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Triple-Targeting Gram-Negative Selective antimicrobial peptides capable of Disrupting the Cell Membrane and Lipid A Biosynthesis

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T. M. Postma[a] and R. M. J. Liskamp[a]*

Early lipid A biosynthesis is a potential antimicrobial target as this is an essential component of lipid A producing Gram-negative bacteria. Herein, we introduce a potent and highly synergistic Gram-negative selective triple-targeting antimicrobial peptide. The peptide contains a dual lipid A biosynthesis inhibiting sequence and an antimicrobial sequence.

Decades of abundant and often overprescribed use of antibiotics has marked a steep global rise in multidrug-resistant bacteria.[1] Especially the emergence of multidrug-resistant Gram-negative (MRGN) bacteria in healthcare settings poses a serious threat to public health.[2] In the United States more than 2 million persons are infected annually with antibiotic resistant bacteria.[3] In Europe, a significant increase in resistance in MRGN bacteria, including resistance to last-line antibiotics such as carbapenems and polymyxins, was observed over the last few years according to the annual surveillance report of the European Centre for Disease Prevention and Control.[4] Resistance to last-line antibiotics clearly illustrates the severity of the problem, where last-line antibiotics are typically only used as emergency treatment when other antibiotics fail. Furthermore, there are few antibiotics and fewer new antibiotic classes in the pharmaceutical pipelines which exacerbates the public health risk to MRGN bacteria. There is an urgent need to discover antimicrobial agents with novel mechanisms of action to address these serious threats to human health and well-being.

Targeting the early lipid A biosynthesis in Gram-negative bacteria has been proposed as a viable antimicrobial strategy with lipid A being essential to bacterial survival in lipid A producing Gram-negative bacteria.[5] Lipid A is the membrane-anchor of lipopolysaccharide, which constitutes the outer layer of the Gram-negative bacterial membrane and is responsible for the endotoxic effects observed in numerous Gram-negative infections. The early biosynthesis of lipid A is characterized by a series of catalytic acetyltransferases that add lipid chains to a molecule of UDP-*N*-acetylglucosamine (Scheme 1). The critical steps in early lipid A biosynthesis are performed by UDP-*N*-acetylglucosamine acetyltransferase (LpxA) and UDP-3-*O*-(*R*-3-hydroxyacyl)GlcN *N*-acetyltransferase (LpxD).

Recently, from a phage display library against both LpxA and LpxD the peptide RJPXD33, a dual targeting 12-residue peptide (TNLYMLPKWDIP) has been identified with inhibition constants (IC_{50}) of 19 μM and 3.5 μM respectively.[6] The bioactivity of this RJPXD33 peptide was determined by inducing a transfected plasmid coding for the peptide in *E. coli*, which led to inhibition of bacterial growth. These results indicate that LpxA and LpxD are essential enzymes for the survival of lipid A producing bacteria. Thus, the RJPXD33 peptide offers a promising sequence for the design of multi-targeting Gram-negative selective antimicrobial agents. Targeting several essential targets inside bacteria may reduce the emergence of resistance against these potential multi-targeting antibiotics since several mutations in critical targets will be required to cause resistance.[7]

The significant inhibitory activity of LpxA and LpxD by the RJPXD33 sequence shows promise for attacking Gram-negative bacteria. However, this dual-targeting peptide sequence lacks the typical characteristics of a cell penetrating peptide, which decreases the likelihood of this peptide to reach the target intracellular acetyltransferases LpxA and LpxD. To enable cell entry and subsequent inhibition of these transferases, conjugation of the RJPXD33 peptide to a cell penetrating peptide sequence was required. Furthermore, by using a cell penetrating sequence that displays potent antimicrobial activity a certain degree of synergy with the RJPXD33 sequence was expected. Thus, we envisioned a "triple-targeting" Gram-negative selective antimicrobial peptide by designing a cell penetrating antimicrobial peptide RJPXD33 conjugate. The RJPXD33 sequence targets the two acetyltransferases LpxA and LpxD, and the cell penetrating peptide increases the permeability of the bacterial membrane acting as both shuttle

