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Activity of antimicrobial peptides in the presence of polysaccharides produced by pulmonary pathogens‡

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Antimicrobial peptides (AMPs) are secreted in the airway and contribute to initial defence against inhaled pathogens. Infections of the respiratory tract are a major cause of morbidity and mortality in preterm newborns and in patients with cystic fibrosis (CF). In this latter group, the state of chronic lung infection is due to the ability of bacteria to grow as mucoid biofilm, a condition characterised by overproduction and release of polysaccharides (PSs). In this study, we investigate the effect of PSs produced by lung pathogens such as *Pseudomonas aeruginosa, Klebsiella pneumoniae* and members of the *Burkholderia cepacia* complex on the antibacterial activity of structurally different peptides. The AMPs tested in this study include the cathelicidin LL-37 and the β-defensin hBD-3 from humans, both released at the alveolar level, as well as peptides from other mammals, i.e. SMAP-29, PG-1 and Bac7(1-35). Susceptibility assays, time killing and membrane permeabilization kinetics experiments were carried out to establish whether PSs produced by lung pathogens may be involved in the poor defence reaction of infected lungs and thus explain infection persistence. All the PSs investigated inhibited, albeit to a different extent, the antibacterial activity of the peptides tested, suggesting that their presence in the lungs of patients with CF may contribute to the decreased defence response of this district upon infection by PS-producing microorganisms. The results also show that inhibition of the antibacterial activity is not simply due to ionic interaction between the negatively charged PSs and the cationic AMPs, but it also involves other structural features of both interactors. **Copyright © 2009 European Peptide Society and John Wiley & Sons, Ltd.**

Keywords: antimicrobial peptides; polysaccharides; cathelicidins; cystic fibrosis; pulmonary pathogens; lung infection; defensins

Introduction

Antimicrobial peptides (AMPs) are an important component of the innate immunity and have been found in all classes of living organisms. Their importance extends beyond their direct antimicrobial properties, and their broad spectrum of biological activities may also derive from their role as effector molecules providing communication between the innate and the adaptive immune systems [1].

In mammals, two principal classes of AMPs have been identified: defensins and cathelicidins [2–4]. Members of both classes are synthesised and stored by phagocytic cells and are expressed at epithelial surfaces, including those lining the bronchoalveolar tree.

The epithelium of the respiratory tract covers a large surface area that is needed for an appropriate rate of gas exchanges. This large surface is in intimate relationship with the environment and may thus encounter a range of microbes and toxic particulates. Despite the significant interplay between host and environment in this compartment, healthy individuals are rarely affected by lung infection, as many different strategies have evolved to prevent these. They include the following: (i) the barrier function of the epithelium, (ii) coughing, (iii) mucociliary clearance, (iv) resident and recruited phagocytes and (v) the presence of a thin layer of airway surface fluid (ASL) in which a number of substances with host defence function are secreted [5–7]. These substances include different antimicrobial proteins (e.g. lysozyme and lactoferrin) and AMPs, such as β-defensins (in particular, hBD-1, hBD-2 and hBD-3) and cathelicidins (LL-37) in humans, mainly produced and secreted by epithelial and phagocytic cells [5–9].

Infectious complications of the respiratory tract are common in various categories of individuals, including preterm newborns and those affected by cystic fibrosis (CF). In these latter patients, dangerous and difficult to eradicate lung infections are frequently caused by opportunistic species such as *Pseudomonas aeruginosa* and members of the *Burkholderia cepacia* complex (Bcc) [10]. Another important cause of respiratory infections is *Klebsiella pneumoniae*, responsible for significant morbidity and mortality in compromised individuals [11,12]. All these bacterial species are characterised by the ability to synthesise and release polysaccharides (PSs) that provide protection against opsonization and phagocytosis, and that are important structural components of the extracellular matrix in biofilms. They contribute to biofilm-related resistance by acting as a diffusion barrier to a variety of antimicrobial agents, thereby rendering eradication of the infection quite difficult [13].

