The Effect of Lipidation and Glycosylation on Short Cationic Antimicrobial Peptides

Elizabeth Grimsey1, Dominic W. P. Collis2, Ralf Mikut3 and Kai Hilpert1\*

1Institute for Infection and Immunity, St. George's University of London, London, UK

2TiKa Diagnostics Ltd, London, UK

3Karlsruhe Institute of Technology (KIT), Institute for Automation and Applied Informatics (IAI), Eggenstein-Leopoldshafen, Germany

**\*Correspondence:**Kai Hilpert

khilpert@sgul.ac.uk

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Abstract

The global health threat surrounding bacterial resistance has resulted in antibiotic researchers shifting their focus away from ‘traditional’ antibiotics and concentrating on other antimicrobial agents, including antimicrobial peptides. These low molecular weight proteins exhibit broad-spectrum activity against bacteria, including multi-drug resistant strains, viruses, fungi and protozoa and constitute a major element of the innate-immune system of many multicellular organisms. Some naturally occurring antimicrobial peptides are lipidated and/or glycosylated and almost all antimicrobial peptides in clinical use are either lipopeptides (Daptomycin and Polymyxin E and B) or glycopeptides (Vancomycin). Lipidation, glycosylation and PEGylation are an option for improving stability and activity in serum and for reducing the rapid clearing via the kidneys and liver. Two broad-spectrum antimicrobial peptides NH2-RIRIRWIIR-CONH2 (A1) and NH2-KRRVRWIIW-CONH2 (B1) were conjugated via a linker, producing A2 and B2, to individual fatty acids of C8, C10, C12 and C14 and in addition, A2 was conjugated to either glucose, N-acetyl glucosamine, galactose, mannose, lactose or polyethylene glycol (PEG). Antimicrobial activity against two Gram-positive strains (methicillin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus faecalis* (*VRE)*) and three Gram-negative strains (*Salmonella typhimurium, E. coli* and *Pseudomonas aeruginosa*) were determined. Activity patterns for the lipidated versions are very complex, dependent on sequence, bacteria and fatty acid. Two reciprocal effects were measured; compared to the parental peptides, some combinations led to a 16-fold improvement whereas other combinations let to a 32-fold reduction in antimicrobial activity. Glycosylation decreased antimicrobial activity by 2 to 16-fold in comparison to A1, respectively on the sugar-peptide combination. PEGylation rendered the peptide inactive. Antimicrobial activity in the presence of human serum of A1 and B1 was reduced 32-fold and 8-fold, respectively. The longer chain fatty acids almost completely restored this activity; however, these fatty acids increased hemolytic activity. B1 modified with C8 increased the therapeutic index by 2-fold for four bacterial strains. Our results suggest that finding the right lipid-peptide combination can lead to improved activity in the presence of serum and potentially more effective drug candidates for animal studies. Glycosylation with the optimal sugar and numbers of sugars at the right peptide position could be an alternative route or could be used in addition to lipidation to counteract solubility and toxicity issues.

Introduction

Since their introduction in the 1930’s, antibiotics have been heralded as the miracle discovery of the 20th century. Undoubtedly, access to efficient antibiotics is of critical importance to society, with numerous procedures including organ transplants, surgery and chemotherapy carrying high, if not prohibitive risks without access to these antimicrobial agents [1]. Unfortunately, the effectiveness of any novel antibiotic is compromised by the presence of drug-resistant organisms, exhibiting resistance to one or more types of antibiotic. In modern society, we face the shortfall between the ever-growing number of antibiotic-resistant bacteria and new antibiotics with novel modes of action [2,3]. If nothing changes in the existing trend, it has been predicted that by 2050 antimicrobial resistance (AMR) will be a bigger killer than diseases such as cancer [4].

Over the last 30 years, more than 100 peptide-based drugs have been released for clinical use against a variety of pathologies including: diabetes, cancer, obesity, cardiovascular disease, inflammation and osteoporosis; generating a net revenue of over US$40 billion annually [5]. Peptide-based drugs have multiple advantages, such as ease of synthesis and scalability as well as known degradation pathways leading to non-toxic by-products. As a result, in the years from 2016-2024 the growth in the development of peptide-based drugs is expected to rise by 9.1% and exceed US$70 billion in revenue by the end of 2019 [6].

AMP’s are produced by a wide range of organisms and exhibit a broad spectrum of antimicrobial activity, contributing to both plant and animal host defense systems [7]. Most of these evolutionarily conserved host defense peptides are composed of 5-100 amino acids and are cationic with a net positive charge of +1 to +9 owing to the excess of positively charged arginine and lysine as well as a high proportion (≥30%) of hydrophobic amino acids [7,8]. A small number of negatively charged anionic AMP’s with net charges of between -1 to -7 also exist [9]. Regardless of charge, length or structure, the key principle underlying these AMP’s is their ability to fold into an amphipathic structure in which they form separate regions rich in charged or hydrophobic residues. This structure enables the peptide to be soluble in an aqueous environment, but also allows their entry into the lipid-rich bacterial membrane [10,11]. However, despite this shared amphipathic structure, AMP’s have such a wide diversity that the same peptide sequence is seldom isolated from different species, even those that are highly evolutionarily conserved. While this diversity has its advantages, it also makes characterizing peptides a challenging process [12]. In the last two decades of AMP research, it became clear that these molecules have multiple biological activities, including antibacterial, antifungal, antiviral, antiparasitic, anticancer and immunomodulatory [13]. During the same time period, multiple bacterial targets of AMP’s were discovered [14], such as binding to RNA, DNA or histones [15–18] blocking DNA-dependent enzymes [19,20], blocking the synthesis of important outer membrane proteins [21], binding to the chaperon DnaK, the ribosome [22–24] and lipid II [25,26]. In order to screen and discriminate different modes of action in a medium-high throughput manner a novel technique using small-angle X-ray scattering was developed [27,28] In addition, considering most AMP’s are administered intravenously, studies were performed to investigate the interaction of AMP’s with blood compounds [29].

