobial peptides found in mammals belong to the defensin (α- and β-defensins) and cathelicidin families. Peptides of the latter family are highly diverse and are synthesized at the C-terminus of precursors characterized by a conserved prosequence [9,10].

cDNA cloning of novel members of this family in sheep led to the identification of a putative peptide of 29 residues, named SMAP-29 [11] or SC5 [12], with a C-terminal glycine likely corresponding to an amidation signal. This highly cationic peptide was predicted to assume an amphipatic α-helical conformation and a corresponding synthetic peptide was shown to exert potent antimicrobial activity against a few bacterial strains (*Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*) and fungi (*Candida albicans*) [12]. Although preliminary, these observations suggested a high potency and broad spectrum of activity for this peptide.

In this study the structure and biological activities of SMAP-29 have been extensively characterized. We show that the peptide adopts an amphipatic α-helical conformation and exerts a highly potent antimicrobial activity in vitro against a broad spectrum of microorganisms, including antibiotic-resistant clinical isolates and fungi that cause serious infections. SMAP-29 acts by rapidly permeabilizing bacterial membranes and inducing remarkable changes in the surface morphology of susceptible microorganisms. Interestingly, the peptide is also hemolytic on human, but not sheep erythrocytes.

2. Materials and methods

2.1. Materials

PAL PEG-PS resin, coupling reagents for peptide synthesis and Fmoc amino acids were purchased from PerSeptive Biosystems (Framingham, MA, USA). Anhydroscan-grade dimethylformamide, *N*-methyl-2-pyrrolidone, dichloromethane and HPLC-grade acetonitrile were from Lab-Scan (Dublin, Ireland). Trifluoroacetic acid, *N*-methylmorpholine and trifluoroethanol (TFE) were obtained from Acros Chimica (Beerse, Belgium). Mueller-Hinton broth (MHB), yeast extract, agar, dextrose, bacteriological and mycological peptone were

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Abbreviations: MRSA, methicillin-resistant *Staphylococcus aureus*; VREF, vancomycin-resistant *Enterococcus faecalis*; CF, cystic fibrosis; TFE, trifluoroethanol; MHB, Mueller-Hinton broth; MIC, minimum inhibitory concentration; OM, outer membrane; IM, inner membrane

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from Difco Laboratories (Detroit, MI, USA). Melittin and *o*-nitrophenyl-β-D-galactopyranoside were purchased from Sigma (St. Louis, MO, USA) and PADAC from Calbiochem (La Jolla, CA, USA). All other reagents were of analytical grade.

2.2. Peptide synthesis

SMAP-29 was synthesized as a 28 residue, C-terminally amidated peptide by the solid phase method, using a Milligen 9050 synthesizer and the Fmoc chemistry. As several couplings were predicted to be difficult, the synthesis was performed at 48°C by heating the jacketed column and the solvent solutions. For each coupling step, the Fmoc-protected amino acid and coupling reagents were added in a 6- to 8-fold molar excess with respect to resin substitution. Couplings (30–60 min) were carried out with *N*-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoro-borate (TBTU), except for residues 8–14 and 20–25, when the highly efficient acylating reagent *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) was used. Following Fmoc deprotection and before addition of the following residue the resin was washed for 15 min with a solution of dichloromethane/dimethylformamide/*N*-methyl-2-pyrrolidone (1:1:1) containing 1% Triton X-100 and 2 M ethylencarbonate ('magic mixture') [13]. Amino acid side-chains were protected as follows: 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Arg), *t*-butoxycarbonyl (Lys), trityl (His) and *t*-butyl (Tyr and Thr). Cleavage from the resin and deprotection of the synthesized peptide were carried out with a solution of 90% trifluoroacetic acid, 3% water, 1% triisopropylsilane and 2% each of phenol, 1,2-ethanedithiol and thioanisole. After repeated precipitation with ether, the peptide was purified by RP-HPLC on a C18 column (Delta-Pak, Waters, Bedford, MA, USA), using an appropriate 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid.

