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Investigating the antimicrobial peptide 'window of activity' using cationic lipopeptides with hydrocarbon and fluorinated tails

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ABSTRACT

To probe the effect of carbon–fluorine bonds on antimicrobial peptide–membrane interactions, 24 cationic lipopeptides were created. The collection of lipopeptides was built from two different peptide sequences, KGK and KKK, with a variety of different lipids selected to probe the effectiveness of both hydrocarbon and fluorinated tails. The antimicrobial activity of each peptide was tested against a mixture of pathogenic and reference bacterial strains, with the cationic disinfectant benzalkonium chloride as a positive control. Non-specific interactions with hydrophobic proteins were assessed by repeating antimicrobial testing in the presence of bovine serum albumin (BSA), and the toxicity of the lipopeptides was assessed by measuring lysis of ovine erythrocytes. Peptide sequence had a moderate effect on activity, with the most active peptide (C16-KGK) inhibiting the growth of two *Staphylococcus epidermidis* strains at $\leq$0.25 μg/mL. Tail composition was less important than the overall hydrophobicity, with the most active fluorinated tails equivalent to moderately active hydrocarbon tails. The activity of all peptides was significantly reduced by the presence of BSA, and haemolysis was closely correlated with antimicrobial activity.

1. Introduction

Over the last few decades, the emergence of drug-resistant bacteria has reduced the efficacy of current antibiotics, relegating first-line treatments to the sidelines [1]. With a reduction in industry investment, the drug pipeline has not kept pace with bacterial evolution, leading many health experts to declare an urgent need for new antibacterials and novel antibiotic scaffolds [2].

One promising avenue of research involves the cationic antimicrobial peptides (CAMPs), used throughout the plant and animal kingdoms to defend against invasive bacteria and viruses [3]. Unlike most antibiotics, CAMPs do not appear to act on any single molecular target. Instead they associate with a large number of anionic, hydrophobic structures such as DNA, folding proteins and the bacterial membrane [4]. This promiscuity is the CAMPs' greatest strength and weakness. Effective resistance mechanisms are rare and are difficult for bacteria to develop in vitro without reducing fitness in the absence of CAMPs [5–7]. Unfortunately, the same tendency for non-specific interactions may lead to significant eukaryotic cell toxicity, limiting therapeutic use of AMPs to topical applications [8,9]. The development and widespread application of CAMPs is further complicated by their long length, as the peptides are impractical to produce synthetically. In vivo production of the 15–30 residue sequences is more cost effective but requires the development of sophisticated production and purification methodology to limit bacterial toxicity [10,11].

Attempts to circumvent the high cost of CAMP production led to the ultrashort lipopeptides, analogues with brief amino acid sequences and a lipid tail [12]. In lipopeptides, the necessary cationic charge is provided by two or more basic residues, whilst the tail provides a strong hydrophobic domain. As with natural CAMPs, large hydrophobic domains increase interactions with bacterial and eukaryotic membranes, leading to depolarisation and lysis of the cell [13,14], whilst smaller domains allow amphiphiles to pass through the membrane and interact with internal targets such as DNA [4]. Larger hydrophobic domains also increase binding to hydrophobic proteins such as bovine serum albumin (BSA), and so the activity of many natural CAMPs and derivatives is sharply decreased in environments similar to human serum [15].

Examining the current literature, we observed that most CAMPs used only carbon–hydrogen bonds in their hydrophobic domains [16–18]. Molecules with carbon–fluorine (CF) bonds are both hydrophobic and lipophobic and as a result may associate primarily with other CF-containing materials. Amphiphiles heavy with CF bonds might therefore prefer to self-associate, reducing the effect of their hydrophobic character whilst in solution and potentially creating areas of high peptide concentration within the

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Table 1
Lipopeptides under consideration.

Compound	Sequence	Molecular mass (g/mol)
BAC	$C_6H_5CH_2N(CH_3)_2RCl$; R = C_8H_{17}–$C_{18}H_{37}$	283.88–424.15
C7-KGK	$CH_3(CH_2)_5CO$-KGK-NH_2	670.64
C9-KGK	$CH_3(CH_2)_7CO$-KGK-NH_2	682.70
C9B-KGK	$(CH_3)_2(CH_2)_7CO$-KGK-NH_2	712.72
C11-KGK	$CH_3(CH_2)_9CO$-KGK-NH_2	726.75
C14-KGK	$CH_3(CH_2)_{12}CO$-KGK-NH_2	768.83
C16-KGK	$CH_3(CH_2)_{14}CO$-KGK-NH_2	796.82
C20-KGK	$CH_3(CH_2)_{18}CO$-KGK-NH_2	852.99
C16OH-KGK	$CH_2OH(CH_2)_{14}CO$-KGK-NH_2	812.88
F7-KGK	$CF_3(CF_2)_5CO$-KGK-NH_2	832.56
F9-KGK	$CF_3(CF_2)_7CO$-KGK-NH_2	932.57
F9B-KGK	$(CF_3)_2(CF_2)_7CO$-KGK-NH_2	982.57
F11-KGK	$CF_3(CF_2)_9CO$-KGK-NH_2	1032.59
C7-KKK	$CH_3(CH_2)_5CO$-KKK-NH_2	855.79
C9-KKK	$CH_3(CH_2)_7CO$-KKK-NH_2	883.84
C9B-KKK	$(CH_3)_2(CH_2)_7CO$-KKK-NH_2	897.87
C11-KKK	$CH_3(CH_2)_9CO$-KKK-NH_2	911.89
C14-KKK	$CH_3(CH_2)_{12}CO$-KKK-NH_2	953.97
C16-KKK	$CH_3(CH_2)_{14}CO$-KKK-NH_2	982.03
C20-KKK	$CH_3(CH_2)_{18}CO$-KKK-NH_2	1038.13
C16OH-KKK	$CH_2OH(CH_2)_{14}CO$-KKK-NH_2	996.09
F7-KKK	$CF_3(CF_2)_5CO$-KKK-NH_2	1017.70
F9-KKK	$CF_3(CF_2)_7CO$-KKK-NH_2	1117.72
F9B-KKK	$(CF_3)_2(CF_2)_7CO$-KKK-NH_2	1167.72
F11-KKK	$CF_3(CF_2)_9CO$-KKK-NH_2	1217.73