[a] *School of Chemistry, Joseph Black Building, University of Glasgow, University Avenue, Glasgow, G12 8QQ, United Kingdom.*
* Email corresponding author: robert.liskamp@glasgow.ac.uk
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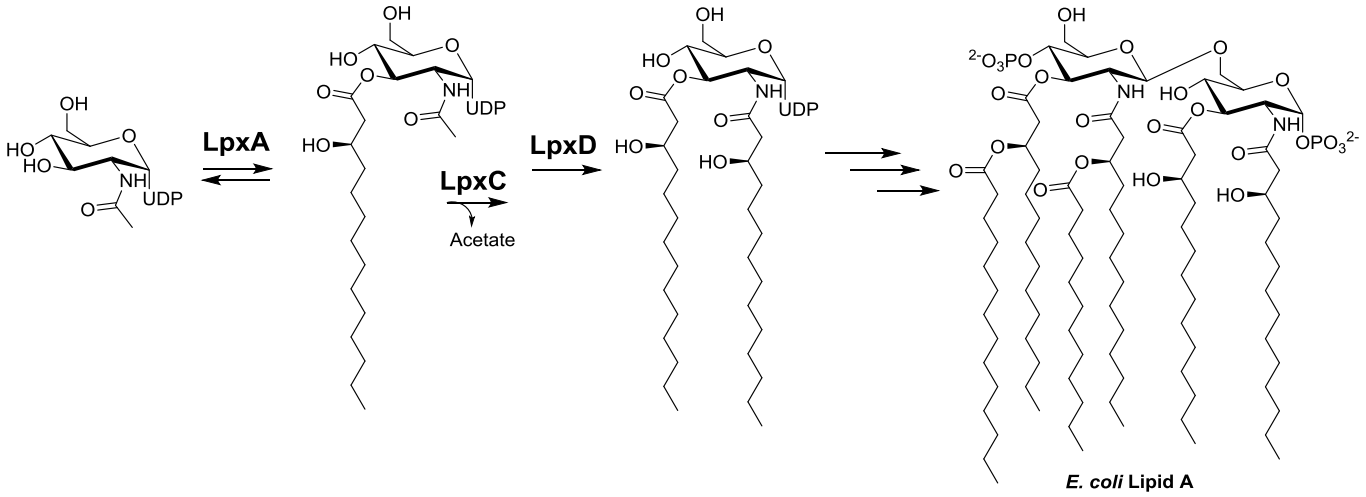

Scheme 1: Lipid A biosynthesis in E. coli[6]

and disruptor of bacterial membrane integrity.

There is significant similarity between cell penetrating and antimicrobial peptides, however not all cell penetrating peptides are antimicrobial. The exact difference is poorly understood as there is frequently a strong sequence overlap, for instance with respect to the presences of several cationic residues.[8] Cationic peptides have a strong tendency to interact with the negative bacterial membrane. There are several mechanisms through which antimicrobial peptides act, most of which primarily damage the bacterial membrane.[9] In the design of a triple-targeting peptide the antimicrobial sequence required a fine balance between antimicrobial activity and ability to shuttle cargo into bacterial cells to ensure synergistic activity on the bacterial membrane and disruption of early lipid A biosynthesis by inhibiting LpxA and LpxD. The cationic bactericidal peptide KFFKFFKFF had shown to significantly increase the permeability of bacterial membranes which resulted in signification potentiation of the antimicrobial effects of several antibiotics in solution.[10] A similar peptide sequence, KFFKFFKFFK, was used in a peptide nucleic acid (PNA) conjugate that demonstrated a significant increase in uptake and potency of a PNA construct.[11] Therefore, we chose the peptide sequence KFFKFFKFFK for the triple-targeting conjugate. To determine suitable placement of the KFFKFFKFFK sequence at the N- or C-terminus of the RJPXD33 sequence the crystal structure of a RJPXD33 LpxA trimer complex was examined.[12] The peptide was bound vertically into a pocket formed in the middle of the LpxA homotrimer allowing sufficient space to fit the N-terminus with the cationic sequence. A glycine spacer was inserted in between to give the complete conjugate sequence KFFKFFKFFKGTNLYMLPKWDIP.

