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Structure-activity and -toxicity relationships of the antimicrobial peptide tachyplesin-1

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Tachyplesin-1 (**1**) is a cationic β-hairpin antimicrobial peptide with a membranolytic mechanism of action. Whilst it possesses broad-spectrum, potent antimicrobial activity, **1** is highly hemolytic against mammalian erythrocytes, which precludes it from further development. In this study we report a template-based approach to investigate the structure-function and structure-toxicity relationships of each amino acid of **1**. We modulated charge and hydrophobicity by residue modification and truncation of the peptide. Antimicrobial activity was then assessed against six key bacterial pathogens and two fungi, with toxicity profiled against mammalian cells. The internal disulfide bridge Cys7-Cys12 of **1** was shown to play an important role in broad-spectrum antimicrobial activity against all pathogenic strains tested. Novel peptides based on the progenitor were then designed, including **5** (TP1[F4A]), **12** (TP1[I11A]) and **19** (TP1[C3A,C16A]). These had 26- to 64-fold improved activity/toxicity indices, and show promise for further development. Structural studies of **5** (TP1[F4A]) and **12** (TP1[I11A]) identified a conserved β-hairpin secondary structure motif correlating with their very high stablility in mouse and human plasma. Membrane binding affinity determined by surface plasmon resonance confirmed their selectivity toward bacterial membranes, but the degree of membrane binding did not correlate with the degree of hemolysis, suggesting that other factors may drive toxicity.

KEY WORDS

Tachyplesin-1; antimicrobial peptide; β-hairpin; antimicrobial activity/toxicity index; amphiphilicity, membrane, antibiotic, resistance

Increasing rates of bacterial resistance to antibiotics has fueled a dire need for the discovery of new replacement drugs for obsolete antibiotics.[1] Antimicrobial peptides (AMPs) are common in nature, being essential components of the innate immune system in the majority of multicellular organisms. AMPs have been well studied over three decades with more than 2000 AMPs reported from eukaryotes (http://aps.unmc.edu/AP/main.php). AMPs, being short, cationic and amphipathic, are characterized with broad range antimicrobial activity resulting from a membranolytic mechanism of action, which consequently offers a low rate of resistance development. Tachyplesin-1 (TP1) **1** is a member of the closely related tachyplesin and polyphemusin families, both originating from the horseshoe crab.[2,3] **1** is a 17 amino acid AMP (KWCFRVCYRGICYRRCR) extracted from the hemocytes of the horseshoe crab, *Tachypleus tridentatus*. **1**, constrained by two disulfide bridges (Cys3-Cys16, Cys7-Cys12), forms a β-hairpin structure (Figure 1), both in aqueous solution and in lipid-mimicking environments.[4,5] **1** exhibits potent activity against Gram-positive (G+ve) and Gram-negative (G-ve) bacteria, as well as fungi. **1** did not induce resistance in short term studies[6], but caused decreased susceptibility under long-term continuous selection conditions[7]. **1** has been shown to compromise the integrity of both the outer and cytoplasmic cell membranes of *Escherichia coli*.[8] In addition to its high antimicrobial activity, **1** unfortunately also displays high toxicity towards mammalian cells, a common detrimental characteristic of many AMPs that renders them unsuitable for therapeutic development. Analogs of **1** have been reported in the past, with amino acid substitutions around the disulfide bridge regions.[9-13] However, no systematic study has been reported to ascertain a clear structure-function relationship for each amino acid in the sequence. In this report, we have conducted an alanine scan of **1** followed by modifications of the overall hydrophobicity by replacement at select positions that the Ala scan indicated were amenable to change. We assessed the effects of amino acid replacements on antimicrobial activity, cytotoxicity, and hemolytic activity. Furthermore, we evaluated the membrane binding affinity of the most interesting peptides by surface plasmon resonance (SPR). We then assessed the stability of the most promising peptides in plasma, and conducted solution phase NMR structrural studies.

