

Esculentin-1a(1-21)NH₂: a frog skin-derived peptide for microbial keratitis

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Abstract *Pseudomonas aeruginosa* is the primary bacterial pathogen causing contact lens related keratitis. Available ophthalmic agents have reduced efficacy and antimicrobial peptides (AMPs) hold promise as future antibiotics. Here we investigated the in vitro and in vivo anti-*Pseudomonas* activity of esculentin-1a(1-21)NH₂, derived from a frog skin AMP. The data revealed a minimum inhibitory concentration between 2 and 16 μM against reference strains or drug-resistant clinical isolates of *P. aeruginosa* without showing toxicity to human corneal epithelial cells up to 50 μM. At 1 μM the peptide rapidly killed bacterial cells and this activity was fully retained in 150 mM sodium chloride and 70 % (v/v) human basal tears, particularly against the virulent ATCC 19660 strain. Furthermore, its dropwise administration at 40 μM to the ocular surface in a murine model of *P. aeruginosa* keratitis (three times daily, for 5 days post-infection) resulted in a significant reduction of infection. The mean clinical score was 2.89 ± 0.26 compared to 3.92 ± 0.08 for the vehicle control. In addition, the corneal level of viable bacteria in the peptide treated animals was significantly lower with a difference of 4 log₁₀ colony counts, compared to 7.7 log₁₀ cells recovered in the control. In

parallel, recruitment of inflammatory cells was reduced by half compared to that found in the untreated eyes. Similar results were obtained when esculentin-1a(1-21)NH₂ was applied prior to induction of keratitis. Overall, our findings highlight esculentin-1a(1-21)NH₂ as an attractive candidate for the development of novel topical pharmaceuticals against *Pseudomonas* keratitis.

Keywords Innate immunity · Ocular surface infections · Antibiotic resistance · Amphibian skin antimicrobial peptides

Abbreviations

AMP	Antimicrobial peptide
BAC	Benzalkonium chloride
CFU	Colony-forming units
Esc-1a(1-21)NH ₂	Esculentin-1a(1-21)NH ₂
hTCEpi	Telomerase-immortalized human corneal epithelial cells
LB	Luria-Bertani
MH	Mueller–Hinton
MIC	Minimal inhibitory concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MPO	Myeloperoxidase
PB	Phosphate buffer
PBS	Phosphate buffered saline

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Introduction

Bacterial keratitis is an ocular surface infection following corneal abrasion or contact lens wear with the potential to seriously threaten vision [1–3]. A particular feature is its

rapid progression to corneal inflammation, ulceration and tissue destruction which can be completed within 24–48 h with some of the most virulent bacteria if not appropriately treated or if unresponsive to treatment [4]. Amongst the culprits, the gram-negative bacterium *Pseudomonas aeruginosa* is a leading cause of keratitis associated with contact lens wear [5–7]. This bacterium can easily adhere to contact lenses forming sessile communities, named biofilms, which constitute a protected mode of growth that is difficult to treat [8–10]. Once a colonized lens is placed on the eye, it is easy for the bacterial community to resist the physical removal due to the tear flow combined with the sweeping action of the eyelids [1]. Furthermore, contact lens wear compromises several innate ocular surface defenses [11–13], while adaptation to the ocular surface microenvironment facilitates bacterial survival by enhancing the production of virulence factors that highly concur with the pathogenesis of *Pseudomonas* keratitis [1, 14]. In this scenario, worsened by the rapid increase of resistant pathogens, including *P. aeruginosa*, to available antibiotics [15–17], which are not even normally capable of eradicating bacterial biofilms [18], the discovery of new ophthalmic antimicrobial agents with a novel mode of action, has become urgently needed.

Gene-encoded antimicrobial peptides (AMPs) represent valid alternatives for the generation of new feasible anti-infective therapeutics [19–23]. They are produced by almost all living organisms throughout the evolutionary scale as a crucial element of innate defense against infection [24, 25]. Furthermore, especially in higher vertebrates, they have shown relevant immunomodulatory properties so leading to the alternate designation of host-defense peptides [26–28]. In Amphibians, AMPs are produced by dermal glands and stored within granules that are released on the skin surface, by a holocrine mechanism, and act to eliminate invading microorganisms [29–31]. Recently, particular attention has been paid to the peptide esculentin-1a(1-21)NH₂ [Esc-1a(1-21)NH₂, GIFSKLAGKKIKNLLISGLKG-NH₂], consisting of the first 20 amino acids of the frog skin AMP esculentin-1a (isolated from the skin of *Pelophylax lessonae/ridibundus*, formerly known as *Rana esculenta* [31, 32]) with a glycine residue at its C-terminus [33]. Notably, it has been found that Esc-1a(1-21)NH₂ has potent and rapid activity against both free-living and biofilm forms of *P. aeruginosa* strains, with a concomitant membrane-perturbation process as the major molecular mechanism underlying its bactericidal activity against both motile and sessile phenotypes [34]. This mechanism is based on an initial electrostatic interaction between the cationic peptide and the negatively-charged microbial membrane followed by its disruption through the formation of local-breakages, presumably limiting the induction of microbial resistance [20, 35]. Indeed, bacterial resistance to AMPs would necessitate modification of the

composition of the cell membrane, which could not be achieved without causing significant harm to the organism itself [36]. Of note, this is in contrast with commonly-used traditional antibiotics that typically interfere with intracellular processes, e.g. DNA, protein, cell wall synthesis, by recognizing and interacting with single and specific targets which are highly sensitive to mutation [37].