BAC, benzalkonium chloride.

membrane. Because the initial interactions between CAMPs and bacterial membranes involve electrostatic interactions between the cationic moieties and the anionic bacterial phospholipids [3], self-association of fluorinated CAMPs is unlikely to alter insertion into bacterial membranes. In contrast, with the zwitterionic eukaryotic membrane, initial CAMP interactions are dominated by hydrophobic interaction effects. Fluorinated CAMPs could thus show significantly reduced toxicity without a similar reduction in their antimicrobial activity, greatly widening the therapeutic window. As interactions with BSA are also largely driven by hydrophobic effects [15], these compounds might even retain their antibacterial activity in the presence of BSA.

Starting with the known lipopeptides C16-KKK and C16-KGK [13], a series of analogues of various tail lengths was prepared to investigate this hypothesis (Table 1). Hydrophobic tails were constructed both of saturated hydrocarbons and fluorocarbons, with a larger selection of hydrocarbons to investigate the difference in tail type from both a length and mass standpoint.

2. Materials and methods

2.1. Materials

Fmoc MBHA Rink Amide resin, TBTU and PyBOP® were purchased from Bachem (Bubendorf, Switzerland). Fluorinated carboxylic acids were purchased from Fluorous Technologies Inc. (Pittsburgh, PA). Carboxylic acids with hydrocarbon tails and all other solvents and reagents were purchased from Sigma-Aldrich (St Louis, MO) at reagent grade and were used without further purification.

2.2. Peptide synthesis

All lipopeptides were synthesised on solid phase using standard Fmoc chemistry [19]. Peptides were purified using reverse-phase flash chromatography and purity was confirmed with a mixture of ^1H and ^{13}C nuclear magnetic resonance on a Bruker AMX-500 spectrometer (Bruker, Billerica, MA) and electrospray ionisation mass spectrometry (ESI-MS) on a Varian 500-MS IT mass spectrometer (Varian, Santa Clara, CA).

2.3. Antimicrobial activity

The activity of purified lipopeptides was tested against a variety of pathogenic and standard laboratory reference bacterial strains according to Clinical and Laboratory Standards Institute (CLSI) macrobroth standards [20]. Stock solutions at 512 μg/mL were prepared, with dimethyl sulphoxide (DMSO) used to increase lipopeptide solubility as required, and testing was performed in glass test tubes using Muller–Hinton broth and bacteria adjusted to 5×10^5 colony-forming units (CFU)/mL. Bacteria were incubated with the lipopeptide of interest for 24 h at 37 °C prior to reading.

The bacteria *Staphylococcus aureus* ATCC 29213, meticillin-resistant *S. aureus* (MRSA) ATCC 33592, *Staphylococcus epidermidis* ATCC 14990, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 27270, *Streptococcus pneumoniae* ATCC 49619, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Klebsiella pneumoniae* ATCC 13883 were acquired from the American Type Culture Collection (ATCC) and were used as quality controls. The clinical strains meticillin-resistant *S. epidermidis* (MRSE) CAN-ICU 61589, *E. coli* CAN-ICU 61714, *E. coli* CAN-ICU 63074, *P. aeruginosa* CAN-ICU 62308, *Stenotrophomonas maltophilia* CAN-ICU 62584 and *Acinetobacter baumannii* CAN-ICU 63169 were obtained from hospitals across Canada as part of the Canadian National Intensive Care Unit (CAN-ICU) studies [21], whilst meticillin-susceptible *S. epidermidis* (MSSE) 81388 was obtained from the 2008 Canadian Ward Surveillance (CANWARD) Study [22].

2.4. Haemolytic activity

Non-specific interactions with eukaryotic membranes were assessed using ovine erythrocytes, a standard model for human cell toxicity. Cells were pre-washed with Tris-buffered saline and were then incubated with a variety of lipopeptide concentrations for 30 min. Following centrifugation, lysis was evaluated by comparing the absorbance measurements at 540 nm, using 0.5% NH_4OH as a positive control [23].

3. Results

3.1. Lipopeptide synthesis

Twenty-four amphiphilic lipopeptides were synthesised on solid phase (Table 1). A variety of hydrocarbon and fluorocarbon lipid tails were used to create strong hydrophobic domains, whilst the amino acid sequences KKK and KGK were used to provide a cationic charge at physiological pH through protonation of the lysine R group. As amides containing fully fluorinated carbon tails were found to be unstable, hydrolysing at room temperature to highly acidic carboxylic acids, fluorinated tails with ethylene spacers between the carboxylic acid and CF bonds were used instead. The two peptides with hydrophilic tails (C16OH-KGK and C16OH-KKK) were produced without issue.

3.2. Antimicrobial activity

Using the cationic disinfectant benzalkonium chloride (BAC) as a positive control [24], the activity of the cationic lipopeptides was assessed against a selection of Gram-positive and Gram-negative bacteria (Tables 2 and 3). The best antimicrobial activity obtained was that of C16-KGK against the MSSE and MRSE strains. Inhibiting bacterial growth at ≤0.25 μg/mL, this peptide was more active than even F11-KGK, despite the long fluorinated tail. Lipopeptide C16-KGK was also the most active compound overall, displaying the