In nature a range of antimicrobial peptides are modified such as; modified amino acids, cyclisation, lipidation and glycosylation are frequent. Clinically, the most successful antimicrobial peptides are cyclic lipopeptides, such as Daptomycin and Polymyxin E and B or cyclic glycopeptides, such as Vancomycin. The landmark publication from Makovitzki et al. showed that short peptides (4mers), when coupled with different lipids can have strong antimicrobial activity [30]. It was shown that both the peptide sequence and the choice of lipid played a role in the antimicrobial and the hemolytic effect of these lipopeptides.

Here, we describe the impact of N-terminal modifications (lipidation, glycosylation and PEGylation) on the antimicrobial activity and toxicity of two peptide sequences which have shown broad spectrum antimicrobial activity against a variety of organisms including methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. These two peptides are part of an ongoing drug optimization project (unpublished, publication in preparation), where one strategy was to modify peptides in order to optimize their therapeutic potential.

Materials and Methods

**Peptides**

Antimicrobial peptides were synthesized by automated solid-phase peptide synthesis (SPPS) on a MultiPep RSI peptide synthesizer (Intavis, Tübingen; Germany) using the 9-fluorenyl-methoxycarbonyl-tert-butyl (Fmoc/tBu) strategy. Reactive side chains were protected by *t*Bu (Tyr and Asp), trityl (Trt, for Asn, Cys, Gln and His), 2,2,4,6,7 pentamethyldihydrobenzofuran-5-sulfonyl (Pbf, for Arg) and *tert*-butoxycarbonyl (Boc, for Lys and Trp). For automated SPPS, four equivalents of Fmoc amino acids (Bachem, Bubendorf, Switzerland) were coupled on TentaGel® HL RAM resin (25 μmol scale, loading 0.3- 0.4 mmol/g, Rapp Polymere, Tübingen, Germany) after *in situ* activation with four equivalents of N,N,N′,N′-Tetramethyl-O (1H-benzotriazol-1-yl) uronium hexafluorophosphate (HBTU; Carbosynth, Berkshire, United Kingdom) and eight equivalents of N-Methylmorpholine (NMM, Sigma, Dorset, United Kingdom). After a double-coupling procedure (2x30 min) the Fmoc group was cleaved using 20% (*v*/*v*) piperidine (Thermofisher Acros Organics, Geel, Belgium) in dimethylformamide (DMF, Jencons-VWR, Leicestershire, United Kingdom). Peptide amides were cleaved from the resin with 95% (*v*/*v*) aqueous trifluoroacetic acid solution (TFA, Fisher Scientific, Loughborough, United Kingdom) containing 5% (*v*/*v*) triisopropylsilane (TIPS, Thermofisher Acros Organics, Geel, Belgium) / water (1:1) scavenger mixture within 3 h. Cleaved peptides were precipitated from ice-cold methyl *tert*-butyl ether (MTBE; Thermofisher Acros Organics, Geel, Belgium). After washing and collection by centrifugation, the crude peptides were dissolved in 20% (*v*/*v*) acetonitrile (ACN, Jencons-VWR, Leicestershire, United Kingdom) / 80% (*v*/*v*) water containing 1% (*v*/*v*) TFA, to a concentration of 15 mg/ml and analyzed by analytical reversed-phase (RP) HPLC on a Shim-pack VP-ODS (120 Å, 150x4.6 mm, Shimadzu, Milton Keynes, United Kingdom) using a Shimadzu LC2010AHT system. The binary solvent system contained 0.1% (*v*/*v*) TFA in H2O (solvent A) and 0.1% (*v*/*v*) TFA in acetonitrile (solvent B). The identity was verified by a liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS) Shimadzu LC2020 system equipped with a Jupiter 4μ Proteo C18 column (90 Å, 250x4.6 mm, Phenomenex, Cheshire, United Kingdom). The binary solvent system contained 0.01% (*v*/*v*) TFA in H2O (solvent A), and 0.01% (*v*/*v*) TFA in acetonitrile (solvent B).

The peptides elongated with the linker structure (A2 and B2) were modified with four fatty acids of increasing hydrophobicity (C8, C10, C12 and C14) under the HOBT/DIC strategy. The reaction conditions were not sufficient to drive the reaction to completion, homogeneities of as low as 18% were produced. Therefore, a second coupling step was required to produce peptides with homogeneities upwards of 85%. Peptide A2 was additionally modified with five individual sugar residues (glucose, galactose, mannose, lactose and N-Acetylglucosamine (GlcNAc)). The high price of the starting material meant that glycosylation proceeded under the HATU/DIPEA strategy. HATU allows a faster coupling reaction with less epimerization than its HBTU and DIC counterparts, allowing the use of a smaller excess of sugar to obtain the same degree of efficiency. Like lipidation, the first coupling reaction produced homogeneities of between 26% and 48%. A second coupling reaction was performed under the same conditions but with a 1.5 excess of sugar as opposed to the two-fold excess used previously. Crude peptides were purified to homogeneity of >89% by preparative RP HPLC on a Shimadzu LC2020 system equipped with a Jupiter 10μ Proteo C18 column (90 Å, 250x21.2 mm, Phenomenex) using a linear gradient system containing 0.01% (*v*/*v*) TFA in H2O (solvent A) and 0.01% (*v*/*v*) TFA in acetonitrile (solvent B). Finally, pure products were characterized by analytical RP-HPLC and LCMS.

**Bacterial Strains**

The bacterial strains used were *Escherichia coli* UB1005 (F-*nal*A37, *met*B1), wild-type *Pseudomonas aeruginosa* PAO1, vancomycin resistant *Enterococcus faecalis* ATCC29212 and wild-type *Salmonella enterica* serovar Typhimuriumall obtained from R.E.W. Hancock (Department of Microbiology and Infection, University of British Columbia, Vancouver) and methicillin resistant *Staphylococcus aureus* ATCC25923 obtained from J. Lindsay (Institute of Infection and Immunity, St Georges, University of London).

