Differential effects of α-helical and β-hairpin antimicrobial peptides against *Acanthamoeba castellanii*

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SUMMARY

In this work we evaluated the ability of different types of antimicrobial peptides to promote permeabilization and growth inhibition of *Acanthamoeba castellanii* trophozoites, which cause eye keratitis. We used cationic α-helical peptides P5 and P6, corresponding to the N-terminus of the pore-forming protein from *Triatoma infestans*, a blood-sucking insect, and a β-hairpin amphipathic molecule (gomesin), of the spider *Acanthoscurria gomesiana* haemocytes. *A. castellanii* permeabilization was obtained after 1 h incubation with micromolar concentrations of both types of peptides. While permeabilization induced by gomesin increased with longer incubations, P5 permeabilization did not increase with time and occurred at doses that are more toxic for SIRC cells. P5, however, at doses below the critical dose used to kill rabbit corneal cells was quite effective in promoting growth inhibition. Similarly, P5 was more effective when serine protease inhibitor was added simultaneously to the permeabilization assay. High performance chromatography followed by mass spectrometry analysis confirmed that, in contrast to gomesin, P5 is hydrolysed by *A. castellanii* culture supernatants. We conclude that the use of antimicrobial peptides to treat *A. castellanii* infections requires the search of more specific peptides that are resistant to proteolysis.

Key words: *Acanthamoeba castellanii*, antimicrobial peptides, amphipathic peptides permeabilization, protease.

INTRODUCTION

Acanthamoeba castellanii is a free-living protozoan that dwells in different habitats. It is found in 2 morphological stages: the mobile trophozoite and the encapsulated cyst. Pathogenesis by *Acanthamoeba* infection includes granulomatous amoebic meningoencephalitis, cutaneous acanthamebiasis, and the most common *Acanthamoeba* keratitis, affecting the cornea. Contact-lens wear is the main risk factor for infection. It causes infectious keratitis and can lead to severe ocular inflammation and loss of vision (Kashiwabuchi *et al*. 2008). The parasite attaches to mannose-rich glycoproteins from the host epithelium via a 136 kDa mannose-binding protein on the surface of trophozoites (Clarke and Niederkorn, 2006*a*) and release several proteases that interfere with the host immune-response (Na *et al*. 2002).

Clinically effective treatment of *Acanthamoeba* keratitis emerged 20 years ago, and several antimicrobial compounds have been evaluated. Cationic antiseptic agents (polyhexametyl biguanide PHMB, chlorhexidine) and aromatic diamidines (hexamidine, propamidine isethionate), are the drugs of choice for keratitis and intensive topical treatment must be rigorously performed (hourly applications during the first 3 days) which may lead to toxicity and poor visual outcome (Gooi *et al*. 2008). These agents can cause allergic conjunctivitis, or toxic corneal epithelial erosions leading to chronic inflammation of the anterior segment of the eye. Moreover, the development of cataract, iris atrophy and refractory glaucoma during therapy has been attributed to drug-related side-effects (Herz *et al*. 2008).

Antimicrobial peptides (AMPs) and cytolytic peptides participate in innate immunity and can be found as several invertebrate toxins (Kuhn-Nentwig, 2003; Boman, 2003; Bulet *et al*. 2004). They either target the plasma membrane causing its lysis, or affect intracellular components affecting cell physiology. They also modulate the immune system (Brown and Hancock, 2006). According to their secondary structure, they can be classified into α-helical (cecropins, magainins) and β-sheeted peptides (defensins, dermaseptins) (Brogden, 2005). AMPs secreted by the ocular surface are from the cathelicidin and defensin families being involved in immuneprotection against cornea infection processes (McIntosh *et al*. 2005; Huang *et al*. 2007). It is notable that upon eye

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infection natural β-defensin expression is reduced (Abedin *et al*. 2008). There are some reports describing the use of AMPs against *A. castellanii* (John *et al*. 1990; Feldman *et al*. 1991; Schuster and Jacob, 1992). For a review see Ondarza (2007). One important issue is that most of AMPs are sensitive to proteases and *A. castellanii* is known to secret large amounts of proteases (Dudley *et al*. 2008).