In the present study, we investigated whether the PSs released by lung pathogens may inhibit the antibacterial activity of different

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AMPs, thus contributing to a decrease in the defence potential of the lung epithelium. To test this hypothesis, polysaccharides produced by *P. aeruginosa, K. pneumoniae* and by members of the *B. cepacia* complex were used to investigate their *in vitro* effect on the antibacterial activity of several AMPs from mammals. These included the α-helical cathelicidin LL-37 and the β-defensin hBD-3 from humans. Both peptides are secreted in the airway and play a role in the lung defence against infectious microorganisms [5,6,8]. AMPs from other mammals were also included in this study to test members of different structural groups, such as the β-hairpin protegrin-1 (PG-1) from pig and Bac7(1–35), the 1–35 fragment of the Pro-rich extended peptide Bac7, from cattle [4,14]. In addition, the α-helical cathelicidin SMAP-29 from sheep was also tested because of its high potency and very broad spectrum of activity [15].

Materials and Methods

Peptides

The cathelicidin-derived peptides LL-37, SMAP-29, PG-1 and Bac7(1–35), and the human β-defensin hBD-3 used in this study, were chemically synthesised by the solid phase method using the Fmoc strategy as previously reported [14–18]. Protected amino acids and the PEG-PS, PAL-PEG and 2-chlorotrityl chloride resins were purchased from Advanced Biotech Italia (Milan, Italy) and Novabiochem (Merck, Darmstadt, Germany). All the peptides were synthesised by using a Pioneer™ Peptide Synthesis System (PE, Foster City, California, USA) and purified by reversed-phase HPLC (ÄKTA Basic, Amersham Biotech, Uppsala, Sweden) using a X-Terra RP C_{18} column (19 × 300 mm; Waters, Massachusetts, USA), eluted with appropriate water/acetonitrile linear gradients from 0% to 60% acetonitrile in 0.05% trifluoroacetic acid. PG-1 was air-oxidised at a concentration of 0.1 mg/ml under gentle stirring in 0.1 M Tris-HCl buffer, pH 7.5, for 24–36 h at room temperature; the defensin hBD-3, a generous gift of Prof. Alex Tossi (Department of Life Sciences, University of Trieste), was oxidised as previously described [17]. The correct arrangement of the disulfide bridges in both peptides was tested by mass spectrometry after proteolytic digestion. The molecular masses of all the purified peptides were determined by ESI mass spectrometry using an API-I instrument (PE SCIEX, Toronto, Canada).

After purification, the peptides were lyophilised in 10 mM HCl, dissolved in double distilled water and stored at −20 °C until use.

Polysaccharides

The following polysaccharides were used: the polymer K40, alginate (ALG) and Cepacian (CEP), produced, respectively, by *Klebsiella pneumoniae* serotype K40 and by two *Pseudomonas aeruginosa* and *Burkholderia pyrrocinia* strains isolated from patients with CF.

The K40 polymer was purified and structurally characterised as previously described [19]. The CEP polysaccharide was chosen because it is produced by most of the strains belonging to the *B. cepacia* complex and its primary structure is reported in [20]. The ALG polysaccharide was a kind gift of Prof. I. W. Sutherland (University of Edinbourgh, Scotland).

Circular Dichroism Measurements

All CD measurements were made on a Jasco J-600 instrument at room temperature. Spectra were recorded in 5 mM sodium phosphate buffer at pH 7.4, using 10 mm quartz cells in the wavelength region from 190 to 270 nm. A correction for solvent baseline contribution was made digitally. The alginate/peptide concentration ratio was 65 and the peptide was used at 10 μM.

Evaluation of Antibacterial Activity

All experiments were performed with the *Escherichia coli* ML-35 strain. The MICs were determined in Mueller–Hinton broth (DIFCO, Becton Dickinson, Sparks, Maryland, USA) by using a microdilution susceptibility assay performed as previously described [14], according to the guidelines of National Committee for Clinical Laboratory Standards, except for LL-37 and hBD-3, whose MICs were determined, respectively, in 20% and 5% Tryptic Soy broth (Acumedia Manufacturers Inc., Baltimore, Maryland, USA) in 10 mM phosphate buffer, pH 7.4. The MIC value of each peptide, in the absence or presence of polysaccharides, was defined as the lowest concentration of peptide that inhibited visible growth of the organism. All the determinations were repeated at least three times in duplicate.