**Bacteriological Media And Culture Conditions**

Mueller Hinton broth (MHb) (Merck) was used for all bacterial cultures. Media was prepared and sterilized according to the manufacturer's’ instructions. Cultures were incubated at 37oC for 18-20 h with aeration and cultured on Mueller Hinton agar (Merck) were incubated at 37oC for 18-24 h.

**Minimal Inhibitory Concentration Determination**

Minimum inhibitory concentrations (MIC) were determined using a broth microdilution assay as previously described [31]. In short, bacteria from an overnight culture grown at 37˚C were diluted in fresh MHb to achieve a concentration of 1 x 106 CFU/ml. 100 µl of the bacterial suspension was added to wells in a 96 well polypropylene microtiter plate that had been preloaded with serial dilutions of antimicrobial peptides in MHb (100 µl), giving a final bacterial concentration of 5 x 105 CFU/ml. Microtiter plates were incubated at 37˚C for 18-20 h before the MIC was determined as the lowest concentration of antimicrobial able to inhibit visible growth. The MIC of the peptides in serum was determined the same way, except an equal volume of 50 % human serum was added to the broth culture in order to obtain 25% serum conditions.

**Hemolytic Activity Assessment**

Erythrocytes from human donors was used to test all peptides for hemolytic activity. The toxic effect was assessed by hemoglobin release from human red blood cells resulting from cell lysis, according to standard procedures, modified to be carried out in 96-well polypropylene microtiter plates for high-throughput screening. The blood was washed and diluted with PBS and transferred into 96 well plates. Peptides were added as a pre-made dilution series and incubated at 37°C for 1 hour. Triton X (1% final concentration) was used for the 100% hemolysis value. Hemoglobin release was monitored chromogenically at 414 and 546 nm using an ELISA plate reader. Untreated wells were used to measure auto-hemolysis and this value was subtracted from all other measurements on the corresponding plate. HC50 values were determined using the Prism software (GraphPad).

Results

Two peptides from a current drug optimization project (unpublished data, manuscript in preparation) were chosen based on the evidence that they killed Gram-positive as well as Gram-negative multi-drug resistant bacteria (Figure 1). Peptides NH2-RIRIRWIIR-CONH2 (A1) and NH2-KRRVRWIIW-CONH2 (B1) were active against methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus faecalis* (VRE) and *Pseudomonas aeruginosa* respectively, bacteria that are problematic to treat due to various resistance patterns. For example, carbapenem-resistant *Pseudomonas aeruginosa* is classified as a priority one organism by the World Health Organization (WHO).

**Peptide design and synthesis**



Figure 1: Schematic representation of the two parent peptides NH2-RIRIRWIIR-CONH2 (A1) and NH2-KRRVRWIIW-CONH2 (B1) using the program PEPDRAW (<http://pepdraw.com/>).

The parental peptide sequences A1 and B1 were modified by elongation at their N-terminus with a spacer consisting of two beta-alanine (β-Ala) and a single Boc-protected lysine (Lys), producing peptides A2 and B2 (Figure 2). The Fmoc protected ε-amine of the lysine could be cleaved to produce a free amine in which to modify upon, whilst the Boc-protected amine of the main chain would be cleavage upon acidolysis.

A

B

C

D

**Figure 2**: A) Peptide design. The peptide backbone was synthesized using automated SPPS, then elongated at the N-terminus by the addition of a spacer consisting of two Fmoc-β-Ala-OH and a single Boc-Lys (Fmoc)-OH. The modifications were manually introduced onto the lysine side chain at the peptide’s N-terminus; B) the introduction of PEG, C) a sugar and D) a fatty acid, at the Lys side chain present at the peptides N-terminus.

The elongation of the peptides A1 and B1 with the described β-Ala spacer and a Lys resulted in peptides A2 and B2 (see Table 1). A2 and B2 were then modified with four different fatty acids of increasing length (C8, C10, C12 and C14), and peptide A2 was additionally modified with five distinct sugars (galactose, glucose, N-acetylglucosamine (GlcNAc), mannose or lactose) or PEG. All sugars were introduced as building blocks pre-attached to serine. All modifications were incorporated onto individual peptide sequences at the side chain of the lysine at the peptides N-terminus and the resulting peptides, with their respective fatty acid, sugar or PEG modifications are displayed in Table 1.

The manual coupling of the alky tail showed low conversion on a single coupling. A double coupling was therefore performed, which showed increased success of the coupling. Single glycosylation showed good conversion for A8-10 and A12, whereas A7 and A11 showed a low conversion and the coupling was therefore repeated to increase conversion. PEGylation required double coupling whilst a change in solvent from DMF to DMF: DCM (1:1 ratio) led to an improvement in conversion. The peptides were cleaved using TFA, however, the peptides that contained a saccharide were first treated with 16% hydrazine hydrate for one hour to deacetylate the hydroxides before acidolysis. The deprotected peptides were purified using HPLC with mass determined by ESI-MS, see Table 1. ).

Table 1: List of N-terminal modification to parent sequences A1 and B1 with purity and mass conformation. a Purity determined by HPLC analysis. b Calculated by amino acid residue masses. c Determine by ESI-MS of product.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Peptide | N-TerminalModification | Final Purity a(%)  | Desired m/z b(Da)  | Detected m/z c (Da) |
| A1 | None | > 99 | 1281 | 1280 |
| A2 | (Beta-Ala)-(Beta-Ala)-Lys | > 99 | 1551 | 1550 |
| A3 | Caprylic acid (C8) | > 99 | 1667 | 1676 |
| A4 | Capric acid (C10) | > 99 | 1705 | 1704 |
| A5 | Lauric acid (C12) | > 99 | 1733 | 1732 |
| A6 | Myristic acid (C14) | > 99 | 1761 | 1760 |
| A7 | Ser-Glucose | > 95 | 1801 | 1800 |
| A8 | Ser- N-Acetylglucosamine (GlcNac) | > 89 | 1801 | 1800 |
| A9 | Ser-Galactose | > 90 | 1801 | 1800 |
| A10 | Ser-Mannose | > 99 | 1842 | 1843 |
| A11 | Ser-Lactose | > 96 | 1963 | 1962 |
| A12 | Meo-PEG | > 99 | 3464 | 3464 |
| B1 | None | >99 | 1311 | 1311 |
| B2 | (Beta-Ala)-(Beta-Ala)-Lys | > 97 | 1582 | 1582 |
| B3 | Caprylic acid (C8) | > 99 | 1708 | 1708 |
| B4 | Capric acid (C10) | > 99 | 1736 | 1736 |
| B5 | Lauric acid (C12) | > 99 | 1764 | 1764 |
| B6 | Myristic acid (C14) | > 99 | 1792 | 1792 |