The following peptides were prepared using Fmoc solid-phase peptide synthesis: H-GTNLYMLPKWDIP-NH_2 (**1**), H-KFFKFFKFFK-NH_2 (**2**) and H-KFFKFFKFFKGTNLYMLPKWDIP-NH_2 (**3**). The antibacterial activity of peptides **1**-**3** was evaluated in a broth microdilution assay to determine the minimum inhibitory concentration (MIC), defined as the lowest concentration of a compound that will visibly inhibit bacterial growth. Prior to determining the MIC values of peptides **1**-**3** we had to choose relevant Gram-negative bacteria. The RJPXD33 peptide was discovered using LpxA and LpxD from *E. coli*, which renders *E. coli* an obvious choice. Moreover, multi-drug resistant *E. coli*, including carbapenem resistance, is spreading rapidly and is a common human pathogen that causes community and nosocomial acquired infections.[13] *E. coli* together with *Klebsiella pneumoniae, Acinetobacter baumannii* and *Pseudomonas aeruginosa* are part of the high priority ESKAPE pathogens and were included in the MIC testing.[14] The MRGNB *Citrobacter freundii*, a common nosocomial cause of infection, can be extremely drug-resistant. The final two bacteria that we chose to test our conjugates against have a rising incidence of resistance, were *Shigella sonnei* and *Salmonella enterica*.[15] As a control we used the Gram-positive bacteria, *Staphylococcus aureus and S. epidermidis*, which do not express the LpxA and LpxD acetyltransferases. The MICs of these bacteria were determined using an established protocol within our group with some minor modifications (Table 1).[16]

The MIC values confirmed our initial expectation that extracellular dual-targeting peptide **1** would prove to be inactive. Peptide **1** caused no inhibition of bacterial growth in all bacterial species tested at high concentration (1mg/mL, 563 µM). In contrast, antimicrobial peptide **2** demonstrated potent inhibition of growth in all bacteria. An inhibitory range between 1 and 32 µM for the Gram-negative bacteria, and between 0.5 and 16 µM for Gram-positive *S. epidermidis* and *S.*

aureus was found. A combination of peptide **1** and **2** gave the same MIC values as for peptide **2** alone. The triple-targeting peptide construct **3** demonstrated very potent synergistic inhibition of growth in all Gram-negative bacteria tested < 2 µM. This is a significant increase in potency over peptide **2** with a 1 – 128 fold decrease of the MIC values. In *K. pneumoniae* the increase in potency was most pronounced with a 128 fold decrease of MIC value from 126 µM to 0.25 µM. In contrast, the only exception with the Gram-negative bacteria was *P. aeruginosa* in which no increase in potency was observed. Conversely, the effect of peptide **3** on Gram-positive bacteria was reversed by a 2 fold increase in the MIC values. This increase in MIC-value was expected as Gram-positive bacteria do not express the LpxA and LpxD acetyltransferases. Thus, the antimicrobial activity of peptide **3** in Gram-positive bacteria is probably primarily due to the membrane damaging cationic KFFKFFKFFK sequence.

Table 1: Evaluation of antibacterial activity of peptides using a broth microdilution assay

Bacterium	Gram	Dual (1) MIC (µM)	AMP (2) MIC (µM)	Dual (1) + AMP (2) MIC (µM)	Triple (3) MIC (µM)
Escherichia coli	-	>563	8	8	0.5
Klebsiella pneumoniae	-	>563	32	32	0.25
Citrobacter freundii	-	>563	4	4	0.5
Shigella sonnei	-	>563	2	2	0.25
Salmonella enterica	-	>563	16	16	2
Pseudomonas aeruginosa	-	>563	1	1	1
Acinetobacter baumannii	-	>563	32	32	0.25
Staphylococcus aureus	+	>563	16	16	32
Staphylococcus epidermidis	+	>563	0.5	0.5	1

Lipopolysaccharide deficient, polymyxin E resistant *A. baumannii* strains with mutations in either LpxA or LpxD, resulting in inactivation of lipid A biosynthesis and loss of lipopolysaccharide, have been reported.[17,18] The strain AL1851 ΔLpxA contains a 445-bp deletion in LpxA, and the strain AL1851 ΔLpxD contains a single base deletion at nucleotide 364 in LpxD. The peptide MIC values were determined for three *A. baumannii* strains to study the effects of the triple-targeting peptide **3** (Table 2).

The results show the same synergistic increase in potency for the parent *A. baumannii* as for the LpxA/D mutated strains. The polymyxin E MIC values have increased significantly for the Lpx mutants, which is caused by loss of lipopolysaccharide. Polymyxin E binds to lipopolysaccharide and this explains the increase in MIC in the Lpx mutants. Loss of the lipopolysaccharide protective barrier greatly increased the potency of ampicillin in the Lpx mutants. This highlights a potentially interesting application of the triple-targeting conjugate on Gram-negative bacteria. In addition to its action it may sensitize bacteria to other antibiotics by inhibiting lipopolysaccharide biosynthesis. However, the increase in potency with triple-targeting peptide **3** in Lpx mutants was unexpected. Especially, when considering that resistance to last-line antibiotics such as polymyxin E is a serious clinical issue, the preservation of potent activity against Lpx mutants of triple-targeting peptide **3** is very interesting. The absence of LPS will significantly impact the membrane integrity of Gram-negative bacteria and increase permeability.[17] Therefore, peptide **3** may retain increased activity due to interaction of the hydrophobic dual-targeting sequence with the hydrophobic membrane environment. This also raises the possibility of additional targets within the cell and

