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High in vitro antimicrobial activity of β-peptoid–peptide hybrid oligomers against planktonic and biofilm cultures of *Staphylococcus epidermidis*

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ABSTRACT

An array of β-peptoid–peptide hybrid oligomers displaying different amino acid/peptoid compositions and chain lengths was studied with respect to antimicrobial activity against *Staphylococcus epidermidis* both in planktonic and biofilm cultures, comparing the effects with those of the common antibiotic vancomycin. Susceptibility and time–kill assays were performed to investigate activity against planktonic cells, whilst confocal laser scanning microscopy was used to investigate the dynamics of the activity against cells within biofilms. All tested peptidomimetics were bactericidal against both exponentially growing and stationary-phase *S. epidermidis* cells with similar killing kinetics. At the minimum inhibitory concentration (MIC), all peptidomimetics inhibited biofilm formation, whilst peptidomimetics at concentrations above the MIC (80–160 μg/mL) eradicated young (6-h-old) biofilms, whilst even higher concentrations were needed to eradicate mature (24-h-old) biofilms completely. Chiral and guanidinylated hybrids exhibited the fastest killing effects against slow-growing cells and had more favourable antibiofilm properties than analogues only containing lysine or lacking chirality in the β-peptoid residues. However, the results of the mature biofilm killing assay indicated more complex structure–activity relationships. Cytotoxicity assays showed a clear correlation between oligomer length and cell toxicity within each subclass of peptides, but all possessed a high differential toxicity favouring killing of bacterial cells. This class of peptidomimetics may constitute promising antimicrobial alternatives for the prevention and treatment of multidrug-resistant *S. epidermidis* infections.

1. Introduction

Infections with the opportunistic human pathogen *Staphylococcus epidermidis* frequently affect immunocompromised and immunosuppressed patients, especially when subjected to prolonged use of indwelling medical devices [1]. The prevalent antibiotic resistance and ability to form biofilms have led to an increase in complications associated with the treatment of *S. epidermidis* infections [2]. *Staphylococcus epidermidis* biofilms on the surface of indwelling devices such as catheters and prosthetic heart valves are difficult to treat with conventional antibiotics [3]. The increased antibiotic tolerance of biofilm-associated cells has been correlated with their slow growth rate, protection mediated by extracellular polymeric substances and the development of tolerant subpopulations [1,4]. Discovery and development of novel agents for the prevention and treatment of *S. epidermidis* biofilm infections are therefore urgently needed.

Natural host defence antimicrobial peptides (AMPs) are produced by most living organisms and, owing to their unique properties and alternative modes of action, they are considered to be effective against multidrug-resistant bacteria [5]. AMPs have also been reported to efficiently kill slow-growing cells from planktonic and biofilm cultures and thus they have been proposed as promising alternative agents in the treatment of multidrug-resistant infections [6]. Peptidomimetics are structural analogues of peptides containing amide bond isosteres or altered peptide backbones that result in higher stability as well as improved pharmacological profiles. Typically, peptidomimetics arise either from modification of an existing active peptide or from the design of structurally similar compounds that mimic peptides, e.g. β-peptides or peptoids. Appropriately designed peptidomimetics have been shown to be capable of maintaining a broad spectrum of antimicrobial activity whilst possessing advantageous properties over natural AMPs, such as stability against proteolytic enzymes and low toxicity towards mammalian cells [7].

Previously we have described a synthetic approach for the design of peptidomimetics consisting of alternating repeats of α-amino acids and β-peptoid residues [8–11]. These studies

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suggested that one strategy to design peptidomimetics with a favourable balance between potency and cytotoxicity involves incorporation of chiral hydrophobic β-peptoids and guanidinylated amino acid side chains whilst keeping the length relatively short.

In the present study we investigated the antimicrobial activity of the simple alternating β-peptoid–peptide hybrid oligomers (i.e. **1a**–**3d**) and the mixed amino/guanidino subtype of peptidomimetics (i.e. **4a**–**4d**) against the biofilm-forming and methicillin-resistant *S. epidermidis* (MRSE) strain RP62A (ATCC 35984) both in planktonic and biofilm cultures.

Their effect is compared with that of vancomycin (VAN), which is commonly used for the treatment of resistant or severe Gram-positive organisms, and thus constitutes the current antibiotic of 'last resort' [12].

2. Materials and methods

2.1. Synthesis of β-peptoid–peptide hybrid oligomers

The four mixed amino- and guanidino-functionalised oligomers **4a**–**d** [13] were synthesised using our previously described solid-phase procedure [8]. The β-peptoid–peptide hybrid oligomers were dissolved in sterile deionised water (5 mg/mL) and aliquots were stored at −20 °C.

2.2. Haemolysis assay

Haemolysis was performed as described previously using human erythrocytes [14], detecting haemoglobin by measuring the optical density at 405 nm. Melittin (400 μg/mL) and Tris buffer (pH 7.2, 150 mM NaCl) defined 100% haemolysis and 0% haemolysis, respectively.