RESULTS AND DISCUSSION

Peptide design

In this study, we systematically determined the importance of each amino acid in **1** in a search for therapeutically valuable analogs of **1**. A schematic representation of **1** (Figure 1) illustrates the structural amphipathicity of the peptide. There are two distinct faces; one β-sheet (top) composed mainly of hydrophobic residues and the other one (bottom) being more hydrophilic and charged. Thus, **1** has been identified as one of the most amphipathic β-hairpin AMPs.[14]

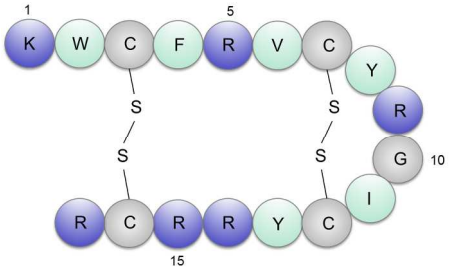

Figure 1 - Schematic representation of 1 (TP1). Hydrophobic residues in green and charged residues in blue.

To modulate the properties of a peptide several methodologies are commonly exploited (i) alteration of the hydrophobicity/amphipathicity by replacing hydrophobic residues with less hydrophobic (e.g. Ala, Gly) or more hydrophilic (e.g. Ser) residues, (ii) modification of the charge by replacing basic or acidic residues, (iii) truncation of the peptide, (iv) combination of (i) to (iii).[15,16] Here we have chosen a panel of peptide modifications that encompass all these methods. An alanine scan is commonly employed to ascertain the importance of individual residues within a peptide. Alanine is the most commonly used amino acid to study the amino acid function in a peptide because it eliminates side-chain interactions

without altering the main-chain conformation. It has the ability to form α-helices and/or β-sheets, is flexible and has low hydrophobicity.

We studied the physicochemical properties of all **1** analogs (see Table 1). The peptides ranged from 2009 Da (**29**, 16 residues) to 2293 Da (**23**, 17 residues). The ExPASy ProtParam tool[17] was used to calculate the physicochemical properties of the peptide analogs. The charge of the analogs varied from +4 (**29** and **33**) to +6 (**1**), while their pI varied from 9.25 (**29** and **33**) to 12.71 (**9** and **14**). GRAVY score (grand average of hydropathicity index) is a method used to calculate the hydrophobicity/solubility of a peptide. A negative GRAVY score indicates hydrophilicity and a positive GRAVY score indicates hydrophobicity. All peptides were hydrophilic except for two, **31** with GRAVY = 0.000, and **33** with a positive hydrophobic GRAVY = 0.224.

Table 1 - Amino acid sequences and physicochemical properties of tachyplesin-1 and its analogs