**Effect of peptide modification on antimicrobial activity**

The antibacterial activities of the parental peptides and their derivatives were determined against a variety of clinical isolates, some of them with a high resistance level to ‘traditional’ antibiotics including two Gram-positive strains (methicillin-resistant *Staphylococcus aureus* (MRSA)and vancomycin resistant *Enterococcus* (VRE)) and three Gram-negative strains (*Salmonella* Typhimurium*, E. coli* and *Pseudomonas aeruginosa*), see Table 2. The MIC values were determined to provide an evaluation of the antimicrobial activities of peptides with various modifications. We confirmed that the parental peptides A1 and B1 exhibit broad-spectrum activity, inhibiting all organisms with MIC values of between 1 µg/ml to 8 µg/ml. No change in antimicrobial activity was observed upon incorporation of the linker structure to peptide A1, with the exception of MRSA*,* where a twofold decrease in activity was noted. Whereas incorporation of the linker onto sequence B1 showed a consistent two-fold improvement in antimicrobial activity against all organisms, except for *E. coli* which showed no change. The introduction of caprylic acid (C8) to A1 (NH2-RIRIRWIIR-CONH2) did not change the antimicrobial activity compared to the parent. In contrast, adding caprylic acid to B1 (NH2-KRRVRWIIW-CONH2) increased the antimicrobial activity especially for the Gram-positive bacteria, for example for MRSA a factor of eight compared to the parent. For the Gram-negative bacteria, there was either no change (*E. coli*) or a two-fold increase in antimicrobial activity. The N-terminal modification with capric acid on A1 did lead to a two-fold improvement in antimicrobial activity, except for *P. aeruginosa* and *E. coli*, where no change was observed. The addition of Capric acid to B1 resulted in the greatest improvement in antimicrobial activity for this series, a 16-fold improvement in the case of MRSA, 8-fold for VRE and 4-fold for *P. aeruginosa* and *S.* Typhimurium, only the MIC for *E. coli* did not change. The introduction of lauric acid on A1 reduced the antimicrobial activity at least 16-fold for VRE and *P. aeruginosa*, 8-fold for *S.* Typhimurium and *E. coli*, but did not alter activity against MRSA.In contrast, lauric acid in combination with B1 decreased the antimicrobial activity for *E. coli* alone, whereas for all other bacteria an improved activity of 2-fold or 4-fold was measured. Myristic acid (C14) in combination with A1 reduced antimicrobial activity against all bacterial strains tested. The combination with B1 showed a more mixed result, for MRSA a 2-fold improvement was observed, for VRE there was no change, whereas for *E. coli* there was a 32-fold reduction in antimicrobial activity.

**Table 2**: MIC values of all lipidated peptides against an array of Gram-positive and Gram-negative organisms. Measurements were performed at least in triplicate and the most frequently observed value is presented.

|  |  |
| --- | --- |
| Peptides | MIC (μg/ml) |
| Peptide Code | **Modification** | **MRSA** | **VRE** | ***E. coli*** | ***P. aeruginosa*** | ***S.* Typhimurium** |
| A1 (Parent) | None | 2 | 8 | 2 | 8 | 8 |
| A2 | Ala spacers and Lys | 4 | 8 | 2 | 8 | 8 |
| A3 | Caprylic acid (C8) | 2 | 8 | 2 | 8 | 8 |
| A4 | Capric acid (C10) | 1 | 4 | 2 | 8 | 4 |
| A5 | Lauric acid (C12) | 2 | >64 | 16 | >64 | 64 |
| A6 | Myristic acid (C14) | 8 | >64 | >64 | >64 | >64 |
| B1 (Parent) | None | 8 | 8 | 1 | 8 | 4 |
| B2 | Ala spacers and Lys | 4 | 4 | 1 | 4 | 2 |
| B3 | Caprylic acid (C8) | 1 | 2 | 1 | 4 | 2 |
| B4 | Capric acid (C10) | 0.5 | 1 | 1 | 2 | 1 |
| B5 | Lauric acid (C12) | 2 | 4 | 4 | 4 | 2 |
| B6 | Myristic acid (C14) | 4 | 8 | 32 | 32 | 8 |

The MIC for the glycosylated and PEGylated peptides were also determined (Table 3). Based on the high price of the building blocks and the workload required we performed glycosylation only for peptide A1 and used a smaller set of bacteria. In general, glycosylation decreased the antimicrobial activity for all tested bacteria and PEGylation rendered the peptides inactive. The degree of decrease was different between *E. coli* and MRSA/*P. aeruginosa;* 2-4-fold reductions in activity were seen against *E. coli* while the activity against MRSA and *P. aeruginosa* was reduced 4-16-fold.

