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Research Paper

End group modification: Efficient tool for improving activity of antimicrobial peptide analogues towards Gram-positive bacteria

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ABSTRACT

Increased incidence of infections with multidrug-resistant bacterial strains warrants an intensive search for novel potential antimicrobial agents. Here, an antimicrobial peptide analogue with a cationic/hydrophobic alternating design displaying only moderate activity against Gram-positive pathogens was optimized. Generally, introduction of hydrophobic moieties at the *N*-terminus resulted in analogues with remarkably increased activity against multidrug-resistant *Staphylococcus aureus* and *Enterococcus faecium*. Interestingly, the potency against *Escherichia coli* strains was unaffected, whereas modification with hydrophobic moieties led to increased activity towards the Gram-negative *Acinetobacter baumannii*. Despite increased cytotoxicity against murine fibroblasts and human umbilical vein endothelial cells, the optimized peptide analogues exhibited significantly improved cell selectivity. Overall, the most favorable hydrophobic activity-inducing moieties were found to be cyclohexylacetyl and pentafluorophenylacetyl groups, while the presence of a short PEG-like chain had no significant effect on activity. Introduction of cationic moieties conferred no effect or merely a moderate activity-promoting effect to the analogues.

1. Introduction

Multidrug-resistant (MDR) Gram-positive bacteria constitute a major health concern worldwide. Although strains such as vancomycin-resistant *enterococci* (VRE) and methicillin-resistant *Staphylococcus aureus* (MRSA) are still treatable by second- or third-line antibiotics, the treatment options for MDR strains in general are declining rapidly [1,2]. Indeed, these pathogens represent a severe threat to the general public health, *e.g.* nosocomial MRSA infections are estimated to kill a similar number of patients in the United States as HIV/AIDS, tuberculosis and viral hepatitis altogether on an annual basis [3,4]. Considerable effort has been devoted to the research in and development of synthetic antimicrobial peptides (AMPs), as these have been shown to readily kill pathogens resistant to conventional antibiotics [5]. Peptide analogues with a design mimicking the activity of AMPs are usually preferred over sequence-modified peptides due to their superior stability towards enzymatic degradation [6]. Previously, we have shown that hybrids consisting of α-amino acids and α-peptoid (*N*-alkylated glycine) or β-peptoid (*N*-alkylated β-alanine) residues exhibit antibacterial [7,8] and antiplasmodial activity [9], significant anti-biofilm [10,11] activity as well as cell-penetrating properties [12–14]. In the present work, we show that *N*-terminal modification may serve as an efficient tool for enhancing the activity against clinically important Gram-positive pathogens. It is envisioned that similar modifications may be of utility in the optimization of antimicrobial peptides or peptidomimetics in general.

Recently, we reported on an *N*-acetylated 12-meric oligomer, having an alternating sequence composed of cationic amino acids and hydrophobic β-peptoid residues (Fig. 1; compound **1**), that exhibited moderate activity against *S. aureus* [8]. A protocol involving dimeric building blocks was employed in the preparation of analogues displaying various modifications at the N-terminus (Fig. 1; compounds **2**–**16**) [15,16]. Cationic properties are essential to the activity of most antimicrobial peptides, and thus modifications increasing the net positive charge of AMPs have been correlated with improved antibacterial activity [17]. Consequently, the chosen α-peptide/β-peptoid template was subjected to atypical

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modification with acyl moieties containing one or more amine functionalities (compounds **2**–**5**). Acylation with fatty acids [17–20] and PEGylation [21] have been associated with increased antibacterial activity and cell selectivity, and thus decanoylation (compound **7**) and introduction of the corresponding polar isosteric PEG-like moiety (compound **6**) were included in the test array. Furthermore, a variety of aliphatic and aromatic hydrophobic groups were appended (compounds **8**–**13**), since similar alterations have been shown to increase antibacterial activity of AMP analogues [22]. In particular, indole moieties (compounds **9** and **10**) have been linked to desirable activity levels [23], and also *N*-terminal tagging with tryptophan has previously been found to increase the activity of AMPs [24]. Finally, the *N*-terminus was conjugated to different aromatic fluorinated moieties (**14**–**16**), as fluorous modifications in the magainin II analog pexiganan and other synthetic AMPs have been shown to improve antibacterial activity [25–28].

2. Materials and methods

Starting materials and solvents were purchased from commercial suppliers (Alfa Aesar, CHEMsolute, Iris Biotech, Sigma Aldrich, VWR, AppliChem, Fluka, ABCR, LabScan and Merck) and used without further purification. Water used for analytical and preparative HPLC was filtered through a 0.22 μm membrane filter (Millipore). Analytical HPLC was used to determine purity and was carried out on a Phenomenex Luna C18 (2) (3 μm) column (150 mm × 4.6 mm) using binary mixtures of eluent A (water–MeCN–TFA 95:5:0.1) and eluent B (water–MeCN–TFA 5:95:0.1). Elution with a flow rate of 0.8 mL/min used a linear gradient of 10–60% B during 30 min with UV detection at λ = 220 nm. All compounds had a purity of at least 95%; retention times (t_R) are given for each compound. Preparative HPLC was performed by using a Luna C18 (2) (5 μm) column (250 mm × 21.2 mm) on an Agilent 1100 LC system with a multiple-wavelength UV detector. Elution was performed with a linear gradient of 10 → 40% B or 10 → 60% B during 20 min at a flow rate of 20 mL/min with UV detection at λ = 220 nm. LC-HRMS was performed with a Phenomenex Luna C18 (2) (3 μm) column (150 mm × 4.6 mm) using binary mixtures of eluent C (water–MeCN–formic acid 95:5:0.1) and eluent D (water–MeCN–formic acid 5:95:0.1); elution was performed with a linear gradient of 10–60% D during 30 min at a flow rate of 0.5 mL/min. HRMS spectra were obtained by using a Bruker MicrOTOF-Q II Quadropol MS detector. The analyses were performed as ESI-MS (m/z): $[M+5H]^{5+}$ with $\Delta M < 2$ ppm.