Number	Peptide	H-Sequence-NH2	MW (Da)	Charge	pI	GRAVY
1	TP1	KWCFRVCYRGICYRRCR	2263.7	+6	12.29	-0.518
2	TP1[K1A]	**A**WCFRVCYRGICYRRCR	2206.7	+5	12.28	-0.182
3	TP1[W2A]	K**A**CFRVCYRGICYRRCR	2148.6	+6	12.29	-0.359
4	TP1[C3A,C16S]	KW**A**FRVCYRGICYRR**S**R	2217.6	+6	12.29	-0.753
5	TP1[F4A]	KWC**A**RVCYRGICYRRCR	2187.7	+6	12.29	-0.576
6	TP1[R5A]	KWCF**A**VCYRGICYRRCR	2178.6	+5	12.12	-0.147
7	TP1[V6A]	KWCFR**A**CYRGICYRRCR	2235.7	+6	12.29	-0.659
8	TP1[C7A,C12S]	KWCFRV**A**YRGI**S**YRRCR	2217.7	+6	12.29	-0.753
9	TP1[Y8A]	KWCFRVC**A**RGICYRRCR	2170.0	+6	12.71	-0.335
10	TP1[R9A]	KWCFRVCY**A**GICYRRCR	2178.6	+5	12.12	-0.147
11	TP1[G10A]	KWCFRVCYR**A**ICYRRCR	2277.8	+6	12.29	-0.388
12	TP1[I11A]	KWCFRVCYRG**A**CYRRCR	2221.7	+6	12.29	-0.676
13	TP1[C7S,C12A]	KWCFRV**S**YRGI**A**YRRCR	2217.7	+6	12.29	-0.753
14	TP1[Y13A]	KWCFRVCYRGIC**A**RRCR	2171.7	+6	12.71	-0.335
15	TP1[R14A]	KWCFRVCYRGICY**A**RCR	2178.7	+5	12.12	-0.147
16	TP1[R15A]	KWCFRVCYRGICYR**A**CR	2178.7	+5	12.12	-0.147
17	TP1[C3S,C16A]	KW**S**FRVCYRGICYRR**A**R	2217.7	+6	12.29	-0.753
18	TP1[R17A]	KWCFRVCYRGICYRRC**A**	2178.7	+5	12.12	-0.147
19	TP1[C3A,C16A]	KW**A**FRVCYRGICYRR**A**R	2203.7	+6	12.29	-0.600
20	TP1[C7A,C12A]	KWCFRV**A**YRGI**A**YRRCR	2203.7	+6	12.29	-0.600
21	TP1[C3A,C7A,C12A,C16A]	KW**A**FRV**A**YRGI**A**YRR**A**R	2140.5	+6	11.84	-0.682
22	TP1[V6R,R9A]	KWCFR**R**CY**A**GICYRRCR	2239.8	+6	12.29	-0.659
23	TP1[K1R]	**R**WCFRVCYRGICYRRCR	2292.7	+6	10.16	-0.553
24	TP1[F4G]	KWC**G**RVCYRGICYRRCR	2174.6	+6	9.93	-0.706
25	TP1[F4S]	KWC**S**RVCYRGICYRRCR	2204.6	+6	9.93	-0.729
26	TP1[Y8G]	KWCFRVC**G**RGICYRRCR	2158.6	+6	10.16	-0.465
27	TP1[I11G]	KWCFRVCYRG**G**CYRRCR	2208.6	+6	9.93	-0.806
28	TP1[F4A,Y8A,I11A]	KWC**A**RVC**A**RG**A**CYRRCR	2054.4	+6	10.16	-0.553

29	TP1[-R5,R17G]	KWC -FVCYRGICYRRC**G**	2009.4	+4	9.25	-0.013
30	TP1[K1A,F4A]	**A**WC**A**RVCYRGICYRRCR	2131.5	+5	9.69	-0.241
31	TP1[K1A,Y8A]	**A**WCFRVC**A**RGICYRRCR	2115.5	+5	9.88	0.000
32	TP1[K1A,I11A]	**A**WCFRVCYRG**A**CYRRCR	2165.5	+5	9.69	-0.341
33	TP1[R9A,R17A]	KWCFRVCY**A**GICYRRC**A**	2094.5	+4	9.25	0.224

Antimicrobial activity, toxicity and cell selectivity

1 was determined previously to possess potent and broad-spectrum antimicrobial activity.[14] Here we assessed the antimicrobial activity of 32 analogs of **1** against a broad range of bacteria, both G-ve and G+ve, as well as fungi. Table 2 reports the results of testing against a panel of ATCC reference strains and drug resistant clinical isolates of *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (strain details in supplemental Table S1). The majority of the analogs tested retained potent activity across the range of microbes, even the most resistant ones, as compared to **1**, though no analog displaying increased antimicrobial activity. All peptides, including **1**, showed a marked reduction in activity against *Candida albicans* as compared to other microbes, with at least 8-fold reduction in antifungal activity as compared to *Cryptococcus neoformans*. Similarly, for G+ve bacteria the peptides had lower potency against *Staphylococcus aureus* compared to *Bacillus subtilis*, with antibacterial activity consistently at least 4-fold lower. For *K. pneumoniae,* all peptides demonstrated a reduction in activity between the reference strain, and the multidrug-resistant (MDR) strains of 2- to 8-fold, with no difference between the various MDR strains. Another 4- to 32-fold reduction in activity was noted when tested against an extensively drug-resistant (XDR) strain across all peptides. This difference in activity was not demonstrated in the case of *A. baumannii* or *P. aeruginosa* with the MIC activity remaining within a tight range (~2-fold, i.e. within accepted assay fluctuation range), between reference strains and resistant strains, including XDR and polymyxin resistant isolates.