Permeabilization Kinetics of the *E. coli* ML-35 Inner Membrane

The permeabilizing effect of selected peptides on the inner membrane of the lactose permease deficient, β-galactosidase constitutive *E. coli* ML-35 strain, in the absence or presence of PSs, was evaluated as previously described [21], using the normally impermeant substrate *o*-nitrophenyl-β-D-galactopyranoside (Sigma, St Louis, Missouri, USA).

Determination of Bacterial Killing Kinetics

To study the *in vitro* killing effect of the peptides in the absence or presence of polysaccharides, mid-logarithmic phase cultures of *E. coli* ML35 were diluted in Mueller–Hinton broth or, in the case of LL-37, in 20% Tryptic Soy broth, at a final density of 5×10^5 colony-forming units (CFU) per millilitre. Peptides were added at the indicated concentrations and the suspensions were then incubated for different times in a shaking water bath at 37 °C. At the end of each incubation time, the samples were serially diluted in buffered saline, plated in duplicate on Mueller–Hinton agar and incubated overnight to allow colony counts.

Results

The putative inhibitory effect of PSs produced by microorganisms typically infecting the lungs (*K. pneumoniae*) or, specifically, the respiratory tract of the patient with CF (*P. aeruginosa* and members of the *B. cepacia* complex) on the antibacterial activity of selected AMPs was tested by carrying out microdilution susceptibility assays with the *E. coli* ML35 as a reference strain. The peptides, either of human or animal origin, were selected based on their different structural features and, at least in the case of those of human origin, for their release in the airway surface liquid. The peptides used in this study included both linear 'molecules', which may either assume an α-helical structure (LL-37 and SMAP-29) or are extended because of a high content of proline (the active 1–35 fragment of Bac7), and heterodetic cyclic peptides because of the presence of two (PG-1) or three (hBD-3) disulphide bridges (the sequence of all the peptides is shown in Table 1). As shown in Figure 1, all the polysaccharides used inhibited the activity of the peptides tested, albeit to a different extent. CEP is clearly

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Table 1. Amino acid sequence, net positive charge and charge per residue ratio of the peptides used in this study

Peptide	Sequence	Net positive charge	Charge/residue
SMAP-29	RGLRRLGRKIAHGVKKYGPTVLRIIRIA-am	11	0.392
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	6	0.16
PG-1	RGGRLCYCRRRFCVCVGR-am	7	0.388
hBD-3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK	11	0.244
Bac7(1-35)	RRIRPRPPRLPRPRPRPLPFPRPGPRPIPRPLPFP	11	0.314

the less inhibiting polysaccharide, with an increase in the relative MIC, which is the ratio between the MIC values in the presence and absence of polysaccharide, of between two and fourfold, even at the highest PS concentration tested (500 µg/ml). ALG and K40 exerted a more potent inhibitory effect, showing also some selectivity towards the different peptides. ALG was highly effective in inhibiting the activity of SMAP-29, LL-37, Bac7(1–35) and hBD-3, with a relative MIC increase between 8- and 32-fold in the 100–500 µg/ml range of concentrations. Conversely, the K40 polysaccharide greatly inhibited the antibacterial activity of PG-1 (relative MIC increase between 8- and 32-fold), whereas its effect on the activity of the other peptides, although still significant, was somewhat lower. These differences likely depend on the structure of both the peptide and the PS considered, as reported by Herasimenka *et al*. [22]. In fact, the presence of PSs can greatly influence the conformation assumed by the peptides, as demonstrated by the results shown in Figure 2, in which the CD spectra of LL-37 are reported both in the presence and in the absence of alginate.