Recent in vivo studies have shown that Esc-1a(1-21)NH₂ is able to prolong survival in mouse models of *P. aeruginosa* sepsis or lung infection, upon intravenous or intra-tracheal administration, respectively [34]. This AMP can also cure inflamed bovine udders in mastitis-affected cows, without inducing irritant effects to the animals, when injected into the teats [33]. Given these promising data, here we investigated both the in vitro anti-*Pseudomonas* activity of Esc-1a(1-21)NH₂ under physiological conditions that better mimic the ocular surface milieu (i.e. at high salt concentration and in the presence of tears) and its in vivo efficacy in a mouse model of bacterial keratitis. Our results suggest that Esc-1a(1-21)NH₂ has great potential for development as a novel pharmaceutical for treatment and/or prevention of *Pseudomonas* keratitis upon topical application to the eye.

Materials and methods

Materials

Synthetic Esc-1a(1-21)NH₂ was purchased from Selleck Chemicals (Houston, TX, USA). The purity of the peptide, its sequence and concentration were determined as previously described [33]. Benzalkonium chloride (BAC) and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Chemical Co, St. Louis MO). Keratinocyte growth medium-2 supplemented with growth factors was from Lonza (Ltd, Basel, Switzerland) and normocin from Invivogen (Toulouse, France). Mueller-Hinton (MH) broth and *Pseudomonas* Isolation agar were from Difco, (Detroit, MI). Myeloperoxidase (MPO) was from Calbiochem (San Diego, CA).

Microorganisms

The invasive *P. aeruginosa* strain ATCC 27853 and the cytotoxic *P. aeruginosa* strain ATCC 19660 were primarily used in this study [38]. Furthermore, the following clinical isolates from human keratoconjunctivitis and conjunctivitis cases and with a different drug-resistance profile (see Table 1) were included: *P. aeruginosa* R1; *P. aeruginosa* 1 Rm; *P. aeruginosa* n. 2 ME; *P. aeruginosa* 3; *Staphylococcus epidermidis* n. 21 (326) ME; *Staphylococcus hominis* n. 1ME; *Staphylococcus aureus* n.6 ME. All of

Table 1 Antimicrobial activity of Esc-1a(1-21)NH₂ against reference and clinical isolates from human ocular surface infections, with varying degrees of antibiotic resistance

Species and strains	Relevant features	MIC (μM)
Reference strains		
<i>Pseudomonas aeruginosa</i> ATCC 27853 ^a	Reference strain, wild type	4
<i>Pseudomonas aeruginosa</i> ATCC 19660	Reference strain, wild type	16
Clinical ocular isolates ^b		
<i>Pseudomonas aeruginosa</i> R1	CAZ, GEN, IPM, TOB	2
<i>Pseudomonas aeruginosa</i> 1 Rm	CAZ, CIP, CTX, FEP, GEN, PIP, SXT, TOB	4
<i>Pseudomonas aeruginosa</i> n. 2 ME	CAZ, IPM	8
<i>Pseudomonas aeruginosa</i> n. 3	IPM	8
<i>Staphylococcus epidermidis</i> n. 21(326) ME	ERY,GEN, OXA,TET, TOB, VAN	8
<i>Staphylococcus hominis</i> n. 1 ME	AMP, ERY, GEN, RIF, TET,TOB	1
<i>Staphylococcus aureus</i> n. 6 ME	TET, TOB,	64

^a Data were taken from ref [34]

^b Relevant resistance traits are indicated as follows: *AMP* ampicillin, *CAZ* ceftazidime, *CIP* ciprofloxacin, *CTX* cefotaxime, *ERY* erythromycin, *FEP* ceftipime, *GEN* gentamicin, *IPM* imipenem, *OXA* oxacillin, *PIP* piperacillin, *RIF* rifampin, *SXT* trimethoprim-sulfamethoxazole, *TET* tetracycline, *TOB* tobramycin, *VAN* vancomycin

these strains were kindly provided by Dr. Anna Rita Blanco, SIFI Pharmaceutical Company, Catania, Italy.

Antimicrobial activity

Susceptibility testing was performed by adapting the microbroth dilution method outlined by the Clinical and Laboratory Standards Institute using sterile 96-well plates. Aliquots (50 μl) of bacteria in mid-log phase at a concentration of 2×10^6 colony-forming units (CFU)/ml in MH broth were added to 50 μl of MH broth containing the peptide in serial twofold dilutions. The range of peptide concentration used was 0.125–64 μM, each in triplicate. Inhibition of microbial growth was determined by measuring the absorbance at 590 nm, after an incubation of 18 h at 37 °C with a microplate reader (Infinite M200; Tecan, Salzburg, Austria). Antimicrobial activities were expressed as the minimal inhibitory concentration (MIC), the concentration of peptide at which 100 % inhibition of microbial growth is observed.

Bactericidal activity of Esc-1a(1-21)NH₂ in the presence of NaCl and human tears

Mid-log phase growth bacteria were centrifuged and resuspended in phosphate buffer (PB, 8.2 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) to an optical density of approximately 1×10^7 CFU/ml. Ten microliters of this suspension were then incubated with vigorous shaking at 37 °C for 20 min with Esc-1a(1-21)NH₂ at the indicated concentration, in the presence or absence of different concentrations of NaCl to give a final reaction mixture

volume of 50 μl. Controls were bacterial cells in PB supplemented or not with NaCl but without addition of the peptide. At the end of the incubation, aliquots of each reaction mixture were withdrawn, diluted in MH broth and spread onto Luria–Bertani (LB)-agar plates. After overnight incubation at 37 °C, the number of CFU was counted and expressed as % of killed bacteria. To further investigate Esc-1a(1-21)NH₂ anti-*Pseudomonas* activity, another set of experiments was performed using pooled tears from human subjects. Tears were collected from 18 consenting adult volunteers after clinical eye exams performed by an ophthalmologist confirmed normal ocular surface. Tears were collected from the inferior tear meniscus using 5 μl microcapillary tubes (Drummond Scientific, Broomall, PA) without anesthesia and stored at –20 °C until used in experiments. Reaction mixtures containing 50 % (v/v) or 70 % (v/v) human basal tears were incubated with 1×10^5 CFU of *P. aeruginosa* in the presence or not of the peptide at the indicated concentration for 30, 90 and 120 min at 37 °C (final volume, 50 μl). Controls were bacterial cells in PB not treated with the peptide. At the corresponding time intervals, aliquots of samples were withdrawn and plated on LB-agar plates for counting.