The alanine scan showed that single Ala replacements of amino acids at positions 1, 2, 4, 5, 6, 8, 9, 10, 11, 13, 14, 15 or 17 (producing peptides **2**, **3**, **5, 6, 7, 9, 10, 11, 12, 14, 15, 16 or 18**, respectively) gave

analogs that retained the broad-spectrum potent antimicrobial activity of **1**; therefore these positions are presumably not key for the antimicrobial activity of the peptide. An NMR and tryptophan fluorescence study performed by Kushibiki *et al*[5] concluded that Trp2 inserted into the hydrophobic acyl chains of LPS and suggested that the residues located at the N- and C-termini of **1** are involved in the binding to LPS, indicating their importance in the mode of action of **1**. Our results do not correlate well with those findings as **3** (TP1[W2A]) showed only 3-fold reduction in antimicrobial activity across all tested strains, with **2** and **18** also showing good activity. Positions 3, 7, 12 and 16 of **1** correspond to Cys residues, forming two disulfide bridges (3-16 and 7-12) that create the disulfide bridge constrained β-hairpin structure as characterized by several earlier studies.[4,5] Removal of the disulfide bridge was achieved via two different approaches: (i) by Ala replacement of one Cys while the partnering Cys residue was replaced by Ser, therefore retaining the same hydrophilic properties of Cys-SH without potential misfolding of the peptide through mismatched disulfide bond formation[18] and (ii) by complete abolition of the disulfide bridge properties by Ala replacement of both Cys residues. The deletion of the internal disulfide bridge (7-12) in **8**, **13** or **20** reduced the antimicrobial activity of **1** by 3- to 12-fold. However, the deletion of the external disulfide bridge (3-16) in **4**, **17** or **19** more significantly reduced the antimicrobial activity of **1**, by 4- to 128-fold. Deletion of both disulfide bridges, **21**, dramatically reduced the overall antimicrobial activity by 64-fold across all tested strains, in accordance with the work performed by Tamamura *et al*.[9] This reduction in antimicrobial activity is presumably due to the subsequent loss of β-sheet stacking, as a direct result of the removal of the disulfide bridges.[7,8,15] In our study, there were several unexpected results. Of the three analogs that removed the external disulfide bond, **19** (TP1[C3A,C16A]) and **17** (TP1[C3S,C16A]) had similar antimicrobial activities across all strains tested, losing only 2 to 16-fold activity as compared to **1**. However, **4** (TP1[C3A,C16S]) showed around 100-fold reduction in antimicrobial activity for most strains tested, except for *B. subtilis* and *C. neoformans* where the reduction was only 10-fold. Cys3 is flanked by hydrophobic residues, Trp2 and Phe4, and Cys16 is flanked by polar residues, Arg15 and Arg17. By inserting the hydrophobic residue

Ala into position 3 and hydrophilic residue Ser into position 16, the amphipathicity of the peptide was increased considerably, potentially creating a repulsive effect between the β-sheets that led to an unfolded structure (1H NMR in supplementary information, Figure S13).

Several positions were selected for further modifications. The modulation of hydrophobicity/amphipathicity at position 4 was studied by mutating Phe with Ala (F4A) **5**, Gly (F4G) **24** or Ser (F4S) **25**, resulting in reduced antimicrobial activity, around 2-fold at each mutation, as hydrophobicity decreased. A selection of five peptides (**4** (TP1[C3A,C16S]), **19** (TP1[C3A,C7A,C12A,C16A]), **22** (TP1[V6R,R9A]), **28** (TP1[F4A,Y8A,I11A]) and **29** (TP1[-R5,R17G])) showed a considerable loss, at least 100-fold, in antimicrobial activity across the full panel of bacteria and fungi. These five peptides were designed with a range of modifications that comprise physicochemical properties from all regions of the spectrum of MW, charge, pI and hydrophobicity. No trend was identified that correlated physicochemical properties with antimicrobial activity, and it is therefore more likely that the overall peptide structure and sites specific to mode of action are the key elements to increasing and decreasing antimicrobial potential of the **1** peptide scaffold.