In addition to the MIC assays, with end-point results measured after 16–18 h incubation, time killing experiments were performed to test the kinetics of peptide's activity inhibition by the three PSs tested (Figure 3). The assays were performed by using a peptide concentration equal to (SMAP-29) or twofold (PG-1 and LL-37) the respective MIC values. The results indicated that the inhibition of the antibacterial activity of SMAP-29, PG-1 and LL-37 is quite fast, particularly in the case of the two former peptides, and confirmed the relative inhibitory potency observed in the microdilution susceptibility assays, with ALG and K40, at 1 mg/ml, capable of fully inhibiting the effect of the AMPs and CEP, at the same concentration, showing only a partial effect.

Similar results were also obtained by following the kinetics of the inner membrane permeabilization on the *E. coli* ML35 strain (Figure 4). This assay monitors in real-time the membrane permeabilization induced by lytic peptides such as SMAP-29 and PG-1, which are among the most active AMPs from mammals [15,23], and LL-37, whose activity is less potent, albeit still significant [18]. Also in this case, the results show that the polysaccharides, when present at a sufficiently high concentration, rapidly inhibit the permeabilizing effect of the peptides, indicating that the interaction of the AMPs with the bacterial membranes is strongly competed by the PSs.

Discussion

The important role that AMPs play in protection of the respiratory tract may be highlighted by the continuous lung inflammation and infection in patients with CF [24,25]. In these individuals, mutations in the CF transmembrane conductance regulator (CFTR) gene cause dehydration of the lung mucosal surface and impaired mucus clearance [24,25]. As a consequence, the resulting airway flow obstruction favours lung inflammation and infection. It has been demonstrated, at least *in vitro*, that the ASL secreted by cultured airway epithelial cells from patients with CF has an increased salt concentration, which determines a decreased antimicrobial activity of the fluid [26], because of the salt-dependent activity of β-defensins and, at least in part, of LL-37 [17,18]. More recently, it has been suggested that the impairment of antimicrobial activity in CF lung may also depend on sequestration of AMPs by components present in the mucus, such as DNA, F-actin and cellular debris [27], and/or by binding to polysaccharides released by microorganisms, such as *P. aeruginosa* and *B. cepacia*, that chronically infect the lung of patients with CF, and that are structural components of the biofilms formed by these pathogens [22]. The ability to sequester AMPs is also shown by PSs released by *K. pneumoniae* [22], which is an important cause of nosocomial infections of the respiratory tract in populations at risk, such as infants and immunocompromised patients.

In this work, we extend our previous biophysical observations aimed to obtain information on the PS/AMP interaction [22], and investigate in depth the *in vitro* effect of three diverse polysaccharides (ALG, CEP and K40) from different species on the biological activity of a panel of structurally diverse AMPs belonging to cathelicidins [LL-37, SMAP-29, PG-1 and Bac7(1–35)] and to β-defensins (hBD-3).

Bacterial PSs are able to reduce, although to a varied extent, the antimicrobial activity of the above-mentioned peptides by forming PS/AMP complexes. Although charge is important, as suggested by the lack of effect of the uncharged PS dextran (data not shown), electrostatic interactions between the negatively charged groups

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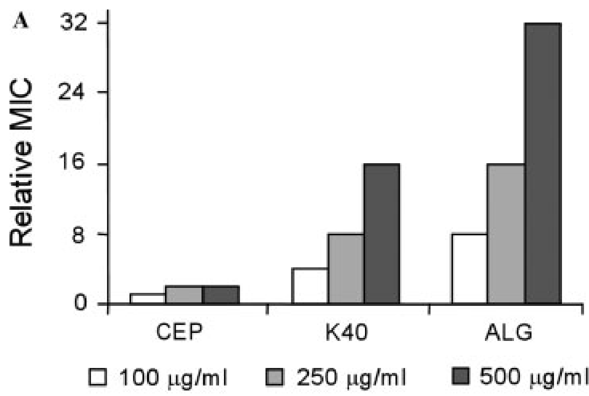