2.3. CD spectroscopy

CD measurements were performed at room temperature on a Jasco J-600 spectropolarimeter, using 0.2 and 2 mm path length cells. Peptide samples (final concentration in the range 10–150 μM) were dissolved in 5 mM sodium phosphate buffer, pH 7.0, in the absence or presence of 15, 30 and 45% TFE. The α-helical content was estimated by using the equation $[\theta]/[\theta]_\alpha$, where $[\theta]$ is the mean molar ellipticity per residue at 222 nm, in ° cm^2 $dmol^{-1}$, and $[\theta]_\alpha$ the estimated molar ellipticity for a 100% helical peptide given by $-40\,000(1-2.5/n)$, where n is the number of residues in the peptide [14].

2.4. Antimicrobial and membrane-permeabilizing activities

The antimicrobial activity of SMAP-29 was evaluated by the broth microdilution susceptibility test as previously described [15]. The activity, expressed as minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), was determined against the following bacterial strains: *Escherichia coli* ATCC 25922, D21 and ML-35, *Salmonella typhimurium* ATCC 14028, *P. aeruginosa* ATCC 27853 and two clinical isolates from CF patients, *Serratia marcescens* ATCC 8100, *Proteus vulgaris* ATCC 13315, *Bacillus megaterium* Bm11, *Staphylococcus epidermidis* ATCC 12228, *S. aureus* ATCC 25923, Cowan 1 and two methicillin-resistant clinical isolates (MRSA), *Enterococcus faecalis* ATCC 29212 and a vancomycin-resistant clinical isolate (VREF). The antifungal activity was evaluated against clinical isolates of *C. albicans*, *Cryptococcus neoformans* and *Rhodotorula rubra*.

The permeabilizing effect of SMAP-29 on the outer and inner membranes of the lactose permease deficient, β-galactosidase constitutive *E. coli* ML-35 strain was evaluated as previously described [15], using the normally impermeant substrates PADAC and *o*-nitrophenyl-β-D-galactopyranoside for the periplasmic β-lactamase and cytoplasmic β-galactosidase, respectively. Erythrocytes were prepared from freshly collected, anticoagulated human or sheep blood. The assays were performed in phosphate buffered saline (PBS) by incubating 10% (vol/vol) erythrocyte suspensions with various amounts of peptide for 30 min at 37°C. The reaction was stopped with cold PBS and, after centrifugation at $10\,000\times g$ for 1 min, the supernatant carefully removed and the release of hemoglobin measured at 415 nm. The percentage of hemolysis was determined as $(A_{pep}-A_{blank})/(A_{tot}-A_{blank})\times 100$, where A_{blank} and A_{tot} correspond respectively to the hemolysis in the absence of the peptide and to 100% hemolysis as obtained by addition of 0.2% Triton X-100. Melittin was used as a positive control.

2.5. Scanning electron microscopy

Midlog phase *E. coli* ML-35 or methicillin-resistant *S. aureus* were resuspended at 10^8 CFU/ml in 10 mM Na-phosphate buffer, pH 7.4, supplemented with 100 mM NaCl (buffer A), and incubated at 37°C with SMAP-29. Controls were run in the presence of peptide solvent. After 30 min the cells were fixed with an equal volume of 5% glutaraldehyde in 0.2 M Na-cacodylate buffer, pH 7.4. After fixation for 2 h at 4°C, the samples were filtered on Isopore filters (0.2 μm pore size, Millipore, Bedford, MA, USA) and extensively washed with 0.1 M Na-cacodylate buffer, pH 7.4. The filters were then treated with 1% osmium tetroxide, washed with 5% sucrose in cacodylate buffer and subsequently dehydrated with a graded ethanol series. After lyophilization and gold coating, the samples were examined on a Leica Steroscan 430i instrument (Leica Inc., Deerfield, IL, USA).

2.6. Analytical assays

Peptide concentration was determined by measuring the absorbance of Tyr at 276 nm using an extinction coefficient of 1450 M^{-1} cm^{-1}. The molecular mass of the purified peptide was determined with an API I ion spray mass spectrometer (PE SCIEX, Toronto, Canada).

3. Results and discussion

3.1. Structural analysis of SMAP-29

SMAP-29 was chemically synthesized as a 28 residue peptide (RGLRRLGRKIAHGVKKYGPTVLRIIRIA) amidated at the C-terminus, as indicated by the presence of C-terminal glycine, a common amidation signal in cathelicidin peptides [9]. The correct peptide was obtained in greater than 60% yield and with a measured mass of 3198.0 ± 0.3 vs. a calculated mass of 3197.99 Da, and was homogeneous after preparative purification, as confirmed by mass and analytical RP-HPLC.