In this study, we evaluated the potential use of 2 types of AMPs against *A. castellanii*. We employed α-helical and β-hairpin amphipathic peptides in permeabilization and growth assays of trophozoites. Peptides P5 and P6 fold in amphipathic α-helices and were derived respectively from amino acids 6–32 and 1–32 of the N-terminus of the pore-forming protein named trialysin, which is found in the saliva of the blood-sucking insect *Triatoma infestans* (Amino *et al*. 2002). These peptides were found to induce bacterial, and protozoal lysis at low concentrations compared to the concentration required to promote mammalian cell lysis (Martins *et al*. 2006). Gomesin was employed as a model of a β-hairpin peptide. It is purified from haemocytes of the spider *A. gomesiana* displaying a large bacterial and fungal activity spectra (Silva, Jr. *et al*. 2000; Mandard *et al*. 2002). It is stable in serum and resistant to proteolytic degradation due to 2 disulfide bonds (Fazio *et al*. 2006). It shows lytic activity against *Plasmodium* spp. (Moreira *et al*. 2007) and *Cryptococcus neoformans* (Barbosa *et al*. 2007). We found that both types of peptides induced *A. castellanii* permeabilization and that proteases secreted by the protozoan impair the effect of the helical peptides, but not of the β-hairpin gomesin.

MATERIALS AND METHODS

Amoeba strain and trophozoite harvesting

An axenic culture of *A. castellanii* (ATCC 30011) was obtained from the American Type Culture Collection (Manassas, VA) and maintained in 25 cm^2 tissue-culture flasks containing 5 ml of modified Neff medium (Neff, 1957) (2·0% peptone, 2·0% yeast extract, 1·8% glucose, 0·1 M sodium chloride, 1 mM sodium phosphate, and 1 mM calcium chloride, pH 6·8) at 25 °C. Trophozoites from logarithmic-phase cultures were collected for the experiments 72–96 h post-inoculum. Culture flasks were gently washed twice with phosphate-buffered saline (PBS) in order to remove non-adherent trophozoites and cysts. Adherent cells were harvested by mechanical agitation. Washed amoebae were suspended in PBS or modified Neff medium, counted in a Neubauer haemocytometer and adjusted to the suitable final concentration.

Mammalian cell culture

The immortalized rabbit corneal cell (Statens Seruminstitut Rabbit Cornea, SIRC) line was obtained from the Rio de Janeiro Cell Bank (ATCC CCL60). The SIRC cells were maintained at 37 °C in 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, non-essential amino acids, 100 U of penicillin/ml, and 100 μg streptomycin/ml.

Permeabilization assay

The synthesis and purification of peptides were previously described for P5 and P6 (Martins *et al*. 2006) and for gomesin (Fazio *et al*. 2006). They were diluted in PBS before use and their concentrations were determined by amino acid analysis. Permeabilization experiments were carried out in 1·5 ml tubes. Twenty μl of trophozoites were resuspended in PBS (1×10^6 trophozoites/ml) and incubated with 20 μl of serial dilutions of each peptide for 1 and 4 h at 25 °C. After adding 100 μl PBS, tubes were centrifuged at 800 ***g*** for 3 min, the supernatant removed and the pellet was stained by addition of 40 μl of 0·4% trypan blue solution (Sigma) diluted with the same volume of PBS. Cells were observed by using a light microscope. The percentage of permeabilized cells was determined by counting stained and non-stained cells in at least 100 cells (see Fig. 1A and B). All experiments were conducted in triplicate and repeated 3 times.

Trophozoite permeabilization was also assessed by flow cytometry analysis after incorporation of propidium iodide (PI) on lysed cells as described previously (Borazjani *et al*. 2000). Trophozoites were exposed to AMPs as indicated above, washed twice with PBS and resuspended in PBS (400 μl) containing 25 μg of PI/ml. Live amoebae and heat-killed amoebae (90 °C for 20 min) stained with PI were used as negative and positive controls respectively. The cell samples were analysed in a FACS Calibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ), using CellQuest software.

Permeabilization assays were also carried out in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), which inhibits serine proteases. Trophozoites were pre-incubated with PMSF 5 min prior to addition of AMPs.