Table 2 - Antimicrobial activity of tachyplesin-1 and its analogs

Compound name	MIC (µg/mL)												
	Bacteria G-ve									Bacteria G+ve		Fungi Yeast	
	E. coli	*K. pneumoniae*				*A. baumannii*		*P. aeruginosa*		*B. subtilis*	*S. aureus*	*C. albicans*	*C. neoformans*
	ATCC 25922 (control strain)	ATCC 13883	ATCC 700603 (MDR)	BAA 2146 (NDM-1 pos)	clinical isolate 100650661:1 (XDR;PmxR)	ATCC 19606	clinical isolate 100734512:2 (XDR;PmxR)	ATCC 27853	FADDI-FA070 (PmxR)	ATCC 6051	ATCC 43300 (MRSA)	ATCC 90028	ATCC 208821
colistin	0.015	0.125	0.03	0.125	>32	0.06	>32	0.125	>32				
meropenem	≤0.03	0.03	0.06	>32	>64	1	>64	0.5	>32				
vancomycin										0.125	1		
fluconazole												0.5	8
amphotericin B												0.25	0.15
1	0.06	0.06	0.25	0.25	2	0.06	0.125	0.125	0.25	0.06	0.25	2	0.125
2	0.03	0.06	0.125	0.125	2	0.125	0.125	0.25	0.5	0.03	0.5	8	0.25
3	0.25	0.5	1	1	>16	0.5	0.5	0.5	4	0.5	8	8	0.5
4	4	16	16	>16	>16	>16	>16	>16	>16	0.5	>16	>16	4
5	0.125	0.5	0.5	0.5	16	0.5	0.5	0.5	2	0.25	4	8	0.5
6	0.125	0.125	0.5	0.5	8	0.06	0.125	0.25	0.5	0.03	0.5	4	0.25
7	0.25	0.25	1	1	16	0.25	0.5	1	2	0.25	4	8	1
8	0.5	0.5	4	2	16	0.25	0.25	2	4	0.25	2	8	0.5
9	0.06	0.125	0.25	0.5	8	0.125	0.5	1	2	0.25	4	8	0.5
10	0.125	0.03	0.25	0.125	2	0.06	0.125	0.25	1	0.125	1	2	0.25
11	0.06	0.03	0.25	0.25	4	0.06	0.06	0.25	0.5	0.03	0.5	2	0.06
12	0.125	0.25	0.5	0.25	8	0.25	0.25	0.25	1	0.125	2	2	0.5
13	1	4	16	8	16	0.5	1	8	16	0.125	16	2	0.5
14	0.25	0.25	0.5	0.5	8	0.125	0.25	0.5	2	0.06	4	4	0.5
15	0.125	0.06	0.5	0.25	1	0.125	0.125	0.125	0.25	0.125	0.5	8	0.25
16	0.25	0.25	2	1	16	0.25	0.25	4	4	0.03	4	2	0.25
17	0.25	1	2	1	16	0.25	0.25	1	4	0.125	4	4	0.25
18	0.015	0.125	0.25	0.25	4	0.125	0.125	0.25	0.5	0.015	0.5	4	0.25
19	0.25	1	1	1	>16	0.25	0.5	2	8	0.125	8	8	1
20	0.125	0.25	2	1	16	0.25	0.25	1	2	0.125	2	8	1
21	8	16	16	16	>16	4	4	>16	>16	1	16	>16	1
22	0.5	>16	16	>16	>16	16	>16	>16	>16	0.25	>16	>16	4
23	0.125	0.125	0.5	0.25	4	0.125	0.25	0.25	0.5	0.125	1	4	0.25
24	0.25	2	1	2	>16	0.5	2	1	16	0.5	16	8	2
25	0.5	2	4	4	>16	0.5	2	4	>16	0.5	16	>16	4
26	0.5	2	8	8	>16	1	2	8	16	0.5	16	>16	4
27	0.25	0.25	0.5	2	8	0.5	0.5	1	4	0.25	4	8	1
28	8	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16	>16
29	8	>16	>16	>16	>16	16	>16	>16	>16	1	>16	>16	4
30	2	4	4	4	>16	2	16	8	>16	16	>16	>16	>16
31	0.125	0.25	0.5	1	8	0.25	0.5	1	4	0.5	8	>16	2
32	0.125	0.125	0.25	0.5	4	0.25	0.25	0.5	2	0.125	4	>16	1
33	0.03	0.06	0.5	0.5	4	0.25	0.5	0.5	2	0.06	2	8	0.5

colistin, meropenem, vancomycin, fluconazole and amphotericin B were used as positive inhibitor controls for G-ve, G+ve and fungi, respectively. Data: n = 4. XDR (extensively drug resistant), PmxR (polymyxin resistant)