**Table 3**: MIC values of all glycosylated and PEGylated peptides against Gram-positive and Gram-negative organisms. Measurements were performed at least in triplicate and the most frequently observed value is presented.

|  |  |
| --- | --- |
| Peptide | MIC (μg/ml) |
| Peptide Code | **Modification** | **MRSA** | ***E. coli*** | ***P. aeruginosa*** |
| A1 (Parent) | None | 2 | 2 | 8 |
| A2 | Ala spacers and Lys | 4 | 2 | 8 |
| A7 | Glucose | 16 | 4 | 32 |
| A8 | GlcNAc | 32 | 8 | 64 |
| A9 | Galactose | 16 | 4 | 32 |
| A10 | Mannose | 16 | 4 | 64 |
| A11 | Lactose | 32 | 8 | 64 |
| A12 | PEG | >64 | >64 | >64 |

**Effect of modification on antimicrobial activity in serum**

Peptides that display potent antimicrobial activity within a media optimized for bacterial growth such as Mueller Hinton (MH), may have reduced potency or target selectivity *in vivo* based on stability, binding and pharmacokinetics*.* In order to further ascertain the therapeutic potential of these peptides modified with lipids, sugars and PEG moieties, an *in vitro* study was designed to assess the antimicrobial activity in 25% human serum [32]. Thus, the activity of increasing concentrations of peptide was assessed against MRSAserum suspensions. The control peptides A1, A2, B1 and B2 showed a decrease in their antimicrobial activity in comparison to the MH*-*only condition for each respective control, with MIC values reported as ≤64 µg/ml (Table 4).

The antimicrobial activities of all glycosylated and PEGylated peptides against MRSAin human serumwere negligible, even at 64 µg/ml, which was the highest concentration assessed (Table 4). This represents a significant reduction when compared to the activity in MH broth only (P=0.0062, comparing MIC in serum and MIC without serum (MH alone)).

Likewise, modification of peptides with fatty acids also displayed a significant reduction in antimicrobial activity in the presence of serum (P=0.001, comparing MIC in serum and MIC without serum (MH alone)) (Table 4). Modification with the fatty acid C8 was unable to recover any of this antimicrobial activity within serum, with negligible effects at 64 µg/ml. In contrast, upon modification with the lipids C10-C14, a change in activity was observed. C10, when applied to the sequence A2, decreased the MIC from 64 to 16 µg/ml, a fourfold increase in activity in comparison to the parental peptide. However, this improvement in activity is peptide sequence-dependent; when the modification is applied to sequence B2 no impact is observed in comparison to the parental control. When the hydrophobicity is further increased by modification with the highly hydrophobic fatty acids C12 and C14, the antimicrobial activity in serum is recovered, regardless of the peptide sequence.

**Table 4**: MIC values of parental and modified peptides against MRSA in Mueller-Hinton media in the presence and absence of 25% human serum. Measurements were performed at least in triplicate and the most frequently observed value is presented. The 50% hemolysis (HC50) values were determined in PBS alone. Measurements were performed at least in triplicate and the mean value is presented. HC50 values were determined using a non-linear regression of the logarithmic concentrations using GraphPad Prism Version 6.01.

|  |  |
| --- | --- |
| Peptides | MIC/HC50 (μg/ml) |
| Peptide Code | **Modification** | **MRSA** | **MRSA *+ Serum*** | ***HC50*** |
| A1 (Parent) | None | 2 | 64 | >250 |
| A2 | Ala spacers and Lys | 4 | > 64 | >250 |
| A3 | Caprylic acid (C8) | 2 | > 64 | 132.0 |
| A4 | Capric acid (C10) | 1 | 16 | 73 |
| A5 | Lauric acid (C12) | 2 | 16 | 35 |
| A6 | Myristic acid (C14) | 8 | 16 | 28 |
| A7 | Glucose | 16 | > 64 | > 250 |
| A8 | GlcNAc | 32 | > 64 | > 250 |
| A9 | Galactose | 16 | > 64 | > 250 |
| A10 | Mannose | 16 | > 64 | > 250 |
| A11 | Lactose | 32 | > 64 | > 250 |
| A12 | PEG | >64 | > 64 | > 250 |
| B1 (Parent) | None | 8 | 64 | > 250 |
| B2 | Ala spacers and Lys | 4 | 64 | > 250 |
| B3 | Caprylic acid (C8) | 1 | 64 | > 250 |
| B4 | Capric acid (C10) | 0.5 | 64 | 50 |
| B5 | Lauric acid (C12) | 2 | 16 | 38 |
| B6 | Myristic acid (C14) | 4 | 8 | 25 |

**Impact of modifications on peptide toxicity**

The hemolytic activity of peptides against human erythrocytes is often used as a measure of their toxicity and to predict their therapeutic potential. The effect of peptide concentration on the percentage of hemolysis was determined and the HC50, the value at which 50% hemolysis was obtained (Figure 2 and Table 4).

 

Figure 2: Hemolytic assay of A1 (NH2-RIRIRWIIR-CONH2) and modified versions (top), B1 (NH2-KRRVRWIIW-CONH2) and modified versions (bottom) at varying concentrations determined against human erythrocytes. The positive control for 100% hemolysis was erythrocytes treated with 0.1% Triton-X-100. The statistical significance of differences was determined using a two-way ANOVA with Bonferroni correction and multiple comparisons in relation to the respective parental control peptide A1 and B1. The values represent the mean of triplicate experiments ±2SD. \*\*\*\* = P<0.0001.

The parental peptides A1, and B1 had minimal hemolytic activity, with HC50 values of >250 µg/ml. Likewise, the glycosylated and PEGylated peptides displayed negligible hemolytic activity, measured by the degree of hemoglobin release, even at 250 µg/ml, the highest concentration assessed (Figure 12). Like the parental peptides, 50% hemolysis was not achieved at this concentration and an exact HC50 value cannot be determined.

In contrast, conjugation with fatty acids significantly increased the toxicity of the lipopeptides in comparison to the parental controls as shown by a decrease in HC50 values. A clear correlation was noted between hemolytic activity and hydrophobicity. In other words, the more hydrophobic the peptide, the higher its toxicity against human erythrocytes. The extent of the peptide’s toxicity was sequence-specific. As reflected by the observation that conjugation of the fatty acid C8 to peptide sequence A2 resulted in a higher degree of toxicity than C8 incorporated into sequence B2, producing a toxicity that is not statistically higher than the parental peptide B2. The most promising peptides determined from the MIC values in MH broth, A4 and B4, both modified with a C10 fatty acid, caused 50% hemolysis at 73.4 µg/ml and 49.6 µg/ml.