Effects of AMPs on amoeba growth

Assays were carried out in 96-well plates as described by Mattana *et al*. (2004). Briefly, 1×10^3 trophozoites were resuspended in 100 μl of modified Neff medium. Then 100 μl of serially diluted peptides in modified Neff medium were added to each well. Plates were maintained at 25 °C for 7 days and daily monitored for amoeba growth at 200× magnification by using an inverted microscope equipped with a CCD camera. Six fields of each well were photographed, and the mean number of

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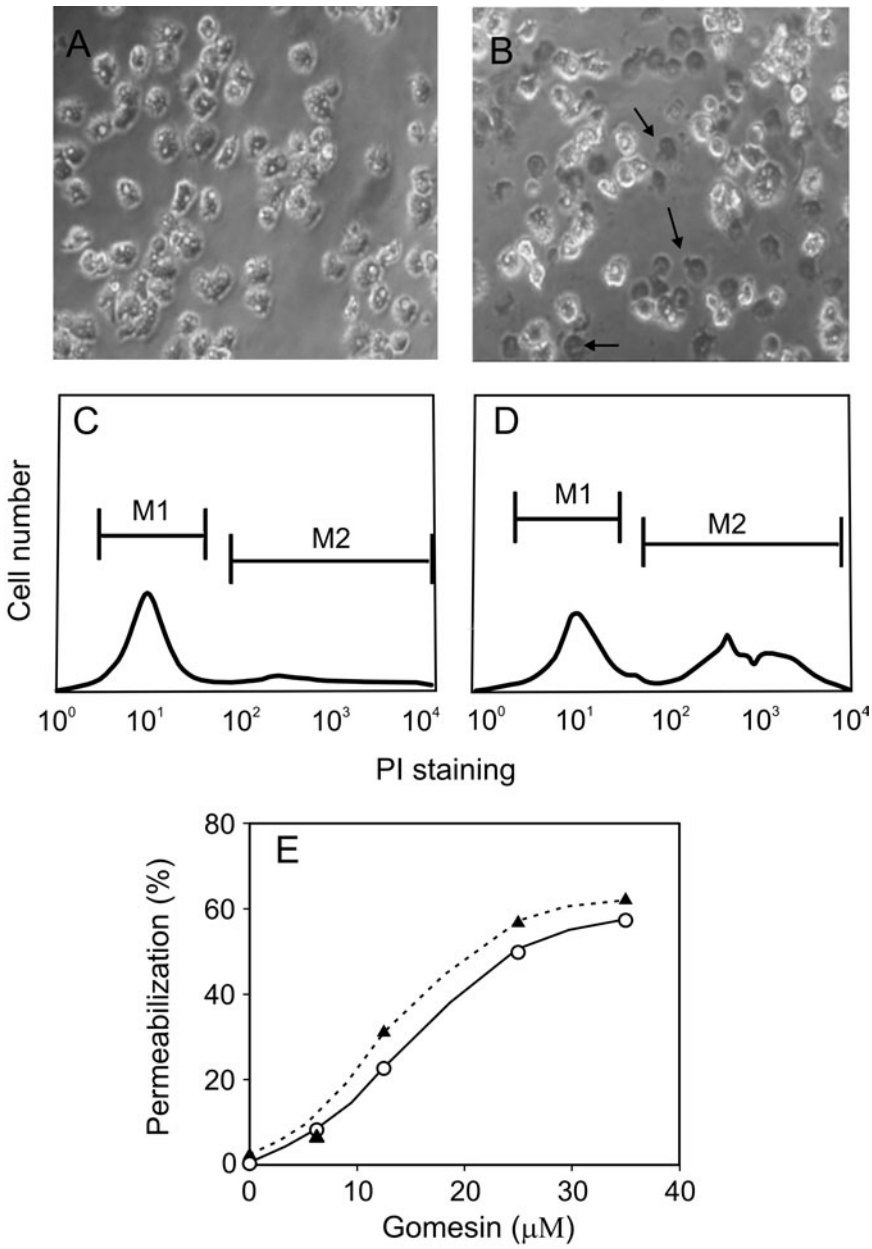

Fig. 1. Permeabilization assays of *Acanthamoeba castellanii* trophozoites induced by gomesin. Trypan blue exclusion (A and B) and flow cytometric analyses (C and D) of control *A. castellanii* trophozoites (A and C) or trophozoites incubated with 25 μM gomesin for 1 h at 25 °C (B and D). Trophozoites stained with trypan blue appear in dark (arrows). Magnification bar = 20 μm. Flow cytometry identified 2 cell populations, M1 and M2. Panel (E) shows *A. castellanii* permeabilization by different concentrations of gomesin measured by trypan blue staining (○) or by PI staining (▲). In both cases the permeabilization was considered as the ratio of labelled (M2) *vs* unlabelled amoeba (M1). Similar results were obtained in 3 different experiments.

trophozoites per field was determined. All experiments were conducted in duplicate and repeated 3 times.