Human corneal epithelial cell culture

Telomerase-immortalized human corneal epithelial (hTC-Epi) cells were maintained in serum-free keratinocyte growth medium-2 supplemented with growth factors and normocin (100 μg/ml) as previously described [39]. Cells were subcultured on T25 tissue culture flasks (Falcon NJ,

USA) incubated at 37 °C in 5 % CO₂ and passaged every 7–10 days.

Cytotoxicity assay

The toxicity of Esc-1a(1-21)NH₂ to hTCEpi cells was assessed using an MTT-based colorimetric assay. hTCEpi cells were seeded in triplicate into 96-well plates and exposed to 0.1, 1, 5, 10, 25, 50 and 100 μM of Esc-1a(1-21)NH₂ for 24 h. Cells exposed to 0.02 % BAC for 15 min acted as a positive control. MTT solution was then added and the incubation continued at 37 °C for another 3 h until purple formazan crystals were seen by light microscopy. The reaction was stopped by addition of isopropanol with 0.04 N hydrochloric acid followed by trituration to solubilize the formazan crystals. The plate was read using a multi-well spectrophotometer (BMG LABTECH, Ortenberg, Germany) at 590 nm.

In vivo infection with *P. aeruginosa*

P. aeruginosa ATCC 19660, a strain previously reported to produce severe microbial keratitis in mice [40, 41] was used in the in vivo studies. A colony of *P. aeruginosa* was grown overnight and expanded. The cultures were centrifuged at 3,100×g for 10 min and the bacterial pellet was resuspended in 2 ml MH broth and the concentration of the bacteria adjusted turbidimetrically to obtain 2 × 10⁸ CFU/ml. A retrospective count of the bacteria was performed by plating the cells.

Infection

All protocols used were approved by the University of Houston Institutional Animal Care and Use Committee and were in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. Male 7–10 week old C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were anesthetized by intraperitoneal injection of ketamine, 100 mg/kg; xylazine 10 mg/kg (Vedco, Inc., St. Joseph, MO). One drop topical proparacaine (Bausch + Lomb, Tampa, FL) was applied then the anesthetized animal was placed beneath a stereomicroscope and the cornea of the right eye scratched with a sterile 27-gauge needle. Three parallel 1 mm scratches were made in the central cornea of the right eye so as to abrade the full thickness of the epithelium and penetrate the superficial stroma. A 5 μl aliquot containing 1 × 10⁶ CFU of *P. aeruginosa* was pipetted directly onto the scarified cornea. The corneas were then topically treated with 40 μM Esc-1a(1-21)NH₂ starting 5 h post-infection (PI). Peptide treatment was repeated twice on the day of infection and

three times a day (6.30 am, 1.30 pm and 8.30 pm) for the next 4 days. Animals receiving the phosphate buffered saline (PBS) vehicle acted as controls. In some animals (the pre-treatment group), Esc-1a(1-21)NH₂ was topically applied, 3 times in 24 h prior to scratching and induction of keratitis.

The clinical progression of infection was monitored and the extent of corneal damage evaluated and documented by digital imaging using a slit-lamp equipped with a camera module CM 01 (Haag Streit USA, Mason, OH) at day 1, 3 and 5 PI. The progression of infection was graded using a previously established scale of 0–4 [42]: grade 0, clear or slight opacity, partially covering the pupil; grade 1, slight opacity, fully covering the cornea; grade 2, dense opacity, partially or fully covering the pupil; grade 3, dense opacity fully covering the cornea; and grade 4, corneal perforation or phthisis.

Bacterial load and myeloperoxidase assay

Control and infected corneas from four to six mice were harvested at day 1, 3 and 5 PI and homogenized (8–10 strokes for 10 s, repeated 3 times until all tissue was uniformly homogenized) on ice in 1 ml of sterile PBS, pH 7.4. A 100 μl aliquot of the homogenate was serially diluted in sterile PBS and duplicate aliquots were plated onto *Pseudomonas* Isolation agar plates which were incubated overnight at 37 °C then the number of CFU counted. The remaining homogenate was processed to quantitate the number of infiltrating inflammatory cells by MPO activity, a standard and well-established method in the study of infectious keratitis [43]. To determine MPO activity, hexadecyltrimethylammonium bromide at a final concentration of 0.5 % (wt/v) in 50 mM phosphate buffer (pH 6.0) was added to 90 μl corneal homogenate. Samples were then freeze-thawed three times and centrifuged at 13,000g for 20 min at 4 °C. Ten microliters of supernatant were pipetted in triplicate into a microtiter plate and the reaction initiated by the addition of 90 μl of 0.0167 % (wt/v) *o*-dianisidinedihydrochloride and 0.002 % (v/v) H₂O₂ in PBS. The absorbance was measured for 90 min at 450 nm and plotted in comparison with a standard curve generated using purified MPO on the same plate [43]. Results are expressed as relative units of MPO activity per cornea (1 MPO unit is proportional to 2 × 10⁵ infiltrating neutrophils).

Statistical analysis

Statistical analysis was performed using Student's *t* test or analysis of variance (ANOVA), with PRISM software (GraphPad, San Diego, CA). A Tukey's HSD test was used for the analysis of the data from in vivo experiments. Differences were considered to be statistically significant

for $p < 0.05$. All experiments were repeated at least three times to ensure reproducibility.