2.3. Cytotoxicity assay

The cytotoxicity assay was performed essentially as reported previously using HeLa cells [11]. Briefly, HeLa cells were incubated at 37 °C for 1 h with peptidomimetics. Incubation was performed using a horizontal shaking table (with a frequency of 50 movements/min) that was placed in a custom-made temperature-controlled polystyrene cabinet. The tested concentration range was 0.1–1000 μM. Dehydrogenase activity was determined as a result of the amount of formazan produced as measured by absorbance at 492 nm.

2.4. Bacterial strains and growth media

Staphylococcus epidermidis RP62A (ATCC 35984) strain was selected as the model organism for this study as it is considered a benchmark strain among the biofilm-producing *S. epidermidis* strains [15]. Tryptic soy broth (TSB) (Oxoid Ltd., Basingstoke, UK) containing 0.25% glucose was used for biofilm cultivation in a static chamber system. Susceptibility assays were carried out in Mueller–Hinton broth (MHB) (Fluka, Steinheim, Germany). SYTO 9 and propidium iodide (LIVE/DEAD® reagents; Molecular Probes, Eugene, OR) were used at a concentration of 1 μM for staining live or dead bacteria in biofilms.

2.5. Bacterial susceptibility assay

Minimum inhibitory concentrations (MICs) were measured using methods described previously by Wiegand et al. [16]. The minimum bactericidal concentration (MBC) was determined as the lowest concentration that resulted in <0.1% survival of the subculture. All MIC and MBC determinations were made in triplicate. For selected compounds, MICs were also measured using the same method in the biofilm medium (TSB with 0.25% glucose).

2.6. Time–kill assay in fresh Mueller–Hinton broth

Staphylococcus epidermidis cells (1×10^7 CFU/mL) were separately treated with β-peptoid–peptide hybrid oligomers at 4 μg/mL or VAN at 4 μg/mL and 20 μg/mL in fresh MHB. Time–kill experiments were performed at 37 °C with shaking at 220 rpm under aerobic conditions. Culture aliquots (50 μL) were taken at different time points (0, 1, 3, 5, 8 and 24 h), serially diluted, plated onto tryptic soy agar and then incubated at 37 °C for 24 h followed by colony counting. Time–kill curves were constructed by plotting the $\log_{10}$ CFU/mL versus time over a 24-h time period. Assays were performed in duplicate on at least two occasions and similar results were obtained. The detection limit for these assays was 5×10^2 CFU/mL.

2.7. Time–kill assay of stationary-phase cells in nutrient-depleted Mueller–Hinton broth

Nutrient-depleted (or spent) MHB (depMHB) was used to arrest cell growth and to keep cells in the slow-growing stationary phase. depMHB was prepared by the method described previously with modifications [17]. Briefly, *S. epidermidis* was cultivated in MHB at 37 °C for 46 h. Cultures were centrifuged at $8000 \times g$ for 30 min at 4 °C and the supernatants were collected and adjusted to pH 7.0, then filtered through a 0.22 μm pore size syringe filter (TPP, Trasadingen, Switzerland). Stationary-phase cells (1×10^7 CFU/mL) were separately incubated in depMHB in the presence of β-peptoid–peptide hybrid oligomers or VAN at 4 μg/mL and 20 μg/mL. The time–kill assay was performed as above.

2.8. Biofilm susceptibility assay

Static chamber *S. epidermidis* biofilms were cultivated in a coverglass cell culture chamber (Nunc, Roskilde, Denmark) as described previously [18]. Briefly, the chamber wells were inoculated with 1.5 mL of diluted overnight cultures (5×10^5 CFU/mL). Following incubation at 37 °C, the 0-, 6- and 24-h pre-formed biofilms in the chambers were washed gently twice with sterile phosphate-buffered saline (1 mL) to remove planktonic cells. Fresh medium containing antimicrobial agents was then added and the biofilm cultures were incubated at 37 °C for 24 h. After removal of the medium, biofilm cells were stained with the LIVE/DEAD reagents and were then observed by confocal laser scanning microscopy (CLSM). This assay was repeated three times and similar results were obtained.

2.9. Microscopy image acquisition and analysis

All microscopy observations and image acquisitions were performed using methods described previously by Qin et al. [18]. For quantification of biofilms, at least six CLSM images from each sample were analysed using the computer program COMSTAT. Statistical differences in comparison with the control (without added antimicrobial agent) were determined by one-way analysis of variance (ANOVA). Differences were considered statistically significant at a P-value of <0.05.

3. Results

In the present study, 16 β-peptoid–peptide hybrid oligomers (**1a**–**4d**) (Table 1; Fig. 1) were investigated for their antimicrobial activity against both planktonic and biofilm-associated *S. epidermidis* cells as well as for their cytotoxicity towards human