SIRC cytotoxicity assay

Exponentially growing SIRC cells (1×10^4) were seeded onto a 96-well plate containing 200 μl of DMEM supplemented with 10% fetal bovine serum and grown overnight at 37 °C prior to exposure to AMP. After 24 h, the medium was removed, and serial dilutions of peptides in 50 μl of medium were added to the wells. After 4 h incubation, the medium was removed, and fresh medium containing 0·5 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma)/ml was added to each well. Cells were incubated for another 4 h and then, 100 μl of 10% sodium dodecyl sulphate in 0·01 M HCl were added and the plates incubated at 37 °C. Following overnight incubation, absorbance was measured at 570 nm using a microplate reader (Fisher Biotech BT 2000, Pittsburgh, PA, USA).

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The percentage of cell cytotoxicity was estimated by the equation:

$$\text{Cytotoxicity percentage} = \left(1 - \frac{A_{570\,\text{peptide}} - A_{570\,\text{medium}}}{A_{570\,\text{cells}} - A_{570\,\text{medium}}}\right) \times 100$$

Absorbance at 570 nm (A_{570}) was recorded in the presence of each peptide, medium, or the cells alone.

HPLC/MS detection of peptides

Trophozoites (1×10^6) were diluted in 1 ml of PBS. After 12 h at 25 °C, amoebae were removed by centrifugation and the supernatant incubated for 1 h with gomesin or P5 at 25 °C. Control samples corresponded to peptides incubated in PBS. The samples were lyophilized and resuspended in 0·1% trifluoroacetic acid and then fractionated by HPLC (Waters Alliance, model 2690, Waters Corporations, Milford, MA, USA) attached to a mass spectrometer (Waters/Micromass instrument, model ZMD). Data were obtained using a Waters Nova-Pak C_{18} column (2·2 × 150 mm, 3·5 μm particle size, 60 Å pore size). For the LC step, elution was performed using a linear gradient of acidified CH_3CN from 3% to 57% in 30 min at a flow rate of 0·4 ml/min. Absorbance was measured between 191 and 400 nm using a Waters photodiode array detector model 996. Mass measurements were performed in a positive mode in the following conditions: mass range between 500 and 3930 m/z; nitrogen gas flow: 4·1 L per h; needle: 4 kV; cone voltage: 35 V; source heater: 140 °C; solvent heater: 400 °C.

RESULTS

P5 and gomesin permeabilized A. castellanii *trophozoites*

A. castellanii trophozoites were incubated for 1 h with different amounts of gomesin and the number of cells stained by trypan blue (Niederkorn *et al.* 1992) and by PI (Borazjani *et al.* 2000). In the presence of gomesin a significant number of cells was stained with trypan blue compared to control trophozoites (Fig. 1A and B). Similarly a large proportion of cells (M2) was stained by PI, as visualized by flow cytometry, when the peptide was added (Fig. 1C and D). Quantitative measurements of the proportion of the cells labelled by the two methods were very similar (Fig. 1E) and suggest that both dyes detect permeable cells. As the trypan blue staining is more convenient to be performed routinely we adopted this method throughout this study.

Next the trophozoites were incubated with peptides P5, P6 and gomesin for 1 and 4 h. As shown in Fig. 2, after 1 h incubation gomesin permeabilized the cells at lower concentrations when compared to P5 and P6. The concentrations causing 50% cell