Secondary structure prediction studies based on the PHD profile network indicate that SMAP-29 can assume an α-helical conformation in the region preceding Gly-18 and Pro-19.

Table 1
Antimicrobial activity of SMAP-29

Organism and strain	MIC (μM)
E. coli ATCC 25922	0.25
E. coli ML-35	0.25
E. coli D21	0.12
S. typhimurium ATCC 14028	0.25
P. aeruginosa ATCC 27853	0.5
P. aeruginosa (isolate from FC patient)	0.25
P. aeruginosa (isolate from FC patient)	2.0
S. marcescens ATCC 8100	0.25
P. vulgaris ATCC 13315	>80
S. aureus ATCC 25923	0.5
S. aureus Cowan 1	0.5
S. aureus (MRSA, clinical isolate)	1.0
S. aureus (MRSA, clinical isolate)	0.5
S. epidermidis ATCC 12228	0.25
E. faecalis ATCC 29212	1.0
E. faecalis (VREF, clinical isolate)	1.0
B. megaterium Bm11	0.25
C. albicans (clinical isolate)	4.0
C. neoformans (clinical isolate)	1.0
C. neoformans (clinical isolate)	1.0
C. neoformans (clinical isolate)	1.0
R. rubra	0.5

MIC was defined as the lowest concentration of peptide preventing visible growth after 18 h (bacteria) and 48 h (fungi) incubation at 37°C. Bacteria and fungi were grown in Mueller-Hinton broth and in Sabouraud, respectively. Results, determined with approximately $1.0–2.0\times10^5$ (bacteria) and $0.2–0.4\times10^5$ (fungi) colony forming units/ml, are the mean of at least three independent determinations with a divergence of not more than one MIC value.

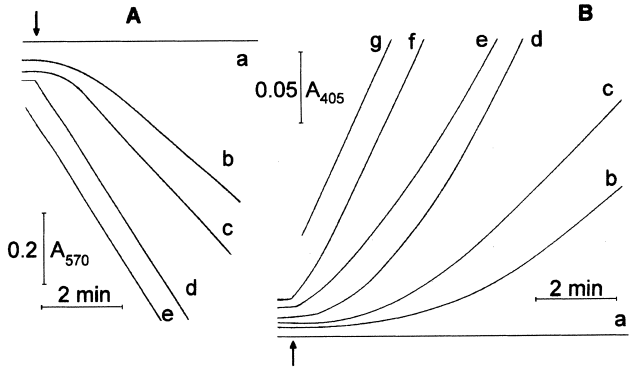

Fig. 1. Kinetics of permeabilization of *E. coli* ML-35 outer and inner membranes by SMAP-29. Permeabilization was determined spectrophotometrically by following the unmasking of the periplasmic β-lactamase (OM permeabilization) and the cytoplasmic β-galactosidase (IM permeabilization) activities. Each assay was performed with approximately 10^7 colony forming units/ml in 10 mM sodium phosphate buffer, pH 7.4, containing 100 mM NaCl. A: OM permeabilization. Trace a: untreated bacteria; traces b–d: 0.05, 0.1, 0.3 μM peptide; trace e: sonicated bacteria. B: IM permeabilization. Trace a: untreated bacteria; trace b: 0.3 μM peptide in the presence of 1 mM Ca^{2+}; traces c, e, f: 0.05, 0.1, 0.3 μM peptide; trace d: 0.3 μM peptide in the presence of 1 mM Mg^{2+}; trace g: sonicated bacteria. The arrows indicate addition of peptide.

These residues likely form a loop followed by an extended and highly hydrophobic C-terminal region, reminiscent of BMAP-27 and -28 from cattle [16]. The predicted helix in the 1–18 region is amphipathic. This is suggested by the helical wheel projection, that shows a striking segregation of polar and non-polar residues, and by a high mean hydrophobic moment per residue ($\mu = 0.861$) calculated according to Eisenberg [17]. This μ value is one of the highest found in a comparative analysis of a number of natural antimicrobial peptides [18] and suggests a potent antimicrobial activity for SMAP-29.