Results

MIC of Esc-1a(1-21)NH₂ against *P. aeruginosa* and *Staphylococcus* genus

The susceptibility of *P. aeruginosa* ATCC 19660 to increasing concentrations of Esc-1a(1-21)NH₂ was determined using the MIC assay as described. Data obtained by visual and spectrophotometric observation were comparable and showed clear/non-turbid wells indicating no bacterial growth with 64, 32 and 16 μM peptide. However, wells containing Esc-1a(1-21)NH₂ at a concentration of 8, 4, 2, 1, 0.5 and 0 μM exhibited considerable turbidity indicating significant bacterial growth and therefore the MIC was 16 μM . As reported in Table 1, a lower MIC (4 μM) was displayed by this peptide against *P. aeruginosa* ATCC 27853. Furthermore, four *P. aeruginosa* clinical isolates from human ocular surface infections (keratitis and conjunctivitis) and with varying degrees of resistance to commonly used antibiotics were included for comparison, as well as other three bacterial strains belonging to the *Staphylococcus* genus (i.e. *S. aureus*, *S. epidermidis*, *S. hominis*) which are relevant not only for cornea, but also for conjunctival infections [44, 45]. Importantly, Esc-1a(1-21)NH₂ was found to be active against the selected clinical isolates with MIC values measured in the range of 2–8 μM for *P. aeruginosa* strains compared with a MIC of 1 or 8 μM for *S. hominis* or *S. epidermidis*, respectively. An exception was given by *S. aureus* toward which a higher MIC (64 μM), was detected (Table 1).

Esc-1a(1-21)NH₂ anti-*Pseudomonas* activity in the presence of NaCl

The bactericidal activity of Esc-1a(1-21)NH₂ against *P. aeruginosa* ATCC 27853 was initially analyzed in the presence of 150 mM NaCl, the physiological salt concentration of the tear film [11]. As reported in Fig. 1a, concentrations of peptide at 0.68 μM and above caused approximately 100 % killing of the bacterial population within 20 min. These data are in keeping with recent studies showing that Esc-1a(1-21)NH₂ provokes a 3-log reduction in the number of viable *P. aeruginosa* ATCC 27853 cells when tested at high ionic strength, i.e. PBS, at 1 μM [34].

In order to determine whether NaCl affected the anti-*Pseudomonas* activity of this peptide by hampering its electrostatic interactions with the anionic bacterial

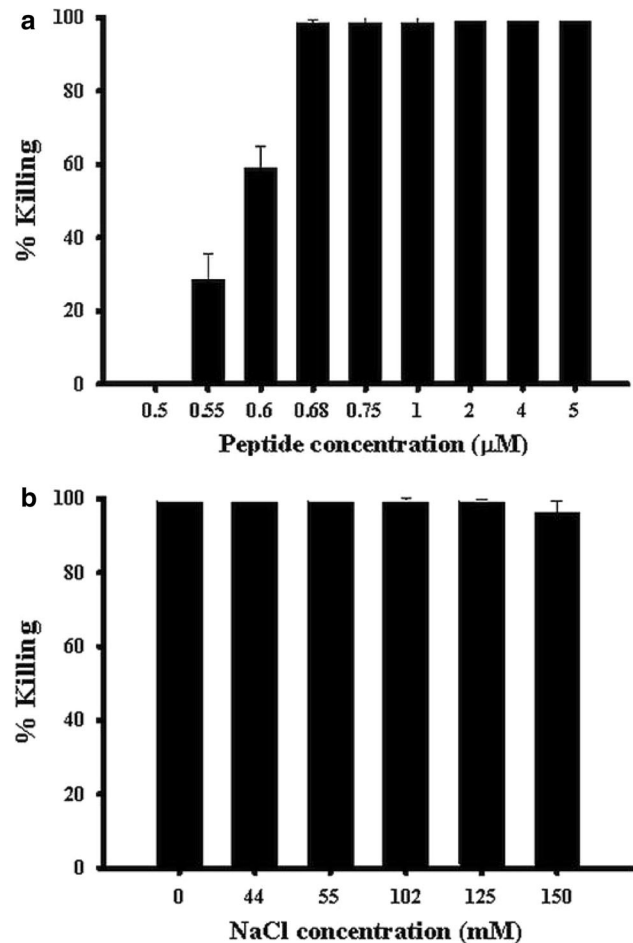


Fig. 1 Effects of NaCl on the bactericidal activity of Esc-1a(1-21)NH₂ against *P. aeruginosa* ATCC 27853. **a** Bacterial cells were incubated with different concentrations of Esc-1a(1-21)NH₂ in the presence of 150 mM NaCl for 20 min at 37 °C. **b** Bacterial cells were incubated with the peptide (1 μM) in the absence or in the presence of varying concentrations of NaCl at 37 °C for 20 min. The data are the mean \pm standard deviation (SD) of three independent experiments and are reported as percentage of bacteria killed by the peptide compared with the corresponding control (see “Materials and methods”)

membrane, we then evaluated the bactericidal activity of Esc-1a(1-21)NH₂ in the presence of different concentrations of salt. The lethal activity of 1 μM peptide against *P. aeruginosa* ATCC 27853 was well-retained after 20 min treatment in the presence of increasing concentrations of NaCl from 44 to 150 mM (Fig. 1b).