The toxicity of the analogs of **1** was tested against Human Embryonic Kidney cells (HEK293) and human red blood cells (RBC). None of the peptides showed cytotoxicity against HEK293 cells at the highest tested concentration, 100 μg/mL. Hemolytic activity (MHC) is reported in Table 3 as the minimum hemolytic concentration that caused 10% hemolysis of fresh RBCs. **1** possessed reasonably potent hemolytic activity (MHC = 0.25 μg/mL) and most of the peptide analogs did not improve this quality by decreasing hemolytic potency. However, nine of the analogs (**3** (TP1[W2A]), **4** (TP1[C3A,C16S]), **5** (TP1[F4A]), **9** (TP1[Y8A]), **12** (TP1[I11A]), **19** (TP1[C3A,C16A]), **20** (TP1[C7A,C12A]), **22** (TP1[V6R,R9A]) and **30** (TP1[K1A,F4A])) did show promising 32 to 160-fold reductions in MHC. Similarly to antimicrobial activity, this did not correlate with the physicochemical properties of the peptides and no trend or correlation could be discerned. Additionally, for some analogs the reduction in hemolytic activity correlated to the loss of antimicrobial activity, meaning these peptide sequences essentially lost all biological activity. This is the case for **22**, a peptide designed to reduce the hydrophobicity of **1** without change of charge, reducing the overall amphipathicity of **1**. This change was not beneficial, emphasizing again the importance of peptide amphipathicity to its activity.

Commonly, the overall therapeutic potential of a peptide is assessed via a cell selectivity or antimicrobial activity/toxicity index (ATI). The ATI, Table 3, can be improved in one of the following ways (i) increase in antimicrobial activity, (ii) decrease in hemolytic activity, or (iii) a combination of both, increasing antimicrobial activity while decreasing hemolytic activity. Here we calculated the ATI from the ratio of the minimum hemolytic concentration (MHC) to the median of the minimum inhibitory concentrations (MIC) determined towards all tested bacteria and fungi (MM). Therefore, a larger ATI value indicates greater desirable cell selectivity. From these ATI calculations, three peptides (**5** (TP1[F4A]), **19** (TP1[C3A,C16A]) and **12** (TP1[I11A])) stood out. These analogs had ATI values that were 26-, 40- and 64-fold improved, respectively, retaining relatively high antimicrobial activity while dramatically decreasing hemolytic activity.

Table 3 - Biological activity and activity/toxicity indices of tachyplesin-1 and its analogs

Peptide	MM[a] (μg/mL)	MHC[b] (μg/mL)	ATI[c]
1	0.125	0.25	2.0
2	0.125	2.1	16.8
3	0.4	8	20.0
4	16	8	0.5
5	0.5	40	80.0
6	0.125	2	16.0
7	0.25	1.2	4.8
8	0.5	1	2.0
9	0.25	8	32.0
10	0.125	2	16.0
11	0.125	0.5	4.0
12	0.25	32	128.0
13	1.5	0.6	0.4
14	0.25	1	4.0
15	0.163	0.5	3.1
16	0.25	0.45	1.8
17	0.5	3	6.0
18	0.125	0.45	3.6
19	0.75	40	53.3
20	0.375	8	21.3
21	8	0.24	0.0
22	16	11	0.7
23	0.163	1	6.2
24	1	0.47	0.5
25	2	0.23	0.1
26	3	0.4	0.1
27	0.5	0.2	0.4
28	16	0.9	0.1
29	16	0.3	0.0
30	6.4	32	5.0
31	0.75	0.4	0.5
32	0.5	0.2	0.4
33	0.375	0.2	0.5

[a]The median of the peptide Minimum Inhibitory Concentrations (MICs) against all bacteria and fungi tested (MM).
[b]The MHC was determined as the minimum hemolytic concentration that caused ≤10% hemolysis of fresh red blood cells.
[c]The activity/toxicity index (ATI) is the ratio of the MHC to the MM. Larger values indicate a greater selectivity of the peptides for microbe cells.