The structural prediction has been confirmed by circular dichroism. Spectra were recorded in 5 mM Na-phosphate buffer at pH 7.0 in the absence or presence of increasing amounts of the helix-inducing solvent TFE. CD spectra of SMAP-29 in aqueous buffer are typical of an unordered conformation. Addition of TFE induces a transition to an α-helical conformation with a helical content of 27.3, 51.5 and 57.6% at respectively 15, 30 and 45% (v/v) TFE (not shown). The helical content did not increase at higher TFE concentrations. The existence of an isodichroic point at approximately 203 nm is consistent with a two-state helix-coil equilibrium. The concentration-dependence and influence of anions on the conformation of SMAP-29 was investigated by recording CD spectra in 5 mM Na-phosphate buffer, pH 7.0, at peptide concentrations up to 150 μM, or at 40 μM in the presence of 15 mM bicarbonate. In both cases the CD spectra are typical of an unordered conformation, suggesting that SMAP-29 is monomeric and does not self-associate into helical oligomers under these conditions. In contrast, other α-helical peptides, e.g. human LL-37 and porcine PMAP-37, may oligomerize and assume an α-helical conformation in aqueous solution in an anion-, pH- and concentration-dependent manner [19,20].

3.2. Antimicrobial activity

The in vitro antimicrobial activity of SMAP-29 was determined as MIC and MBC values. A wide panel of Gram-negative and Gram-positive bacteria and of fungi was used, including clinical isolates of MRSA, VREF, mucoid

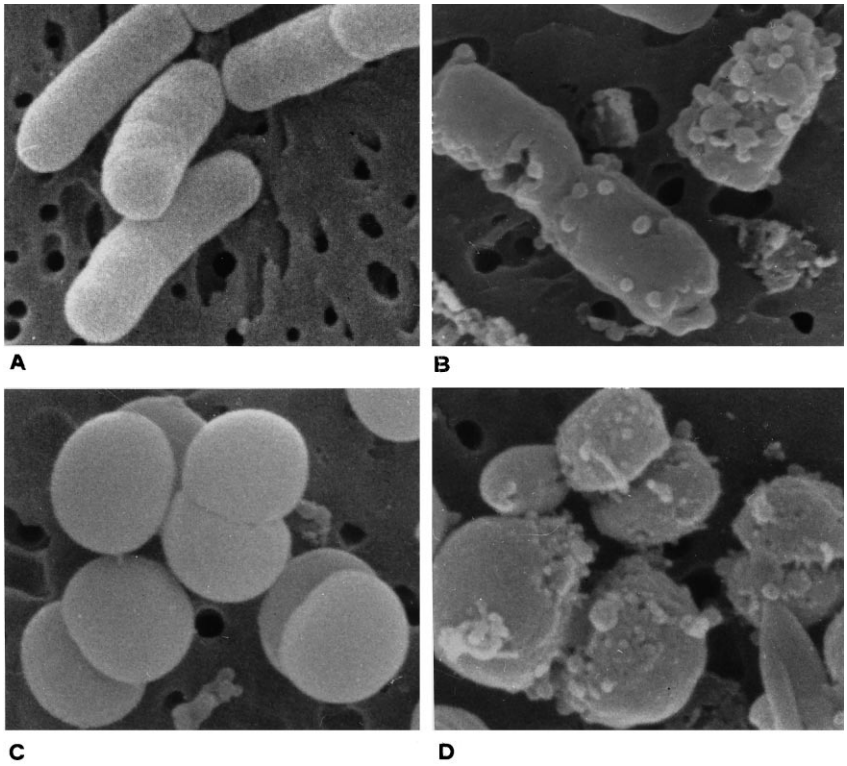

Fig. 2. Scanning electron micrographs of untreated (A and C) and after treatment for 30 min at 37°C with 2 μM (B) and 5 μM (D) SMAP-29 of *E. coli* (top) and *S. aureus* (bottom).