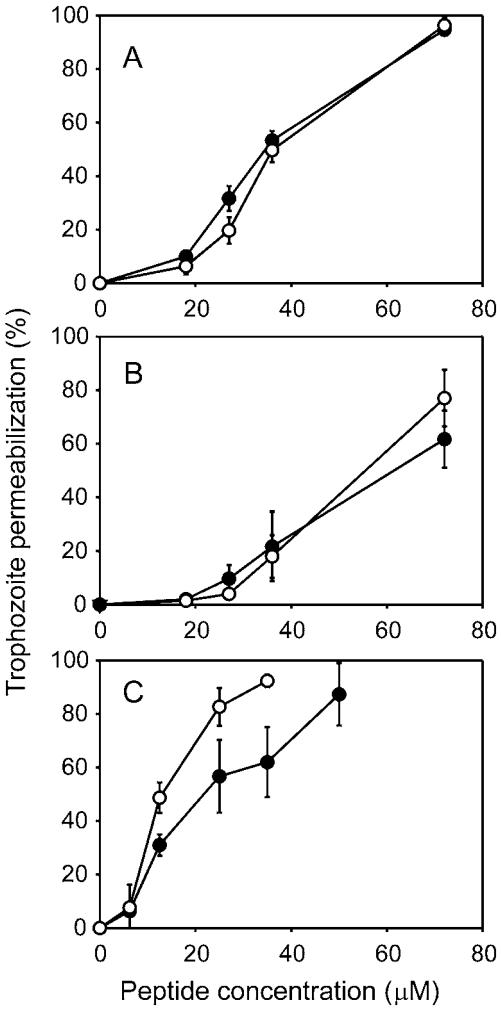

Fig. 2. Permeabilization of *Acanthamoeba castellanii* by different AMPs. Trophozoites were incubated with different P5 (A), P6 (B) or gomesin (C) concentrations for 1 h (closed symbols) or 4 h (empty symbols) at 25 °C and stained by trypan blue dye. Cells were counted in a Neubauer haemocytometer under light microscopy. Values are means ± standard deviations of 3 independent experiments.

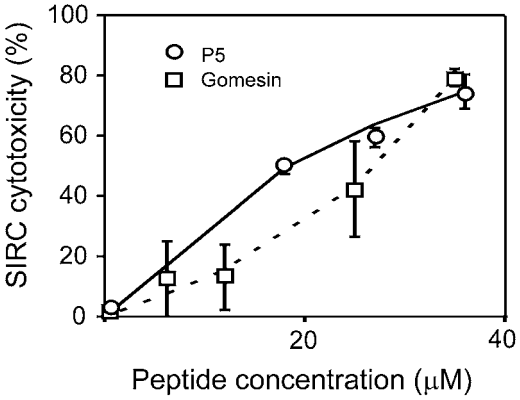

Fig. 3. Effect of AMPs on SIRC cells. The cells were incubated with different concentrations of P5 (○) and gomesin (□) for 4 h at 37 °C. The cytotoxicity was determined as described in methods section and the values correspond to means ± standard deviations of triplicate experiments.

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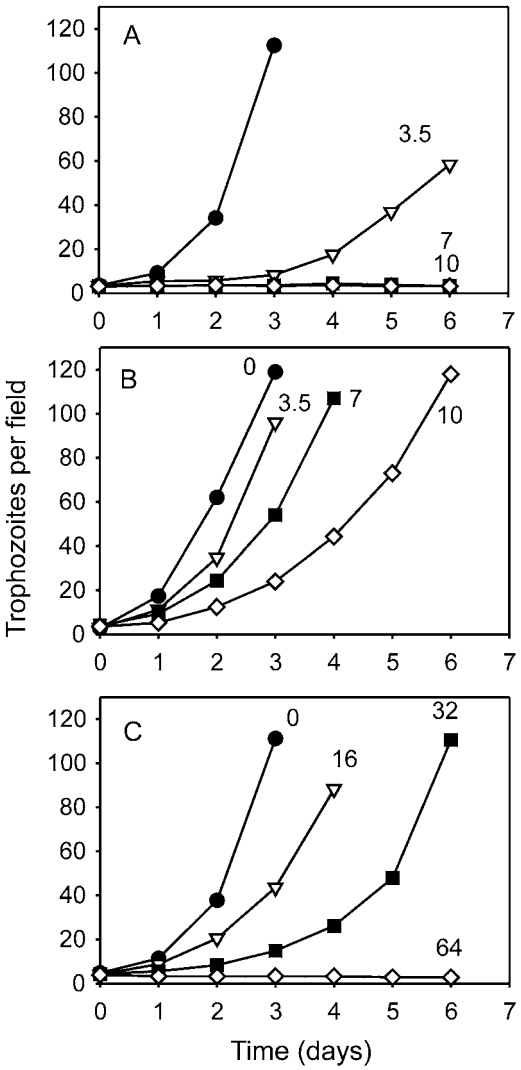

Fig. 4. Effect of AMPs on *Acanthamoeba castellanii* growth. Trophozoites were resuspended in modified Neff medium and maintained at 25 °C with various concentrations of P5 (A); P6 (B) and gomesin (C) in a 96-well plate for up to 6 days. Six fields from each well were photographed daily to determine the mean number of live trophozoites per field. Peptide concentrations (μM) are indicated at each curve.

