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A class IIa peptide from *Enterococcus mundtii* inhibits bacteria associated with otitis media

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Abstract

Peptide ST4SA, produced by *Enterococcus mundtii* ST4SA, inhibits the growth of *Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterococcus faecium*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and Gram-positive bacteria isolated from patients diagnosed with middle ear infections. The peptide adsorbed at a level of 94% to *S. pneumoniae* 40, *Pseudomonas aeruginosa* 25 and *E. faecium* HKLHS. Low concentrations of peptide ST4SA (51 200 arbitrary units (AU)/mL) caused DNA and enzyme leakage from target cells, whilst 1 638 400 AU/mL caused cell lysis. No decrease in antimicrobial activity was observed when tested on solid medium with human blood as base. Peptide ST4SA revealed a similar level of activity compared with tetracycline (30 μg), but much higher activity compared with nasal sprays, aminoglycosides, cephalosporins, fluoroquinolones, lincosamides, macrolides, nitroimidazole, penicillin, quinolones, sulphonamides, chloramphenicol, furazolidone, fusidic acid, rifampicin, trimethoprim, trimethoprim/sulfamethoxazole and vancomycin when tested *in vitro*. Peptide ST4SA dissipates the proton-motive force and may be used in the treatment of multidrug-resistant strains where antibiotics are excluded from cells by efflux pumps dependent on the membrane proton gradient.

Keywords: Otitis media; *Enterococcus mundtii*; Peptide; Antibacterial activity

1. Introduction

Otitis media is one of the most common diseases diagnosed in children under the age of 2 years [1]. Pathogens associated with the disease are β-lactamase-producing *Haemophilus influenzae*, *Moraxella catarrhalis*, multidrug-resistant (MDR) *Streptococcus pneumoniae*, group A streptococci, *Staphylococcus aureus*, *Streptococcus pyogenes* and Gram-negative rods such as *Pseudomonas aeruginosa* [1,2]. In general, infection is treated with amoxicillin [1–3]. Although broad-range antibiotics are effective against a variety of Gram-positive and Gram-negative bacteria, they also affect commensal microflora and should not be used on a routine basis [4–6].

In more recent papers, ribosome-synthesised peptides, known as bacteriocins, have been considered as an alternative to antibiotics [4,6–9] and may be used in synergy with antibiotic peptides or even as antiviral agents [10–13].

Peptide ST4SA is positively charged, hydrophobic, 43 amino acids long and corresponds to class IIa bacteriocins [14]. The aim of this study was to determine the mode of activity of peptide ST4SA and to evaluate it as an alternative to antibiotics in the treatment of otitis media.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Enterococcus mundtii ST4SA was cultured in de Man, Rogosa and Sharpe (MRS) broth (Biolab, Biolab Diagnostics, Midrand, South Africa) at 30 °C. The target organisms, growth media and growth conditions are listed in Table 1.

2.2. Production and partial purification of peptide ST4SA

Ten millilitres of an 18-h-old culture of *E. mundtii* ST4SA was inoculated into 500 mL of MRS broth and incubated

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at 30 °C for 20 h without agitation. The cells were harvested (8000 × *g*, 10 min, 4 °C) and the pH of the cell-free supernatant was adjusted to 6.5 with 1N NaOH and treated for 10 min at 80 °C. The peptide was precipitated from the cell-free supernatant with 80% saturated ammonium sulphate [15], dialysed against sterile distilled water, the pH adjusted to 6.5 with sterile 1N NaOH and then lyophilised. The crude extract was re-suspended in 0.1 mL of sterile Milli-Q® water (Millipore, Billerica, MA) and treated for 10 min at 100 °C. Antimicrobial activity of peptide ST4SA was determined using the agar spot test method described by Ivanova et al. [16]. Activity was expressed in arbitrary units (AU)/mL, with 1 AU defined as the reciprocal of the highest dilution showing a clear zone of growth inhibition [16].