**Therapeutic index**

The therapeutic index of antimicrobial peptides, calculated by determining the ratio of HC50 over the MIC (µg/ml) in the absence of serum, further illustrates their potential as therapeutic agents (Table 5). The higher the therapeutic index, the higher the peptides’ specificity towards prokaryotic cells and hence a higher therapeutic potential. None of the modifications increased the therapeutic potential in comparison to the parental peptide with the exception of C8 applied to peptide sequence B2, where the ratio is slightly increased upon modification. The extent of this increase is bacterial species-dependent, with the highest observation noted against MRSA*,* where the therapeutic index increases from 62.5 to 500.

Table 5: Therapeutic index of the parental peptides and all modifications. Therapeutic index for each peptide against a respective organism calculated by determining the ratio of the HC50 value over the MIC value. Where a HC50 value could not be determined, a value of 500 µg/ml was used instead.

|  |  |  |
| --- | --- | --- |
| Peptide | Modification | Therapeutic index |
| **MRSA** | ***E. coli*** | ***P. aeruginosa*** | **VRE** | ***S*. Typhimurium** |
| A1 | None | 250 | 250 | 62.5 | 62.5 | 62.5 |
| A2 | Ala spacers and Lys | 125 | 250 | 62.5 | 62.5 | 62.5 |
| A3 | Caprylic acid (C8) | 65.8 | 65.8 | 16.4 | 16.4 | 16.4 |
| A4 | Capric acid (C10) | 73.4 | 36.7 | 9.2 | 18.4 | 18.4 |
| A5 | Lauric acid (C12) | 17.7 | 2.2 | < 0.6 | < 0.6 | < 0.6 |
| A6 | Myristic acid (C14) | 3.5 | < 0.4 | < 0.4 | < 0.4 | < 0.4 |
| A7 | Glucose | 31.3 | 125 | 15.6 | - | - |
| A8 | GlcNac | 15.6 | 62.5 | 7.8 | - | - |
| A9 | Galactose | 31.3 | 125 | 15.6 | - | - |
| A10 | Mannose | 31.3 | 125 | 7.8 | - | - |
| A11 | Lactose | 15.6 | 62.5 | 7.8 | - | - |
| A12 | PEG | < 7.8 | 15.6 | < 7.8 | - | - |
| B1 | None | 62.5 | 500 | 62.5 | 62.5 | 125 |
| B2 | Ala spacers and Lys | 125 | 500 | 125 | 125 | 250 |
| B3 | Caprylic acid (C8) | 500 | 500 | 125 | 250 | 250 |
| B4 | Capric acid (C10) | 99.2 | 49.6 | 24.8 | 49.6 | 49.6 |
| B5 | Lauric acid (C12) | 19.1 | 9.5 | 9.5 | 4.8 | 19.1 |
| B6 | Myristic acid (C14) | 6.35 | 0.8 | 0.8 | 3.2 | 3.2 |

Although higher hydrophobicity resulted in a lower antimicrobial activity, presumably due to its self-association ability, this does not impact on the peptides ability to interact with human erythrocytes. The longer the fatty acid chain, the more hemolytic the peptide, but only at concentrations far higher than their MIC’s. In fact, all of the peptides have a far reduced activity in comparison to the well-documented lytic peptide melittin, which results in 100% hemolysis at concentrations as low as 1-3 µg/ml under comparable conditions [33].

Discussion

The significant rise in the appearance of multi-drug resistant pathogens in recent years has become a global public health concern, prompting the development of alternative antimicrobial agents to replace “traditional antibiotics”. Recently, antimicrobial peptides (AMP’s) have received substantial attention as a potential new class of antimicrobial agents, with many natural and synthetic AMP’s exhibiting potent broad-spectrum antimicrobial activity against multi-drug resistant bacteria [34]. However, the application of AMP’s to the therapeutic market has been hindered by a multitude of physiological disadvantages including; low serum stability, fast renal and hepatic clearance as well as relatively high manufacturing costs compared to many “first generation” antibiotics [35]. It is therefore essential to develop highly active AMP’s that can overcome these barriers, facilitating their development for commercial therapeutic agents. Lipidation, glycosylation and PEGylation have been identified as key chemical modifications that have the potential to not only increase the biological activity of AMP’s, but also to increase their stability in human serum and reduce their toxicity.

When designing or optimizing a peptide with the aim of increasing the therapeutic potential, it is important to choose a progenitor peptide sequence with an already high therapeutic index; the ratio of antimicrobial activity to hemolytic activity [36]. We showed that A1 (NH2- RIRIRWIIR-CONH2) and B1 (NH2-KRRVRWIIW-CONH2) demonstrated strong antimicrobial activity against methicillin-resistant *Staphylococcus aureus (MRSA)* and *Pseudomonas aeruginosa* respectively, with negligible hemolytic activity against human erythrocytes. Peptides A1 and B1 therefore have a high therapeutic index and thus were chosen as the parent peptides for subsequent chemical modification with a respective fatty acid, sugar or polyethylene glycol (PEG) moiety.

A number of studies have revealed that antimicrobial activity is correlated with a peptide’s overall amphipathicity, hydrophobicity and flexibility [37]. Alanine is one of four amino acid residues (Gly, Ser, Ala and Thr) that are preferred as natural linkers [38]. While glycine is usually the spacer of choice, being the most flexible amino acid, it is non-polar, whereas alanine is a hydrophobic amino acid that will increase the peptide’s hydrophobicity, potentially allowing for an increase in antimicrobial activity. Furthermore, the beta form of alanine is more flexible than its alpha counterpart, having the same degree of flexibility as glycine [39]. This study revealed that the addition of two flexible β-Ala spacers and one positive-charged lysine had conflicting effects when applied to either sequence A1 or B1. Applied to sequence A1, there was no change in the antimicrobial activity against any organism with the exception of MRSA, where a two-fold drop in activity was observed. Applied to sequence B2, there was a two-fold increase in antimicrobial activity against all organisms excluding *E. coli.* It has been suggested that this correlation between flexibility and antimicrobial activity can be explained by the peptide’s mode of action [40]. It is possible that the addition of two β-Ala spacers to peptide sequence A1 increases the flexibility to a degree where there is no beneficial impact on the peptide’s activity.