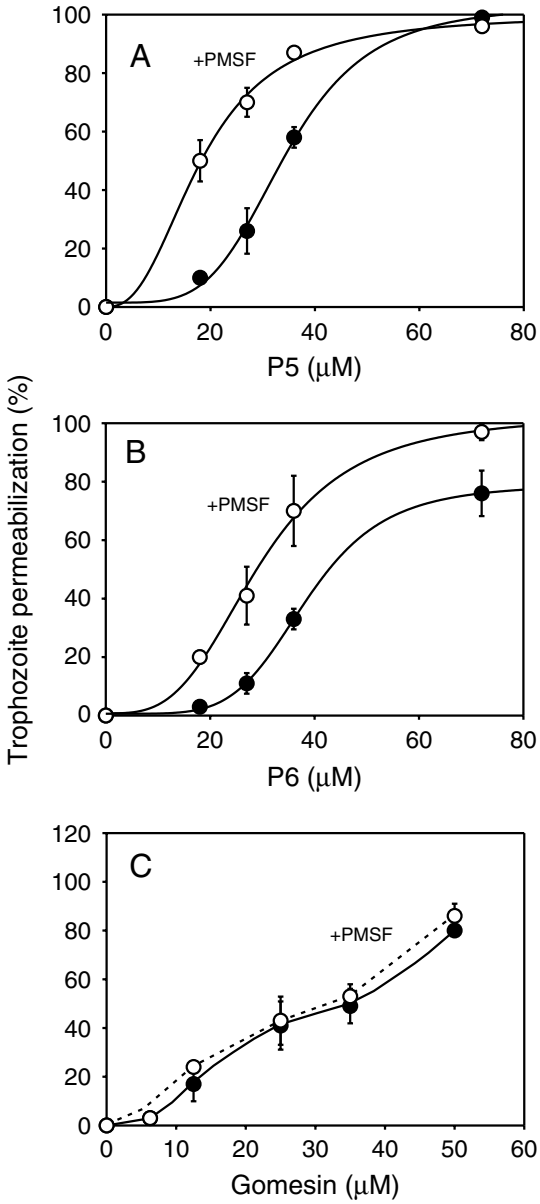

Fig. 5. Effect of a serine-protease inhibitor on *Acanthamoeba castellanii* permeabilization. Trophozoites were resuspended in PBS (1×10^6 per ml) and pre-treated with 1 mM PMSF (○) or without it (●) for 5 min before addition of P5 (A); P6 (B) and gomesin (C) and incubated for 1 h at 25 °C. Permeabilization was determined by the trypan blue assay.

permeabilization (PC_{50}) were $34 \pm 5\,\mu$M for P5, $59 \pm 6\,\mu$M for P6 and $23 \pm 3\,\mu$M for gomesin. Increasing the incubation time to 4 h enhanced the gomesin effect, decreasing the PC_{50} to $13 \pm 3\,\mu$M, while it did not affect P5 and P6 activity.

P5 is more toxic to corneal cells than gomesin

The effects of P5 and gomesin were assessed on rabbit corneal cells (SIRC) because *A. castellanii* is a major ophthalmological problem. Peptides were added to the cells and the toxicity was examined by MTT assay. Both were toxic to SIRC cells (PC_{50} for P5 = $22 \pm 3\,\mu$M, and for gomesin = $26 \pm 5\,\mu$M), as seen in Fig. 3. As gomesin is more effective against the amoeba, it yields a safety index of 2 *vs* a safety index of 0·6 for P5. The safety index is the ratio between the PC_{50} of the peptide against the host cell (in this case, SIRC cells) and the PC_{50} of the same peptide against the target cell (trophozoites). The results therefore suggest that only gomesin-like peptides could be suitable for the treatment of *A. castellanii* ocular infections.

P5, P6, and gomesin affect amoeba growth

We next investigated whether the peptides also affected trophozoite growth. For this, 1×10^3 amoebae