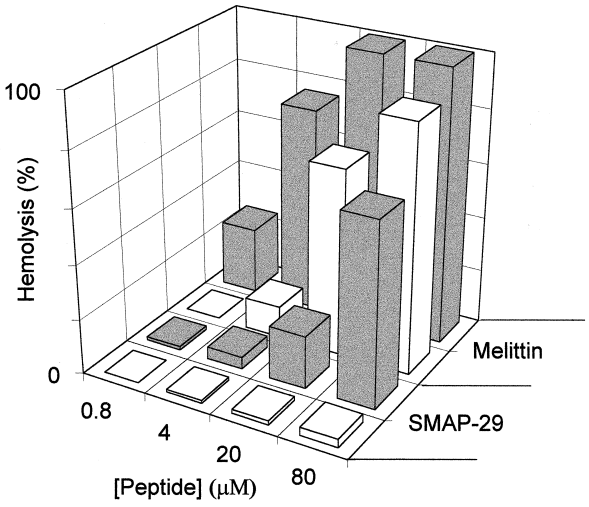

Fig. 3. Hemolytic activity of SMAP-29 and melittin on human and sheep erythrocytes. Hemolysis was evaluated by reading the absorbance at 415 mm of the supernatants of 10% (v/v) suspensions of sheep (open columns) or human (gray columns) erythrocytes incubated with the indicated peptide concentrations for 30 min at 37°C. Results are the mean of three to seven independent experiments with S.E.M. values ranging from ±0.1 to ±3.6.

P. aeruginosa from CF patients, and *C. neoformans* from patients with AIDS. All the bacterial strains tested are highly susceptible to SMAP-29 with MIC values in the 0.12–2 μM range of concentration (Table 1). The only exception is *P. vulgaris*, which is resistant even at 80 μM peptide. Interestingly, SMAP-29 is highly and equally active against antibiotic-resistant and antibiotic-susceptible clinical isolates of the same species, e.g. methicillin-resistant vs. methicillin-susceptible *S. aureus*. MBC values are in general identical to, or most 2-fold higher, than the MIC value, indicating that SMAP-29 is bactericidal and not only bacteriostatic (not shown). The peptide is also active against fungi such as *C. albicans*, *C. neoformans* and *R. rubra* at MIC values of 0.5–4 μM (Table 1). A comparison of the activity of SMAP-29 with that of other cathelicidin-derived peptides (e.g. BMAP-27, BMAP-28, PMAP-37 and BMAP-34) [15,16,20], tested with the same strains under the same conditions, clearly shows that this peptide is the most potent and displays the broadest spectrum of activity.

3.3. Membrane permeabilization and scanning electron microscopy

The ability of SMAP-29 to permeabilize the outer (OM) and inner (IM) membranes of the *E. coli* ML-35 strain was tested by real-time spectroscopy, following respectively the unmasking of the periplasmic β-lactamase and of the cytosolic β-galactosidase activities to normally non-permeant substrates. In buffer A, SMAP-29 at 0.3 μM caused an immediate permeabilization of the OM with a kinetics of PADAC hydrolysis superimposable to that of sonicated bacteria (100% permeabilization) (Fig. 1A). At 0.05 and 0.1 μM, the rate of hydrolysis was 49 and 56% that of sonicated bacteria, with a steady-state attained at respectively 4 and 2 min after peptide addition. Under the same conditions, a similar extent of IM permeabilization was obtained, with a slightly longer lag time (Fig. 1B). IM permeabilization was also tested in buffer A supplemented with Ca^{2+} or Mg^{2+} and in MHB, that was used for MIC determinations. At 0.3 μM SMAP-29, the presence of 1.0 mM Ca^{2+} caused an inhibition of approximately 60% in the extent of permeabilization and prolonged the lag time to attain the steady-state from 0 to 6 min (Fig. 1B). This inhibition likely depends on the stabilizing effect of Ca^{2+} on bacterial membranes through interaction with anionic sites on LPS and competition with the peptide for membrane binding. Interestingly, when Ca^{2+} was added a couple of minutes after the peptide no inhibition was observed in IM permeabilization (not shown). Mg^{2+} ions at 1.0 mM had the only effect of slightly prolonging the lag time (Fig. 1). An inhibitory effect was also observed when the assays were performed in MHB. At 0.5 μM peptide, the rate of IM permeabilization decreased from 100% in buffer A to about 65% in MHB, and the lag time to attain the steady-state was prolonged from 0 to 9 min (not shown). This effect might in part depend on the presence of Ca^{2+} in MHB and, in addition, of polyanionic peptides derived from the acidic hydrolysate of casein, the major component of MHB. These polyanions could complex the cationic peptides, thereby inhibiting their action, as shown for the human LL-37 and pig protegrin-1 [21].