Here we have shown that fatty acid conjugation affects the activity of cationic AMP’s in a more complex way than initially thought. The resulting antimicrobial effect is not only dependent on the overall hydrophobicity (fatty acid length), but also on the bacterial species and the peptide sequence. Interestingly, the antimicrobial activity increases on incorporation of a shorter fatty acid chain and decreases for longer fatty acids. The increase in antimicrobial activity was also observed in previous studies [33,41,42]. Surprisingly, here, the C14 conjugation leads to a strong decrease in antimicrobial activity. However, Makovitzki et al. have shown that for many short peptides the conjugation of palmitic acid (C16) was the most active variant [30]. This underlines our observation that the peptide sequence plays an essential role.

Most AMP’s are attracted to the bacterial cell membrane through electrostatic interactions. The amphiphilic structure of AMP’s is crucial for this behavior, allowing interactions between the positively charged regions of the peptide and the negatively charged components of the bacterial surface, as well as interactions between the peptide’s hydrophobic component and the hydrophobic lipids of the bacterial bilayer. It is assumed that an increase in peptide hydrophobicity could allow for an increased hydrophobic interaction between the peptide and the bacterial cell surface, enabling the peptide to more efficiently reach the negatively charged bacterial membrane. In addition, this increased attraction is also accompanied by an increase in the peptide’s surface area which allows an enhanced region of contact between the peptide and the bacterial cell membrane [43]. Upon the initial interaction between the peptide and the outer membrane, the increased surface area of peptides conjugated with fatty acids also leads to an increase in the volume and surface area of the bacterial outer leaflet, while leaving the inner leaflet unaffected [44]. This discrepancy can generate a curve in the bilayer which causes generalized membrane thinning and disruption of the membrane integrity, facilitating the peptide’s ability to penetrate the bacterial membrane resulting in a higher antimicrobial activity [45]. The MIC results in our study indicate that an optimum threshold for hydrophobicity for each peptide exists, and a further increase in hydrophobicity above this threshold causes a reduction in the peptide’s antimicrobial activity.

There are at least two explanation for this observation:

First, lipopeptides could have a higher tendency to self-associate or aggregate, either upon contact with the membrane surface or within an aqueous solution. This would reduce the concentration of peptide able to interact with the cell membrane, resulting in a lower antimicrobial activity [46]. If the capability of the peptide to self-associate is too strong, it can reduce the peptides’ ability to dissociate [47], endowing them with a large surface area and making it difficult for the peptide to insert itself into and pass through the bacterial capsule and cell wall, where it can penetrate the cytoplasmic membrane to cause cell death [33].

Secondly, the length of the lipid could influence the ability to integrate and interact with the bacterial membrane and in consequence influence the activity. Where a too short or too long fatty acid may not integrate well into the membrane bilayer, an optimal length may exist that will be dependent on the composition of the membrane and the outer layer of each bacterium. Interestingly, if the reduction in antimicrobial activity was only due to self-association then the degree to which the activity is reduced should be equal regardless of the bacterial strain. Instead, this study showed a far lower reduction in activity depending on the bacterial strain.

It is important to note that for each bacterial species antimicrobial activity was determined against one strain only. Given that strains can vary considerably, particularly amongst clinical isolates, so may the antimicrobial activity of the peptides described here. In preliminary (non-published results) we have shown that the MIC variation between clinical strains (sensitive and multi-drug resistant) are small, however larger studies need to be performed.

It was also shown that increasing the hydrophobicity of the peptides’ non-polar face increases hemolytic activity [47]. In the current study, we showed that hemolytic activity was correlated with peptide hydrophobicity. The higher the peptide hydrophobicity, the greater its toxicity against human erythrocytes. This is consistent with prior results that show the HC50 value increases upon conjugation with hydrophobic fatty acids and decreases when a hydrophilic sugar or PEG moiety is introduced [47–49]. On the other hand, for the majority of ultrashort peptides coupled to the longer fatty acid chains (C14 and C16), no increase in hemolytic activity was observed, highlighting that the sequence of the peptide also plays a role [50]. Like prokaryotic cells, the initial peptide interaction with erythrocytes is believed to be dependent on electrostatic and hydrophobic interactions, whereby increased hydrophobicity supports a stronger interaction between the peptide and the lipophilic membrane components of erythrocytes [49]. This allows deeper penetration of the peptides into the erythrocyte’s hydrophobic core, thus resulting in a stronger degree of hemolysis [47].

Beyond a certain threshold of hydrophobicity, the peptides have a stronger ability to self-associate and aggregate leading to a reduction in antimicrobial activity while still maintaining strong hemolytic activity. The reason for this discrepancy between the peptide’s ability to act on prokaryotic and eukaryotic cells is most probably a result of a ‘membrane discrimination’ mechanism in that the peptide’s specificity depends on the compositional difference between the two membranes [49]. In contrast to prokaryotic cells, eukaryotic erythrocytes do not contain a polysaccharide cell wall and are instead composed mainly of zwitterionic phospholipids, acidic cholesterol and sphingomyelin forming a highly hydrophobic bilayer. This highly hydrophobic bilayer encourages the dissociation of peptide oligomers to monomers [47], allowing the peptide to perturb the lipid bilayer and cause cell death by colloid osmotic lysis [51].