Table 2: MIC determination in *A. baumannii* strains

Bacterium	AMP (2) (µM)	Dual (1) + AMP (2) (µM)	Triple (3) (µM)	Polymyxin E (µM)	Ampicillin (µM)
A. baumannii (ATCC 19606)	32	32	0.25	0.4	337
A. baumannii (AL1851 – ΔLpxA)	16	16	0.25	3	3

A. baumannii (AI1852 – ΔLpxD)	32	32	0.25	>400	3

Table 3: Haemolysis of sheep erythrocytes

Compound	Percentage haemolysis at 64 μM
Peptide **2**	0.2 ± 0.03
Peptide **3**	0.7 ± 0.2
1% Triton X-100	100 ± 0.1

warrants further study of this type of multi-targeting peptide.

The MIC determination of triple-targeting peptide **3** demonstrated very potent and selective inhibition of growth in Gram-negative bacteria. Triple-targeting peptides show significant promise for application in Gram-negative bacteria with emerging antibiotic resistance. However, cationic peptides that have antimicrobial activity may act as strong cationic detergents.[19] The detergent-like properties of such cationic peptides can lead to strong cytotoxicity, which is unacceptable in any *in vivo* animal or human applications. Therefore, the cytotoxicity of peptides **2** and **3** was determined in a haemolysis assay using sheep erythrocytes.[20] In this assay the extent of haemolysis was determined by measuring the concentration of released haemoglobin into solution compared to 100% haemolysis (1% Triton X-100) after 1h at 37 °C. The percentage of haemolysis at 64 μM is presented as this is at least 10 times higher than the MIC concentrations for peptide **3** in Gram-negative bacteria (Table 3).

Haemolysis was determined in a concentration range of 0.065 – 128 μM for peptides **2** and **3**. The percentage of haemolysis for peptide **2** at 64 μM was less than 1% compared to 100% haemolysis with Triton X-100. With peptide **3**, haemolysis at 64 μM was less than 1% as well. Peptides **2** and **3** showed very low haemolysis, especially given that the MICs against Gram-negative bacteria for peptide **3** are below 5 μM. Less than 1% haemolysis was observed at 14 to 128 times the MIC concentrations. These results clearly demonstrate that peptide **3** had a very low level of haemolysis with no haemolysis observed at the required active antimicrobial concentrations.

Conclusions

A Gram-negative selective triple-targeting antimicrobial peptide conjugate (**3**), based on a lipopolysaccharide biosynthesis inhibiting sequence and an antimicrobial peptide, was prepared and evaluated for biological activity and cytotoxic properties. For comparison, the LpxA and LpxD targeting peptide (**1**) as well as the antimicrobial cell penetrating peptide (**2**) were prepared and evaluated. Peptide **1** showed no inhibition of bacterial growth at high concentration. Potent antimicrobial activity was observed with peptide **2**. A mixture of peptide **1** and **2** did not increase potency. Triple targeting peptide **3** demonstrated very potent synergistic antimicrobial effects in Gram-negative bacteria, with MIC values below 2 μM, corresponding to a 2 – 128 fold decrease in MIC compared to antimicrobial peptide **2**. Towards Gram-positive bacteria the MIC values of peptide **3** were increased because these bacteria do not express acetyltransferase enzymes LpxA and LpxD. Peptide **3** showed and unexpected increase in potency with lipopolysaccharide deficient Lpx mutants and may indicate additional targets. The cytotoxicity of triple-targeting peptide **3** was found to be very low in a haemolytic assay. Overall, this work demonstrates that peptide **3** shows a significant synergistic increase in potency compared to the individual peptides **1** and **2**. This highly promising multi-targeting strategy will be further studied in our subsequent work. The intracellular binding of peptide **3** to LpxA and LpxD will be studied to confirm the exact mechanism of antimicrobial action. By employing multi-targeting antimicrobial peptides, we envision the possibility of decreasing the emergence of new resistance mechanisms.

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