2.1. General procedure for synthesis of dimeric building blocks

Dimeric building blocks were prepared as previously described [29]. In brief, an intermediate *tert*-butyl ester was prepared from the appropriate amine via alkylation with *tert*-butyl acrylate followed by amide bond formation with Fmoc-Lys(Boc)-OH. Upon treatment with TFA the protected cationic functionality was obtained either via Boc protection or by conversion into a bis(-Boc)-protected guanidine group. In both cases the crude product was purified by using vacuum liquid chromatography (VLC). Purity was confirmed by analytical HPLC, while structural identity was confirmed by NMR and LC-HRMS as reported previously [29].

2.2. General procedure for synthesis of peptide analogues

The peptide analogues were prepared as previously described [15]. In brief, Rink amide resin (loading: 0.70 mmol/g) and Teflon reactors (10 mL) were used for all compounds. Fmoc deprotection conditions: Excess 20% piperidine–DMF (2 × 10 min under shaking at room temperature). Washing conditions: DMF, MeOH, and DCM (each 3 × 3 min). Coupling conditions: Building block, PyBOP, and DIPEA (each 2.0 equiv. for loading; 2.5 equiv for coupling #1, and #2; 3.0 equiv for all subsequent couplings; in all cases: ⩾2 h coupling time under shaking at room temperature). Capping was applied after coupling #4: excess Ac_2O–DIPEA–NMP 1:2:3 (10 min at room temperature). Final Fmoc deprotection was followed by attachment of the *N*-terminal end groups. The corresponding carboxylic acid version of each moiety was added under conditions identical to those applied for the above coupling procedure used for the dimeric building blocks. Cleavage and simultaneous side chain deprotection: Excess TFA–water 95:5 (1 h under shaking at room temperature). The filtrate was collected and the resin was eluted with DCM (2 mL), MeOH (2 mL), TFA–water 95:5 (2 mL) and DCM (2 mL). The combined filtrates were concentrated *in vacuo*, and then co-evaporated with toluene and MeOH (each 3 × 5 mL). The crude product was purified by using preparative HPLC and concentrated *in vacuo* as previously described [7]. Finally, the product was dissolved in water (1 mL) and lyophilized.

Compound 1: Analytical HPLC; t_R = 20.0 min (97.0%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 402.0617, found 402.0621; ΔM = 1.0 ppm.

Compound 2: Analytical HPLC; t_R = 20.1 min (99.7%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 405.0639, found 405.0640; ΔM = 0.2 ppm.

Compound 3: Analytical HPLC; t_R = 19.5 min (95.3%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 413.6723, found 413.6725; ΔM = 0.5 ppm.

Compound 4: Analytical HPLC; t_R = 18.9 min (96.6%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 442.1006, found 442.1004; ΔM = 0.5 ppm.

Compound 5: Analytical HPLC; t_R = 20.0 min (96.5%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 393.6586, found 393.6586; ΔM = 0.0 ppm.

Compound 6: Analytical HPLC; t_R = 21.3 min (98.4%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 425.6740, found 425.6740; ΔM = 0.0 ppm.

Compound 7: Analytical HPLC; t_R = 26.2 min (97.8%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 424.4865, found 424.4865; ΔM = 0.0 ppm.

Compound 8: Analytical HPLC; t_R = 22.9 min (98.4%, at 220 nm). HRMS: calcd for $[M+5H]^{4+}$ 419.6679, found 419.6678; ΔM = 0.2 ppm.

Compound 9: Analytical HPLC; t_R = 22.4 min (99.1%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 425.0687, found 425.0686; ΔM = 0.2 ppm.

Compound 10: Analytical HPLC; t_R = 24.0 min (95.7%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 462.2851, found 462.2848; ΔM = 0.6 ppm.

Compound 11: Analytical HPLC; t_R = 23.2 min (98.3%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 418.4762, found 418.4766; ΔM = 1.0 ppm.

Compound 12: Analytical HPLC; t_R = 24.8 min (96.4%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 428.8837, found 428.8845; ΔM = 1.9 ppm.

Compound 13: Analytical HPLC; t_R = 25.3 min (95.7%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 471.7147, found 471.7145; ΔM = 0.4 ppm.

Compound 14: Analytical HPLC; t_R = 23.7 min (99.2%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 435.2574, found 435.2572; ΔM = 0.5 ppm.

Compound 15: Analytical HPLC; t_R = 26.6 min (96.9%, at 220 nm). HRMS: calcd for $[M+5H]^{5+}$ 482.6637, found 482.6645; ΔM = 1.7 ppm.