Peptide glycosylation is a ubiquitous post-translational modification with the capability to alter the peptides’ structural and functional properties, profoundly impacting their stability, folding propensities and antimicrobial activity [52]. Removal of the naturally occurring monosaccharides or disaccharides that are linked to threonine residues of drocosin [53], pyrrhocoricin [54], lebocins [55] and formaecins [56] results in a significant decrease in antimicrobial activity. The glycopeptide antibiotic vancomycin contains a peptide core consisting of seven amino acid residues, the side chains of which covalently attach to form a macrocyclic structure that is decorated with disaccharide chains at various positions [57]. It has been shown that the glycan structure facilitates a glycopeptides ability to self-associate as dimeric complexes in solution, increasing the degree of multivalence effects and allowing for an increase in antimicrobial activity [58]. Incorporation of additional sugar units intensifies this increase in activity by leading to a tighter dimer association and a further increase in multivalency effects [58]. While the effects of glycosylation on antimicrobial activity are not well described in literature, a number of studies have demonstrated that incorporation of sugar units can induce a conformational change in the peptide backbone, resulting in an improvement in the peptide’s biological activity in comparison to the non-glycosylated derivative [52,59]. However, this study has shown the opposite effect with glycosylation reducing the antibacterial activity against all microbial species (Table 3 and 4).

Loss of antimicrobial activity upon glycosylation is likely due to two factors. First, the introduction of the hydrophilic sugar reduces both the hydrophobicity and the overall positive charge in comparison to the parental peptide. This results in a higher zwitterionic peptide content and increases the formation of both intra- or inter-chain associations that can cause conformational changes in the peptide backbone, thus impacting the peptide’s overall amphiphilicity and its ability to interact with and insert itself into the anionic membranes [60]. Second, the reduction in hydrophobicity upon glycosylation will reduce the extent to which the peptide can bind to both the lipid A component of lipopolysaccharides (LPS) and the teichoic acids of Gram-negative and Gram-positive bacteria. The reduction in the peptide’s propensity to bind to these outer leaflet components will reduce the extent to which the bacterial membrane will be disturbed [61]. Both factors result in lower antimicrobial activity [62] and are highlighted by the observation that an increase in the sugar size (GlcNAc and Lac) intensifies these effects resulting in a further loss of antimicrobial activity in comparison to the smaller sugar residues (Glc, Man and Gal).

Peptides that display potent antimicrobial activities within an artificial media such as MH, may have reduced activity or selectivity within a more complex biomatrix such as whole blood, serum, plasma and intercellular environments. This reduction in its biological properties is usually a result of blood components, such as blocking or binding proteins and peptidases which may inactivate or degrade the peptide within a short period of time, or change in the pH or salt concentration [63]. The first step involved in progression from *in vitro* activity measurements to a more *in vivo* assessment, is to determine the peptide stability in human serum, a useful secondary screening assay to detect peptides that are unstable in human bodily fluids [64]. Human serum mimics the *in vivo* environment and provides an indication regarding the peptide’s suitability (high stability and activity) for animal models [64,65]. The results of our study show that unsurprisingly, the MIC values drastically increase in serum by up to 32-fold. This is presumably due to inactivation through the presence of various salts, non-specific or specific interactions with serum proteins, most likely human serum albumin, or to proteolytic degradation by proteases present in serum. Incorporation of a longer fatty acid (C10, C12 and C14) can recover some of the antimicrobial activity lost when assessed against an array of microbial species with serum (Table 4). The most likely scenario is that the fatty acids change the interaction with human serum albumin and also shield the peptide from proteolytic attack [66]. While it is theorized that the introduction of a glycan can also result in a degree of steric hindrance which should theoretically increase the glycopeptide’s stability and activity in human serum, the results from this study contradict this, with no difference in activity noted in the presence of serum compared with the parental controls. Glycans are short, polar, bulky modifications and it is possible that the alanine and lysine spacer creates a large intramolecular distance between the glycan and peptide backbone. This means that, unlike the aliphatic fatty acid chain which is flexible and able to move and interact with the peptide, the glycan is restrained in terms of conformation and its ring-like compact structure is not able to contact the peptide backbone [67]. It may instead be more advantageous to either use a short linker, or directly incorporate the sugar modification into the peptide backbone.

The therapeutic potential of AMP’s is compromised by rapid clearance from the blood by the renal or hepatic routes and proteolytic degradation or inactivation by serum proteins and proteases. Incorporating the molecule PEG is frequently employed as a strategy to improve the *in vivo* efficacy of many pharmacological agents and at least nine PEGylated therapeutic proteins have been approved for clinical use by the FDA [68]. PEGylation dramatically increases the molecular weight of a peptide and can influence its physiological properties including hydrophobicity, degree of steric hindrance and conformation [69,70]. The reduction in antimicrobial activity observed on PEGylation in our study is consistent with previous studies that noted a reduction in antimicrobial activity of the peptide nisin by about two-fold when a single PEG molecule of 5 KDa was introduced at the N-terminus [69]. This same study determined that while the peptides mode of action against the bacterial lipid bilayer was unchanged, there was a reduction in both the PEGylated peptides ability to interact with and bind to the bacterial membrane and the degree of membrane permeabilization, both presumed to result from an alteration in the peptide’s secondary structure [69].

In conclusion, despite the fact that most clinically used antimicrobial peptides are either lipidated or glycosylated, the results of such modifications for a new peptide sequence for their biological activity are rather complex. Our data suggests that the effect is dependent on the peptide sequence, the bacteria and the lipid/sugar. More systematic investigations are needed to study the effect on various lipids, sugars, combinations of lipids and sugars as well as the position of the modification. In particular, finding the best peptide-sugar combination might lead to compounds with low toxicity, high solubility and consequently high therapeutic value.

# CRediT Author Statement

# EG: Investigation, Data curation, Writing - Review & Editing; DWPC: Writing - Original Draft, Visualization; RM: Software, Validation, Writing - Review & Editing, Funding acquisition; KH: Conceptualization, Validation, Resources, Writing - Review & Editing, Supervision, Funding acquisition

# Conflict of Interest

DWPC and KH Hilpert declare their affiliation with TiKa Diagnostics Ltd. KH is Founder and Director of Tika Diagnostics Ltd. The company did not influence the design, conduction, interpretation, or evaluation of this study. The peptides described here are submitted for a patent application. All the other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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