The Ala scan peptides that showed an improved ATI, **5** (TP1[F4A]), **9** (TP1[Y8A]) and **12** (TP1[I11A)], were subsequently substituted with Gly instead of Ala, to see if further reduction in side chain

hydrophobicity was beneficial. The F4G substitution **24** had similar antimicrobial activity to F4A **5** but almost 100-fold increase in hemolytic activity. Similarly, while Tyr8 and Ile11 substituted by Ala (**9** and **12**) retained **1**'s antimicrobial activity and reduced its hemolytic activity; the analogs with Gly (**26** and **27**) had reduced antimicrobial activity while retaining similar levels of hemolytic activity. In a study by Panteleev *et al*[19], Tyr8 and Ile11 of **1** were substituted by Arg or Ser, in both cases dramatically reducing the hemolytic activity without detriment to the antimicrobial activity, hence making the compounds very promising for development as antibiotic candidates. In our study, the replacement of the Tyr8 or Ile11 by a less hydrophobic amino acid was also shown to be highly beneficial for the cell selectivity of the peptide. However a fine balance needs to be found, as if the amino acid composition of the peptide becomes too hydrophilic the toxicity increases significantly. This may be influenced by the overall conformation of the peptide as well as the specific conformation of the peptide turn. Residues Tyr8 and Ile11 both form part of the β-turn and the replacement of either residue to a Gly would change the type of structural turn.

The beneficial mutations seen in analogs **5**, **9** and **12** were then combined to see if the effects were additive. Unfortunately, none of **28, 30**, **31**, **32** and **33** showed the desired improvements; while they kept relatively potent antimicrobial activity (except for **28** and **30**), they also concomitantly retained high hemolytic activity (except for **30**).

Another method to increase the activity/toxicity index of **1** was reported by Saravanan *et al*,[12] who deleted the four Cys residues of **1** without replacement (peptide named CDT). In this case, the β-hairpin structure was less rigid but conserved in solution and in the presence of LPS, due to hydrogen bonding and packing interactions across the sheets, allowing the peptide to maintain potent antimicrobial activity while reducing its hemolytic activity. Wood *et al*,[20] studied the SAR of CDT, finding that two analogs [des-Arg^{12,13}] CDT and [Ile^{2,3,6,10}] CDT increased the selectivity index.

Peptide membrane binding followed by Surface Plasmon Resonance

To investigate the cell selectivity, we determined the peptide membrane binding affinity of select peptides by SPR. We selected two model membranes, one mimicking the outer leaflet of the G-ve bacteria *E. coli* (taken as representative of the AMP antimicrobial activity) and the other one mimicking the outer leaflet of the human red blood cell (taken as representative of the AMP hemolytic activity). Bacterial membranes are more negatively charged than mammalian cells due to their lipid composition. Moreover, the cell composition of the outer leaflet of bacterial membranes differs between classes. *E. coli* is predominantly composed of palmitoyloleoyl-phosphatidylethanolamine (POPE), palmitoyloleoyl-phosphatidylglycerol (POPG) and cardiolipin, and was therefore modelled as POPE/POPG/cardiolipin (15:4:1) in our study.[21] In contrast, about half of the mass of the RBC membrane is comprised of protein and the other half is lipids, predominantly phospholipids with the addition of cholesterol.[22] Palmitoyloleoyl-phosphatidylcholine (POPC), a zwitterionic phospholipid forming a fluid bilayer at room temperature, is the major component of eukaryotic cells. However, cholesterol also plays a key role in the membrane composition of RBCs. Therefore, POPC/cholesterol (4:1) was used here as a simplistic model to reflect the charge and fluidity of the outer leaflet of RBCs. Matsuzaki *et al.*[23] stated that hemolytic peptides exhibited strong interactions with zwitterionic phospholipids, whereas non-hemolytic peptides did not. We thus investigated the relative membrane binding affinities of the three peptides possessing the highest ATI values (**5**, **12** and **19**), as well as less active **1** and **27**, to assess this hypothesis. In our study (Figure 2), all five peptides exhibited a stronger interaction to POPE/POPG/cardiolipin, modelling the outer leaflet of *E. coli*, as opposed to POPC/cholesterol, modelling the outer leaflet of RBCs. The cell selectivity was relatively similar amongst the five peptides. These data do not correlate with the findings of Matsuzaki *et al*.[23] and did not correspond with our observed antimicrobial and hemolytic activity and hemolytic activity testing, which showed that all five peptides had similar antimicrobial potency but differed by their hemolytic activity. **1** and **27** exhibited high hemolytic activity compared to the other three peptides. We would therefore have expected to see an increase in binding affinity to RBC with **1**