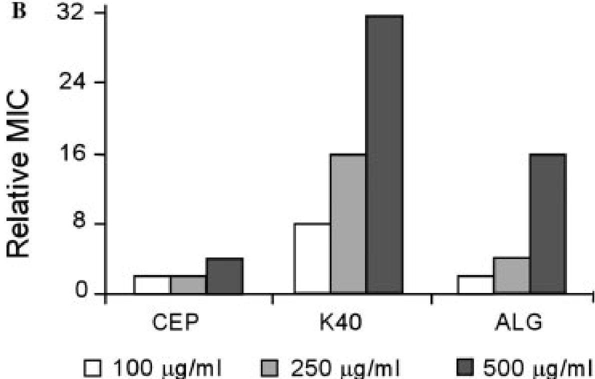

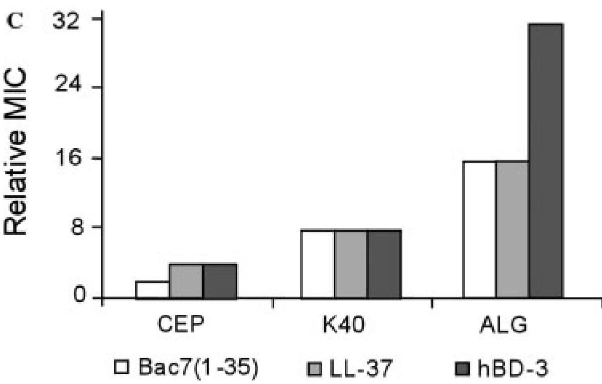

Figure 1. Relative MIC values against *E. coli* ML-35 for SMAP-29 (A) and PG-1 (B) in the presence of different concentrations of the indicated polysaccharides, and for Bac7(1–35), LL-37 and hBD-3 (C) in the presence of 500 µg/ml of the indicated polysaccharides. The relative MIC is the ratio between the MIC value in the presence and in the absence of the polysaccharide. The MIC values in the absence of polysaccharides were 0.5 µM for SMAP-29, 1 µM for PG-1, 0.5 µM for Bac7(1–35), 2 µM for LL-37 and 2 µM for hBD-3. The values were obtained from three independent experiments.

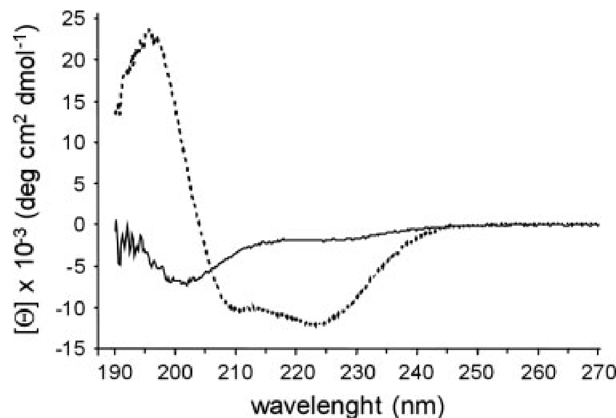

Figure 2. Circular dichroism spectra of LL-37 (10 µM) in the absence (solid line) or in the presence of alginate (dotted line). The spectra were recorded in 5 mM Na-phosphate buffer, pH 7.4, and the alginate/peptide concentration ratio was 65.

of the bacterial PSs and the cationic residues of AMPs are not the only driving force contributing to the formation of peptide–PS complexes.

Specific chemical and conformational motifs, present or induced in the structure of the PSs, such as pockets with increased hydrophobicity, likely also contribute to the intermolecular interactions. In this respect, some light is shed by biophysical analyses of the interaction of alginate from *P. aeruginosa* with a series of novel designed peptides (α-CAPs), which consist of a nonamphipathic hydrophobic core sequence with two and four lysines at, respectively, the *N*- and the *C*-termini [28,29]. As we have shown also with LL-37 and SMAP-29 (Figure 2 and ref. 22), these peptides fold into α-helices in the presence of alginate, although the assumed helical conformation is not amphipathic [28,29]. Spectroscopic studies indicated that hydrophobic interactions between alginate and α-CAPs may be mediated by hydrophobic microdomains induced at the level of pyranosyl C-H groups upon charge neutralisation between the interacting species [28,29]. These results suggest an explanation for the lack of correlation observed here between PS charge density and inhibitory activity on AMPs. In our experiments, the inhibitory activity of the PSs does not simply correlate with the density of negative charges, which decreases in the order ALG $>$ CEP $>$ K40. In this respect, although ALG exhibits the highest negative charge density and, on the whole, is also the most effective in inhibiting the antibacterial activity of the peptides, this correlation is no longer observed for the CEP and K40 polysaccharides. The inhibitory effect of CEP on the peptides' activity is actually lower than that of K40, despite this PS having a higher charge density than K40. This observation suggests that peptide/polysaccharide binding does not merely depend on electrostatic interactions between the two partners. Thus, future studies on the investigation of how different bacterial PSs can form hydrophobic pockets or other structures that favour their interaction with AMPs will be certainly interesting. In this respect, it should also be taken into account that while ALG and K40 are linear polysaccharides, CEP is composed of a branched repetitive unit. This feature might weaken the interaction with AMPs and could contribute to the reduced capacity of CEP to inhibit the antibacterial activity of the peptides (Figure 1).