Anti-*Pseudomonas* activity in the presence of human tears

In addition to salt, mucins in tears have also been shown to hinder the antimicrobial activity of some AMPs, such as hBD2 [38]. Surprisingly, in the present experiments,

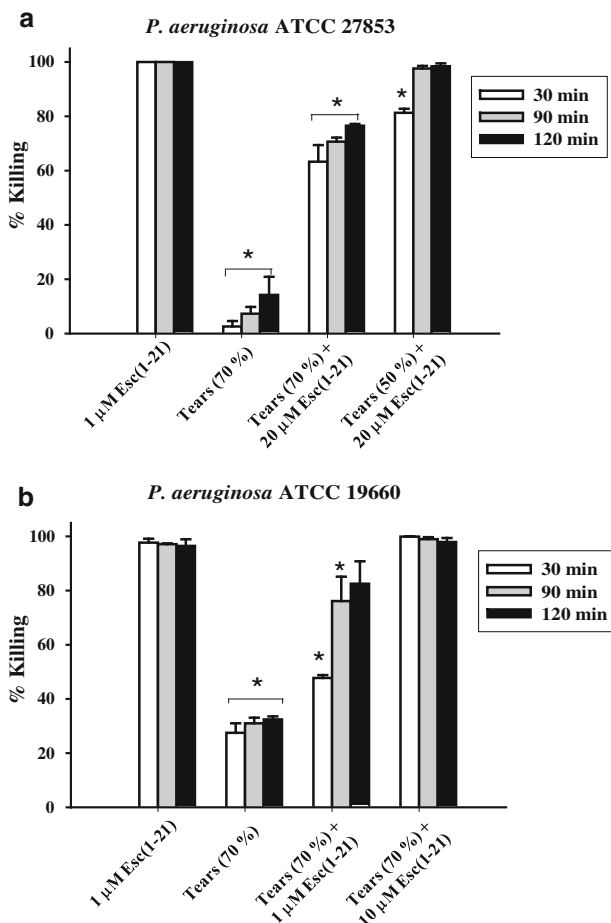


Fig. 2 Effects of human basal tears on the bactericidal activity of Esc-1a(1-21)NH₂ against *P. aeruginosa* ATCC 27853 (a) and ATCC 19660 (b). Bacterial cells were incubated with the peptide in the presence of 70 % (v/v) or 50 % (v/v) basal tears, as indicated, for 30, 90 and 120 min at 37 °C. Cells treated with 1 μ M peptide [indicated as Esc(1-21) in the figure] in PB or with 70 % (v/v) tears were included for comparison. After incubation, aliquots were withdrawn for cell counting. The data are the mean \pm standard deviation (SD) of three independent experiments and are reported as percentage of bacteria killed compared with the control (see “Materials and methods”). Significance was assessed using values of all groups compared with those of peptide treated samples in the absence of tears by *t* test. *Indicates significant difference, $p < 0.05$

70 % or 100 % killing of bacterial cells (strain ATCC 27853) was obtained after 90 min incubation with 20 μ M Esc-1a(1-21)NH₂ in the presence of 70 % (v/v) or 50 % (v/v) human basal tears, respectively (Fig. 2a). For comparison, only approximately 10 % killing of *Pseudomonas* was previously demonstrated by 100 μ g/ml hBD2 (~20 μ M), 2 h after treatment in 70 % (v/v) human basal tears [38].

Most importantly, when Esc-1a(1-21)NH₂ was studied against the cytotoxic strain of *P. aeruginosa* ATCC 19660, which is capable of inducing severe ocular infection in experimentally infected mouse models of bacterial keratitis [41, 46], we found even more promising results. Indeed,

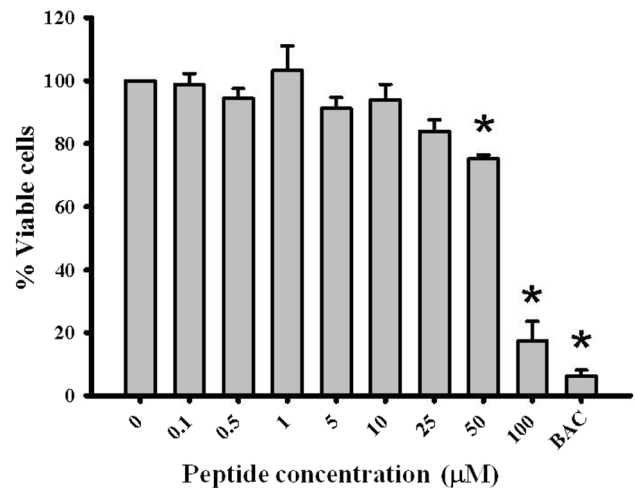


Fig. 3 Esc-1a(1-21)NH₂ toxicity to human corneal epithelial cells. hTCEpi cells exposed to Esc-1a(1-21)NH₂ for 24 h were tested for cytotoxicity using an MTT assay. Data shown are the average from 3 independent experiments ($n = 3-4$ wells/experiment). BAC benzalkonium chloride, the positive control. *Indicates $p < 0.0002$ using ANOVA with Tukey’s HSD for post hoc analysis

despite Esc-1a(1-21)NH₂ displaying a higher MIC against this strain with respect to the invasive ATCC 27853 (Table 1), 10 μ M peptide was sufficient to induce the complete killing of *P. aeruginosa* ATCC 19660 within 30 min in the presence of 70 % (v/v) basal tears (Fig. 2b). Note also that, compared with the results obtained with 1 μ M of peptide alone, only ~20 % reduction in killing efficacy was observed 90 min after addition of Esc-1a(1-21)NH₂ in basal tears at the same concentration of 1 μ M (Fig. 2b). However, in comparison with the invasive ATCC 27853, the cytotoxic strain was more susceptible to the toxic activity inherent to basal tears themselves, which gave rise to approximately 30 % microbial death (Fig. 2b), whilst ~10 % was recorded in the case of *P. aeruginosa* ATCC 27853 (Fig. 2a).

Cytotoxic effects of Esc-1a(1-21)NH₂ on hTCEpi cells

Cytotoxicity of Esc-1a(1-21)NH₂ to hTCEpi cells was examined by MTT assay. As shown in Fig. 3, cell viability after 24 h exposure at concentrations of peptide 0.1–10 μ M, was 93.77–100 %, and at 25 μ M, viability was 83.93 %. At 50 μ M peptide, viability was statistically significantly reduced to 75.73 % and at 100 μ M Esc-1a(1-21)NH₂ this was reduced even further to 17.35 %. Exposure to the positive control BAC resulted in a viability of only 6.18 %. These results revealed that Esc-1a(1-21)NH₂ became cytotoxic to hTCEpi cells at concentrations of 50 μ M and above and guided the choice of peptide concentration for the in vivo studies.