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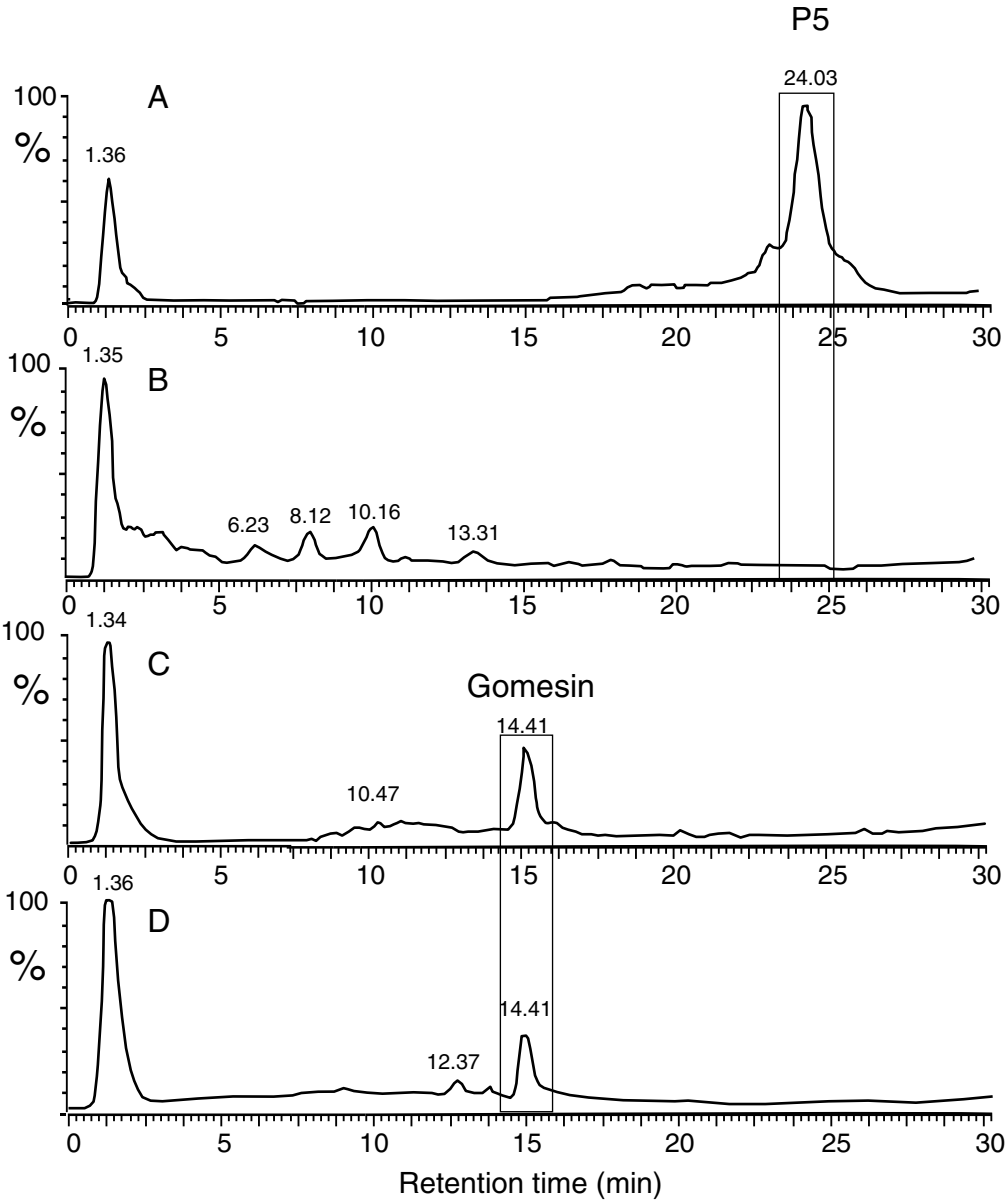

Fig. 6. *Acanthamoeba castellanii* supernatants degrade P5 but not gomesin. Cell culture supernatants were incubated with P5 or gomesin and submitted to reverse phase HPLC. Chromatogram peaks had their compositions analysed by MS (not shown). (A and C) synthetic P5 and gomesin respectively in PBS; (B and D) P5 and gomesin incubated with trophozoites supernatants respectively.

were seeded in the presence of different concentrations of peptides and growth was followed for 6 days. Surprisingly, peptide P5 and even P6 were more effective in preventing *A. castellanii* growth. P5, up to 7 μM, was capable of totally inhibiting trophozoite multiplication while the same effect could only be obtained at 64 μM gomesin (Fig. 4). Although this result may be considered controversial, it can be explained by the lower number of trophozoites used to follow growth, and the amount of secreted proteases, as explained in the next section. To start the cultures, 10^3 cells/ml were used, while 2×10^4 amoebae/ml were used in the permeabilization assays.