Further features that have to be considered are the density of positive charge in the AMPs as well as their structure. The peptides here tested are either linear or cyclic because of the presence of disulphide bridges. In particular, SMAP-29 and LL-37 can assume an amphipathic α-helical conformation in an anisotropic environment [15,18], whereas the Bac7(1–35) fragment likely has a polyproline type II–like structure [30]. PG-1 forms a β-hairpin structure stabilised by two disulphide bridges [31], and hBD-3 is characterised by the presence of a three-stranded antiparallel β-sheet and a short α-helical loop at the *N*-terminus. Interestingly, hBD-3 has the ability to form amphipathic symmetrical dimers through intermolecular interactions of residues with opposite charge in the second strand of the β-sheet [32,33]. As for the

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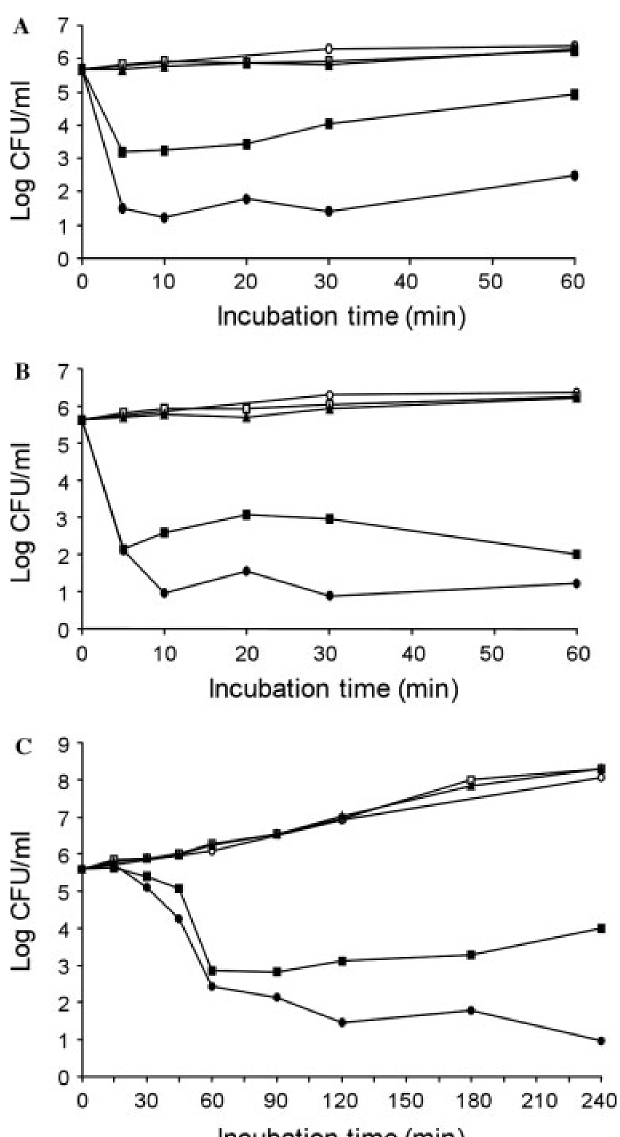
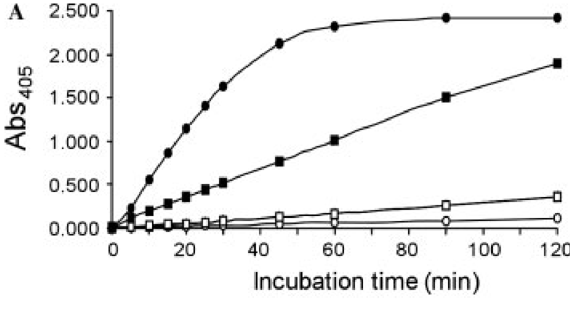
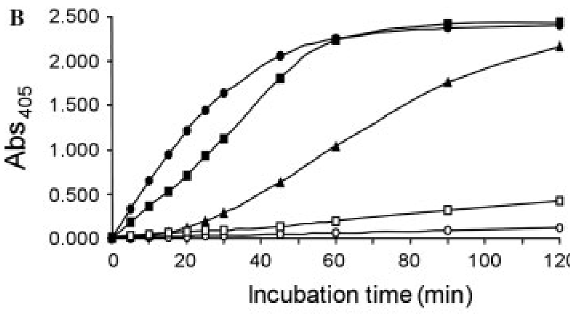