2.3. Adsorption of peptide ST4SA

Adsorption of peptide ST4SA to pathogens (Table 1) was studied as described by Todorov and Dicks [17]. Suspensions of the target cells (0.7 mL) were mixed with an equal volume of peptide ST4SA (819 200 AU/mL). Samples were incubated at 37 °C for 1 h, the cells were harvested (10 000 × *g*, 15 min, 4 °C) and the activity of unbound peptide ST4SA was determined as described previously. Cells suspended in MRS medium without peptide ST4SA were used as controls. Adsorption of peptide ST4SA to target cells was calculated according to the following formula:

$$\%\text{Adsorption} = 100 - \left[\left(\frac{\text{bacteriocin activity after treatment}}{\text{original bacteriocin activity}}\right) \times 100\right]$$

2.4. Mode of action

Target strains (Table 2) were cultured overnight as described previously. Cells were harvested (10 000 × *g*, 15 min, 4 °C) and washed twice with sterile 5 mM phosphate buffer (pH 6.5). Cell-free supernatant containing peptide ST4SA was added to washed target cells at a final concentration of 5160 AU/mL and incubated at 37 °C for 1 h. Changes in optical density readings were recorded at 260 nm. Controls were target cells suspended in 5 mM phosphate buffer and not treated with peptide ST4SA.

In a separate experiment, cells were treated with peptide ST4SA (final concentration 25 600 AU/mL) and extracellular levels of β-galactosidase were monitored as described by Todorov et al. [18]. Controls were cells prepared in the same way but not treated with peptide ST4SA.

In another experiment, *Enterococcus faecium* HKLHS and *S. pneumoniae* 40 were cultured overnight in MRS and brain–heart infusion (BHI) media, respectively, as described previously. Ten millilitres of peptide ST4SA (1 638 400 AU/mL) was added to both cultures at mid exponential growth and incubated for a further 12 h. Optical density readings at 600 nm were recorded every hour.

The effect of peptide ST4SA on cell morphology was determined by atomic force microscopy as described by Todorov et al. [19]. Peptide ST4SA was added to an 18-h-old cell suspension (final concentration 49 152 AU/mL) and incubated for 1 h at 30 °C.

2.5. Comparison of peptide ST4SA with antibiotics and otic drop suspensions

Enterococcus faecium HKLHS and the middle ear pathogens *S. pneumoniae* 27 and *P. aeruginosa* J were grown

Table 1
Antimicrobial activity of peptide ST4SA crude extract

Pathogen	Growth medium, incubation	Origin	Antimicrobial activity[a]
Acinetobacter baumannii 16	BHI, aerobic, 37 °C	HME	+
A. baumannii 19	BHI, aerobic, 37 °C	HME	–
Enterococcus faecalis 20	BHI, aerobic, 37 °C	HME	+++
E. faecalis 21	BHI, aerobic, 37 °C	HME	+
Enterococcus faecium HKLHS	MRS, aerobic, 30 °C		++++
Haemophilus influenza C	CBA, aerobic, 37 °C	HME	–
Pseudomonas aeruginosa 8, 14, 25	BHI, aerobic, 37 °C	HME	–
P. aeruginosa G, BG	BHI, aerobic, 37 °C	HME	(+)
P. aeruginosa I, J	Bld, aerobic, 37 °C	HME	(+++)
P. aeruginosa B	BHI, aerobic, 37 °C	NC	(+++)
P. aeruginosa E	BHI, aerobic, 37 °C	NC	(+)
Staphylococcus aureus 13	BHI, aerobic, 37 °C	HME	–
S. aureus 36	BHI, aerobic, 37 °C	HME	+++
Staphylococcus carnosus LMG 13567	BHI, aerobic, 37 °C	HME	–
Streptococcus pneumoniae A, D, 10	BHI/Bld, aerobic, 37 °C	HME	–
S. pneumoniae 29	BHI, aerobic, 37 °C	HME	+++
S. pneumoniae 27, 40	BHI, aerobic, 37 °C	HME	++++
Unidentified Gram-positive bacteria A, BW, DW, F, G, H	BHI, aerobic, 37 °C	HME	++++

BHI, brain–heart infusion; MRS, de Man, Rogosa and Sharpe; CBA, chocolate blood agar; Bld, blood agar; HME, human middle ear; NC, nasal cavity.

[a] –, no zone; +, zone diameter 1–11 mm; ++, zone diameter 12–16 mm; +++, zone diameter 17–21 mm; ++++, zone diameter ≥22 mm. Results in parentheses refer to activity with some resistance.