Effect of Esc-1a(1-21)NH₂ on *P. aeruginosa* keratitis in C57BL/6 mice

Clinical progression of corneal infection, inflammatory cell recruitment and bacterial load with and without treatment with 40 μ M Esc-1a(1-21)NH₂ were examined in C57BL/6 mice infected with *P. aeruginosa* ATCC 19660. Figure 4a shows the mean clinical scores from mice examined by slit-lamp biomicroscopy. Corneas of the PBS treated control animals demonstrated severe disease with rapid progression beginning with a dense opacity fully covering the pupil at day 1 PI to corneal perforation or phthisis in some animals at day 5 PI. The mean clinical scores for this group at days 1, 3 and 5 PI were 2.43 ± 0.17 , 3.63 ± 0.17 and 3.92 ± 0.08 . Infection was significantly less severe at all time points ($p < 0.009$, 0.005 and 0.009 at day 1, 3 and 5 PI) for mice treated (or pre-treated) with Esc-1a(1-21)NH₂, although as with controls, the severity of the infection was progressive. Figure 4b shows the appearance of *P. aeruginosa* keratitis infection at day 3 PI in the peptide and PBS control treated groups. In the latter, there was a very dense opacity fully covering the cornea with significant edema whereas the peptide treated animals had a much smaller opacity partially covering the cornea. Data from the Esc-1a(1-21)NH₂ pre-treated and treated animals were not significantly different at day 1, 3 or 5 PI ($p < 0.95$, 0.83 and 0.92 respectively). At day 1 PI, the corneas of mice in the peptide pre-treatment and treatment groups appeared cloudy with a slight opacity and a mean clinical score of 1.37 ± 0.07 and 1.38 ± 0.22 respectively. At day 3 PI, corneas of the peptide treated and pre-treated animals exhibited a dense opacity covering up to 40 % of the cornea with a mean clinical score of 2.29 ± 0.17 and

2.36 ± 0.24 . At day 5 PI, a dense opacity covering about 50–60 % of the cornea was observed in the Esc-1a(1-21)NH₂ treated animals with a mean clinical score of 2.89 ± 0.26 (2.86 ± 0.20 in the pre-treated animals). The severity of ocular disease was significantly greater at day 3 PI and day 5 PI compared to day 1 in all groups ($p < 0.05$), but not at day 3 compared to day 5.

Inflammatory cell recruitment to the site of infection was determined by measuring the relative MPO activity in corneas harvested at day 1, 3 and 5 PI (Fig. 5a). MPO activity for the control PBS treated animals at days 1, 3 and 5 PI was 2.5 ± 0.36 , 4.53 ± 0.22 and 4.2 ± 0.12 respectively. These values were significantly higher than for both groups of peptide treated animals at days 3 and 5 PI ($p < 0.0004$ and 0.0012 for the pre-treated group and 0.0006 and 0.005 for the Esc-1a(1-21)NH₂ treated group), indicating a substantially lower influx of inflammatory cells into peptide treated corneas than corneas receiving the vehicle PBS. The latter demonstrated a significantly higher recruitment of inflammatory cells and hence higher MPO activity at day 3 and 5 PI compared to day 1 ($p < 0.008$ and 0.008), although there was no significant difference between days 3 and 5 PI. The pattern was different for peptide treated animals in that the level of inflammatory cell recruitment was similar at all time points with mean MPO values for the pre-treatment and Esc-1a(1-21)NH₂ treated group of 1.55 ± 0.4 and 1.69 ± 0.40 at day 1; 1.66 ± 0.13 and 1.84 ± 0.16 at day 3 and 1.55 ± 0.28 and 1.82 ± 0.42 at day 5 PI. There was no significant difference in MPO values at day 1, 3 and 5 PI among peptide pre-treated and peptide treated animals ($p < 0.83$, 0.42 and 0.62 respectively).

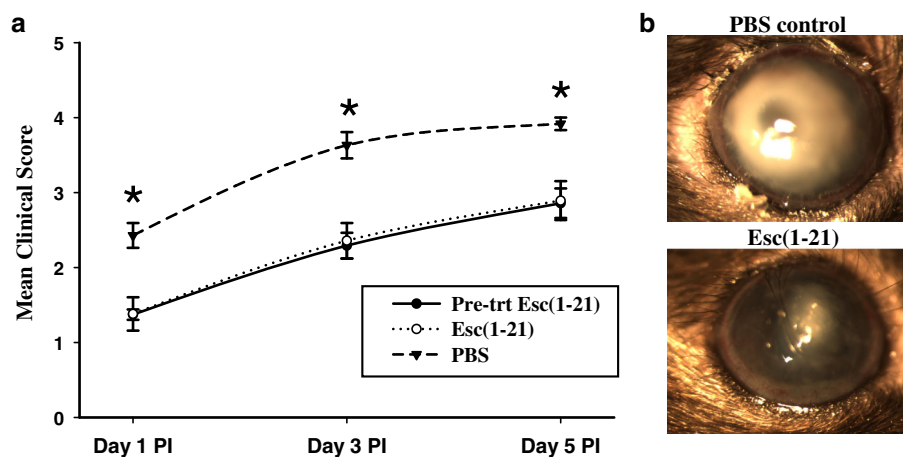


Fig. 4 Effect of Esc-1a(1-21)NH₂ on *P. aeruginosa* keratitis in C57BL/6 mice. **a** Clinical scores indicated that severity of infection was significantly less at all time points in mice treated (or pre-treated) with the peptide [indicated as Esc(1-21) in the figure] compared to controls. Data are mean clinical scores from 4 independent

experiments with 4–6 mice per experiment. *Indicates significant difference, $p < 0.05$, among PBS control and peptide treated groups. **b** Representative photographs of infected animals in the control and peptide treatment group at day 3 PI