Figure 3. Kinetics of the bactericidal activity of 0.5 μM SMAP-29 (A), 2 μM PG-1 (B) or 5 μM LL-37 (C) against *E. coli* ML-35 in the absence (●) or in the presence of K40 (□), CEP (■) or ALG (▲) at 1 mg/ml. The control in the absence of peptides is indicated by (○). At the concentrations used, the polysaccharides alone had no bactericidal activity (data not shown). Results are representative of three independent determinations.





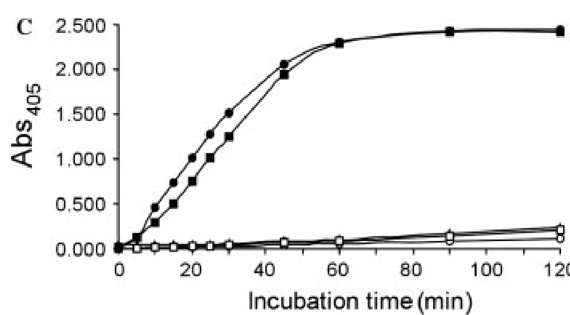


Figure 4. Kinetics of permeabilization of the *E. coli* ML-35 inner membrane by 0.5 μM SMAP-29 (A), 2 μM PG-1 (B) or 5 μM LL-37 (C) in the absence of polysaccharides (●) and peptides (○), or in the presence of K40 (□), CEP (■) or ALG (▲) at 1 mg/ml. At the concentrations used, the polysaccharides alone showed no permeabilizing effect (data not shown). Results are representative of three independent determinations.

charge density, here defined as the charge content per residue at neutral pH, it is comprised between 0.39 and 0.16 and ranks the following order: SMAP-29 ≈ PG-1 > Bac7(1–35) > hBD-3 > LL-37 (Table 1). The tested PSs display a similar inhibitory effect on all the peptides tested, with the possible exception of K40, which is slightly more effective on the AMPs with higher density charge, i.e. SMAP-29 and PG-1. These results overall indicate that the PS have no preference in inhibiting linear or cyclic peptides, so that features other than charge may play a role also for peptides.

Although not providing a detailed understanding of the fine structural features involved in PS/AMP interactions, the present work suggests that sequestration by PSs of natural antimicrobial agents present in the ASL, such as LL-37 and β-defensins, may render ineffective this form of antimicrobial defence in CF and in other pulmonary pathologies. This may contribute to the progressive pulmonary deterioration observed in patients with CF and adds to other putative causes of this problem, such as airway surface dehydration and binding of antimicrobial agents to components of mucus [34]. In addition, this work suggests the usefulness of SAR studies to design novel synthetic AMPs, with a decreased tendency to interact with polysaccharides, which could be effective in the presence of biofilm and developed into novel pulmonary therapeutic agents.

Acknowledgements

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