P5 and P6, but not gomesin permeabilization increased in the presence of a serine-protease inhibitor

One explanation for the difference in the effective concentration required to permeabilize amoebae versus the concentration required to prevent growth might be due to peptide degradation by proteases secreted by *A. castellanii* known to be involved in adherence and tissue invasion (Na *et al.* 2002; Kim *et al.* 2006; Sissons *et al.* 2006; Dudley *et al.* 2008). The secreted proteases are serine proteases sensitive to PMSF. Therefore, trophozoites were pre-incubated with PMSF for 5 min prior to exposure to AMPs. After 1 h incubation, the permeabilization

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of trophozoites was assessed by the trypan blue assay. As shown in Fig. 5, PMSF only increased P5 and P6 activity, while the effect of gomesin remained the same. This result suggests that PMSF acts by inhibiting degradation of P5.

To verify whether P5 was in fact sensitive to proteases released by the trophozoites, P5 and gomesin were incubated with the supernatant of trophozoites cultures for 5 h and the samples analysed by HPLC/MS. The peak corresponding to P5 seen in the sample incubated in medium alone (Fig. 6A) disappeared after incubation with the trophozoites supernatant (Fig. 6B). On the other hand, the gomesin peak did not change (Fig. 6C and D), indicating that the secreted serine proteases only degrade P5 and P6.

DISCUSSION

Here we showed that AMPs from distinct structural groups are capable of interfering with growth and cellular permeability of *A. castellanii* trophozoites. Gomesin, a β-hairpin amphipathic peptide, known to be resistant to proteases is quite effective in permeabilizing trophozoites, compared to mammalian cells. In contrast, peptides corresponding to trialysin that form amphipathic α-helices are only more effective when proteases are inhibited, i.e. at low amoeba densities or in the presence of protease inhibitors. It is likely that gomesin resistance to proteases is due to its rigid structure, stabilized by 2 disulfide bonds (Fazio *et al*. 2006). Conversely, the structural flexibility of the α-helical P5 and P6 (from random structure in water solution to α-helices in the presence of membrane-mimetic agents) confers the susceptibility to protease degradation in the culture medium (Martins *et al*. 2006).

These conclusions are based on the results showing that differently from gomesin, *A. castellanii* permeability is not increased at longer incubation times with P5 and P6, and that addition of PMSF increased their lytic effect. The hypothesis that PMSF renders the cells more susceptible to α-helical peptides was excluded by the observation that trophozoites pre-incubated with PMSF were not more susceptible to lysis induced by P5, or P6, than untreated ones. Moreover, degradation of P5 by culture supernatants of *A. castellanii* was also clearly seen for P5, but not for gomesin.

The increased ability of P5/P6, compared to gomesin, to block the growth of trophozoites can also be explained by the fact that there were fewer cells in the assay producing proteases, probably increasing the time for the former peptides to exert their actions. More importantly, this result also indicates that permeabilization induced by P5, and to a less extent by gomesin, also produces cell damage since both decrease growth. We cannot exclude, however, that P5-induced growth arrest could be unrelated to cell permeabilization, as measured by trypan blue staining. Some intracellular targets could also be affected by P5. Nevertheless, gomesin affects both growth and permeability suggesting that these processes are linked to each other.

Proteases secreted by *A. castellanii* trophozoites have been found to play important roles in infection (Clarke and Niederkorn, 2006*b*) and are associated with cytopathic effects on mammalian cells (Taylor *et al*. 1995). Importantly, *A. castellanii* infection decreases the amount of DEFB-109, an ocular defensin, which could favour parasite survival (Abedin *et al*. 2008). Whether proteolytical degradation is involved in this process should be further investigated. In fact, proteolysis of AMPs has been found in other parasites. For example, AMP-mediated death is circumvented by proteases localized at the surface of *Leishmania major* (Kulkarni *et al*. 2006).

Treatments used for keratitis caused by *A. castellani* employ very toxic compounds even for SIRC cells. For example, chlorhexidine gluconate which is used at 0·02% (can be used up to 0·06%) kills SIRC cells in less than 4 min (Xuguang *et al*. 2006). Also, the *in vitro* effects do not correlate with the clinical outcome. In some cases very toxic drugs provide satisfactory results in no more than 50% of the patients (Perez-Santonja *et al*. 2003). It is also important to note that biguanides are used at very high concentrations in patients, although lower concentrations are effective *in vitro* for polyhexamethylene biguanide. In this case toxicity is very high for SIRC cells (Lee *et al*. 2007). Therefore, although the AMPs used here presented some *in vitro* toxicity to rabbit corneal cells, our results suggest that the association of AMP treatment with specific protease inhibitors, or with drugs already being used to treat *A. castellanii*-infected patients should be further exploited. A similar use of gomesin associated with fluconazole has been proposed to treat cryptococcosis (Barbosa *et al*. 2007).

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