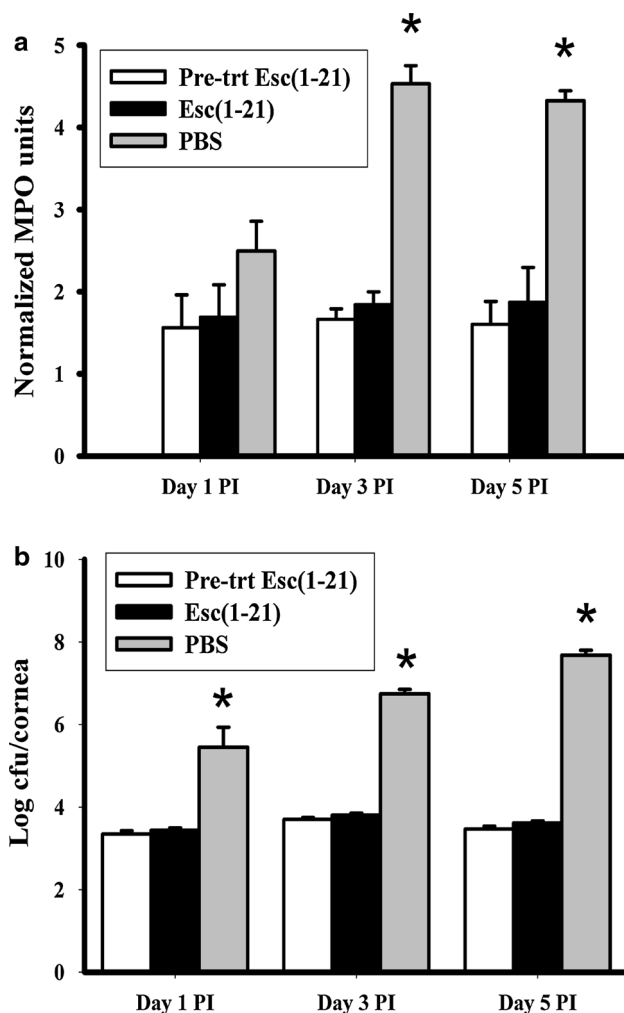


Fig. 5 Effect of Esc-1a(1-21)NH₂ on corneal neutrophil infiltration and bacterial load in *P. aeruginosa* keratitis. **a** MPO activity revealed a significantly greater inflammatory cell recruitment in infected corneas of the PBS treated control animals compared to the pre-treatment and peptide [indicated as Esc(1-21) in the figure] treated groups. **b** Viable bacterial count in the infected cornea was significantly greater in the PBS control animals compared to peptide treated or pre-treated animals. Data represent averages from 3 independent experiments with 2–4 mice/group. *Indicates significant difference, $p < 0.05$, among PBS control and peptide treated groups

The viable bacterial load that was determined by quantifying *P. aeruginosa* colonies at day 1, 3 and 5 PI is shown in Fig. 5b. The levels of recoverable viable bacteria obtained in the PBS control group were significantly greater than for the pre-treated or Esc-1a(1-21)NH₂ treated groups at day 1, 3 and 5 PI ($p < 0.01$, 0.0001, 0.0001, respectively) with a difference in the number of CFU of ~2–3 log₁₀ at days 1 and 3, and 4 log₁₀ at day 5 PI. There was no significant difference between the pre-treated and Esc-1a(1-21)NH₂ treated groups ($p < 0.41$, 0.15 and 0.15 at days 1, 3 and 5 PI respectively). The pre-treated corneas demonstrated a significantly higher CFU at day 3 PI (3.7

log₁₀ CFU) compared to day 1 (3.35 log₁₀ CFU) and day 5 PI (3.47 log₁₀ CFU) ($p < 0.01$, 0.04 respectively). The peptide treated infected corneas demonstrated a similar pattern, with statistically significant differences ($p < 0.008$, 0.04 at day 1 and 5 PI respectively). However, in the PBS control animals there was a much greater increase in the viable bacteria recovered at day 3 PI (6.76 log₁₀ CFU) compared to day 1 PI (5.8 log₁₀ CFU) and for day 5 PI (7.7 log₁₀ CFU) as compared to day 3 PI ($p < 0.02$).

Discussion

P. aeruginosa, a common cause of contact-lens associated bacterial keratitis [47–49], induces rapid destruction of the cornea, initially owing to bacterial proteases and toxins [40, 50]. Although host reactions to *Pseudomonas* keratitis rapidly occur after disease is initiated, they are not adequate to defeat this pathogen and contribute significantly to corneal damage. Further, the antimicrobials currently used, e.g. aminoglycosides and fluoroquinolones, are problematic, due to their side effects and the growing emergence of resistant bacterial strains [51, 52]. Of note, AMPs hold promise as future therapeutics. Thus, our current investigation aimed at studying the potential of the amphibian AMP-derived Esc-1a(1-21)NH₂ as a novel pharmaceutical agent for bacterial keratitis.