The morphological changes induced by SMAP-29, peptide-treated *E. coli* ML-35 and *S. aureus* (MRSA strain) were examined by scanning electron microscopy. Untreated cells had a normal, smooth surface (Fig. 2A and C). In contrast, cells treated for 30 min with SMAP-29 showed surface roughening and blebbing (Fig. 2B and D). In *S. aureus*, blebs were more frequent at the division septum and were accompanied by long, filamentous projections (Fig. 2D). Cells often showed large holes in their surface and cellular debris likely arising from cell lysis were also observed. The SEM observations provide morphological evidence of the potent permeabilizing activity of SMAP-29. The membrane alterations are similar to those induced by protegrins [22] and the α-helical peptide PGYa, designed using a 'sequence template' approach [23].

Although expected the permeabilizing activity of SMAP-29 is considerably higher than that of other antimicrobial peptides evaluated under similar conditions, including defensins [24], the Pro- and Arg-rich peptides Bac5 and Bac7 [25], and various α-helical peptides such as PMAP-37 [20] and LL-37 [21]. Only BMAP-27 and BMAP-28 from cattle display a comparable permeabilizing activity [16]. These peptides have in common with SMAP-29 a hydrophobic C-terminal tail following the conserved Pro-19.

3.4. Hemolytic activity

The potential lytic activity of SMAP-29 on human and sheep erythrocytes was monitored by following hemoglobin release from 10% (v/v) cell suspensions. The peptide showed a significant hemolytic activity towards human cells, although at concentrations relatively higher than those effective against microorganisms. As shown in Fig. 3, SMAP-29 at 4, 20 and 80 μM caused lysis of respectively 4.4, 19.4 and 67% of the cells. When compared to other peptides with an amphipathic α-helical conformation, SMAP-29 is more active than magainins and cecropins, that are virtually not hemolytic [26,27], but less than melittin, a peptide used as a positive control (Fig. 3). The hemolytic activity of SMAP-29 likely depends on the balance of several factors that include a high hydrophobic moment, a relatively narrow angle subtended by the cationic residues, and the presence of a highly hydrophobic

region, as previously shown for both natural, such as BMAP-27 and -28 from cattle and LL-37 from humans [16,19], and model [28] antimicrobial peptides.

Unlike human, sheep erythrocytes are resistant to SMAP-29 with only 3% lysis even at 80 μM peptide. This likely depends on different contents of sphingomyelin (53% of total phospholipids in sheep vs. 25% in human) and phosphatidylcholine (< 2% of total phospholipids in sheep vs. 31% in human) in the red blood cells of the two species [29]. Decreased membrane fluidity due to high content of sphingomyelin has been suggested as a possible explanation for the lower susceptibility of sheep erythrocytes to several lytic agents [30], including peptides such as melittin (Fig. 3).

3.5. Conclusions

The above results show that SMAP-29 is a potent and broad spectrum peptide and suggest that it may be a good candidate as a lead compound for the development of novel antiinfective agents. Targets susceptible to this peptide include antibiotic-resistant clinical isolates that are a major worldwide problem [22] and *P. aeruginosa* associated with chronic respiratory inflammation in CF patients. In addition, SMAP-29 is also active against fungi that in the last few years have emerged as a major complication in immunocompromised patients [31]. On the other hand, the potent antimicrobial activity of this peptide is associated to toxicity towards mammalian cells. This undesirable feature might compromise its therapeutic use and should be reduced. Dissociation of antimicrobial and hemolytic activities has in other instances been obtained by modifying parameters such as hydrophobicity, amphipathicity and helicity [28], by removing hydrophobic regions [16,19], or by synthesizing diastereomer peptide analogs [32]. Work is in progress to synthesize SMAP-29 analogs retaining the antimicrobial, while decreasing hemolytic activity.

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