Prior studies have characterized AMPs for their activity against ocular pathogens in vitro [38, 53–55], a preservative for eye bank corneal storage media [56] and as contact lens disinfectants [57]. Efficacy of two AMPs has also been investigated in in vivo studies in a rabbit model of *P. aeruginosa* keratitis. In one study, a synthetic AMP COL-1, administered topically at 10 or 50 μg/ml, resulted in greater inflammation than that observed in controls, and despite its potent antimicrobial activity in vitro showed no efficacy in vivo [49]. In another study, infected rabbit eyes topically treated with a hybrid cecropin A-melittin peptide showed higher clinical scores after 6 h than those from control animals, likely owing to a toxic effect of the peptide [58]. After 24 h however, more promising results were seen as clinical scores for the peptide treated animals were lower than the control group, although not as low as the gentamicin treated positive control. Possibly contributing to the lack of robust AMP efficacy in these two studies is the fact that the antimicrobial activity of some AMPs is significantly compromised by physiological salt concentrations and other tear components such as mucins [38, 54, 59]. Thus, to determine if an exogenously applied AMP is potentially active at the ocular surface it is important to establish if its activity is hampered by salt and tears. Here, we found that the bactericidal activity of Esc-1a(1-21)NH₂

against *P. aeruginosa* is fully preserved at increasing salt concentrations. Notably, this response clearly differs from that previously reported for some endogenously expressed mammalian AMPs, e.g. hBD-1 and hBD-2 produced by corneal and conjunctival epithelial cells [54, 60, 61]. Indeed, their bacterial killing activity is reduced by at least 30 % in reaction mixtures containing NaCl solutions at an osmolality equivalent to that of tears [38, 62, 63]. In addition, in contrast with what has been observed for mammalian AMPs, e.g. rabbit NP-3a [59] and human hBD-1 and -2 [38, 54], whose ability to kill *Pseudomonas* in the presence of 70 % (v/v) tears is completely abolished or very markedly diminished, Esc-1a(1-21)NH₂ significantly retains bactericidal activity in basal human tears, especially against the cytotoxic strain *P. aeruginosa* ATCC 19660. Furthermore, our previous studies demonstrated the ability of Esc-1a(1-21)NH₂ to be similarly active against both reference and clinical strains of *P. aeruginosa*, with a multi-drug resistance or mucoid phenotype (isolated from cystic fibrosis sufferers), with a MIC value measured in the range of 4–8 μM [34]. This is now strengthened by the findings that the antimicrobial activity of this peptide is preserved also against a range of *P. aeruginosa* isolates from keratitis and conjunctivitis cases and with varying degrees of antibiotic resistance (MIC value from 2 to 8 μM).

All of these promising *in vitro* data prompted us to test the efficacy of this peptide in a mouse model of *P. aeruginosa* keratitis.

Results from our *in vivo* study established that Esc-1a(1-21)NH₂ treated C57BL/6 mice demonstrated significantly less severe keratitis compared to their PBS treated counterparts at all time points. This improved clinical appearance is accompanied by reduced inflammatory cell infiltration and reduced bacterial counts compared to PBS treated mice. The peptide does not appear to lead to an inflammatory process, suggesting that the clinical benefit is presumably due to a direct effect of Esc-1a(1-21)NH₂ on the bacterial load rather than being mediated by additional events e.g. recruitment of neutrophils to the site of infection which may result in severe damage to the corneal tissue. To better explore whether this peptide had immunomodulatory activity, as discovered for other mammalian AMPs [25–28], we also investigated if initiating treatment with Esc-1a(1-21)NH₂ before infection may affect the clinical outcome. However, we found that this was not the case, with results from mice where treatment of the peptide was started before infection and those where Esc-1a(1-21)NH₂ administration was started post-infection being virtually identical. This suggests that pre-treatment with Esc-1a(1-21)NH₂ does not induce innate defenses at the ocular surface, supporting the notion that the amelioration of keratitis by Esc-1a(1-21)NH₂ is mainly due to direct

antibacterial activity of the peptide once applied to the eye. Note, however, that in our study, although Esc-1a(1-21)NH₂ treatment, at the concentration tested, significantly reduced severity of the infection compared to PBS treated controls, the disease was still progressive. This may be related to our use of the ATCC 19660 cytotoxic strain. Thus, while peptide treatment was associated with reduced inflammatory cell infiltration and viable bacteria within the cornea, there may have been sufficient bacterial toxin produced early on to cause persistent damage.

In the experiments, a balance needed to be achieved among antimicrobial efficacy and lack of toxicity. Thus, we selected 40 μM for the topical treatment as this was below the concentration at which the Esc-1a(1-21)NH₂ began to show cytotoxicity *in vitro* and was higher than the MIC for ATCC 19660. It should also be noted that the peptide was only applied topically three times per day. This is in stark contrast to typical treatment regimens for bacterial keratitis in human patients in which antibiotic drops every 15 min to 1 h are recommended for the first 2 days of treatment. Thus, more frequent application likely would result in greater efficacy. Further, other studies from our lab have shown that tethering of AMPs to surfaces and nanoparticles greatly enhances their antimicrobial efficacy compared to an AMP in solution [64, 65]. Formulating Esc-1a(1-21)NH₂ for delivery in such a fashion would therefore also be expected to increase its effectiveness while not causing significant toxicity, as much lower amounts can be utilized.

In summary, our findings highlight that simple topical application of Esc-1a(1-21)NH₂ in solution or likely its attachment to a “surface” (e.g. contact lens or nanoparticles) may represent a very promising mode of drug delivery for the prevention/treatment of *P. aeruginosa* keratitis. In regards to development of an AMP based-antimicrobial contact lens, Willcox and colleagues have already shown the feasibility of this with a hybrid melittin-protamine peptide, named melimine, which when conjugated to contact lenses, reduced bacterial colonization [66–68], as well as the degree of ocular inflammation and corneal infiltrates in animal models of contact lens related adverse events [68]. Despite some frog-skin AMPs having already been shown to have anti-*Pseudomonas* activity at high salt concentration [69], our study is the first to report such an activity by a frog skin AMP-derived peptide in the presence of either high ionic strength or human tears (in contrast with what found for some mammalian AMPs) as well as when administered, in solution, in an animal model of bacterial keratitis, with clear resulting signs of beneficial effect. Overall, our data, coupled with the wide spectrum of activity of Esc-1a(1-21)NH₂, either against reference microbial strains [33, 34] or clinical isolates from human ocular surface infections (Table 1), and with the low risk of development of bacterial resistance, due to the mechanism

of action of this peptide [34], suggest Esc-1a(1-21)NH₂ as an excellent candidate for development as a novel drug against microbial keratitis.

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