and **27**. This lack of correlation between the model membrane binding affinities and the biological data could be due to several hypotheses (i) our RBC model is too simplistic and does not reflect the full complexity of what occurs *in vitro* with live cells, as the RBC membrane is a very complex ensemble of two asymmetric layers,[24,25] composed mainly of phosphatidylcholine (PC), sphingomyelin (SM) and cholesterol, or (ii) the affinity for the peptide to bind to the membrane is only the first step in the mode of action for activity. It is likely that the peptide not only needs to bind to the membrane but must also act to disrupt the cell to exhibit activity and cause cell death. This is in accordance with the findings of Imura *et al*,[26] suggesting a lipid flip-flop with toroidal pore formation mechanism of action.

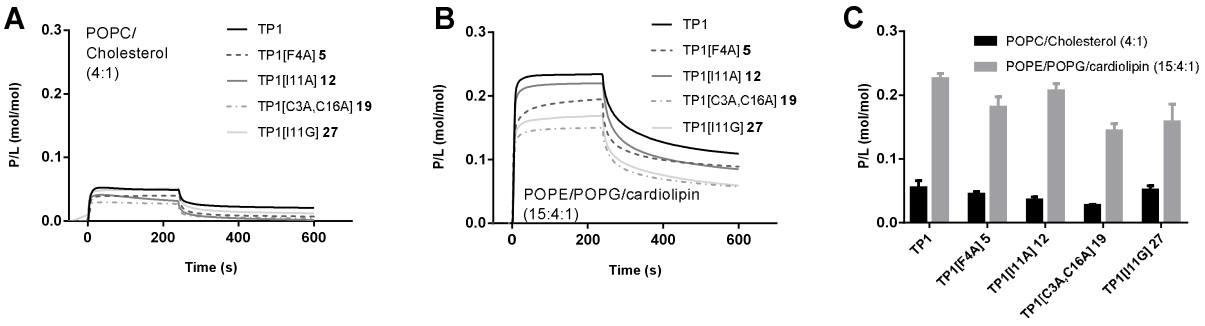

Figure 2 - Membrane binding affinity of tachyplesin-1 and its analogs as monitored by surface plasmon resonance. The response to the total amount of lipid deposited on the chip surface was normalized by calculating the peptide-to-lipid ratio (P/L mol/mol) (1RU = 1 pg.mm^{-2}). Sensorgrams obtained upon injection of 64 µg/mL of peptide over (A) POPC/Cholesterol (4:1) [RBC] or (B) POPE/POPG/cardiolipin (15:4:1) [*E. coli*] bilayers. (C) Comparison of the P/L obtained for the five peptides at the reported point at the end of the association phase over both model bilayers. Error bars represent standard error of the mean of three replicates.

Plasma stability

Plasma stability is an important parameter for potential peptidic drug candidates. Compounds with certain functional groups are more susceptible to hydrolysis by plasma enzymes than others. These include esters, amides, lactones, lactams, carbamides, sulfonamides and peptide mimetics. The plasma stability assay is designed to focus on these classes of compounds. To evaluate the stability of the three peptides possessing the highest ATI values (**5**, **12** and **19**), we performed a plasma stability assay against mouse

and human plasma over 2 hours. The percentage of peptide remaining in plasma was followed by LC-MS, as shown in Figure 3. Interestingly, **1**, **5** and **12** remained fully stable in both mouse and human plasma, however, **19** was rapidly degraded to leave only 13% or 52% after two hours, in mouse and human plasma, respectively. Loss of the disulfide bridge in **19** TP1[C3A,C16A] improved the activity/toxicity relationship but made **19** TP1[C3A,C16A], more susceptible to hydrolysis in the plasma environment.

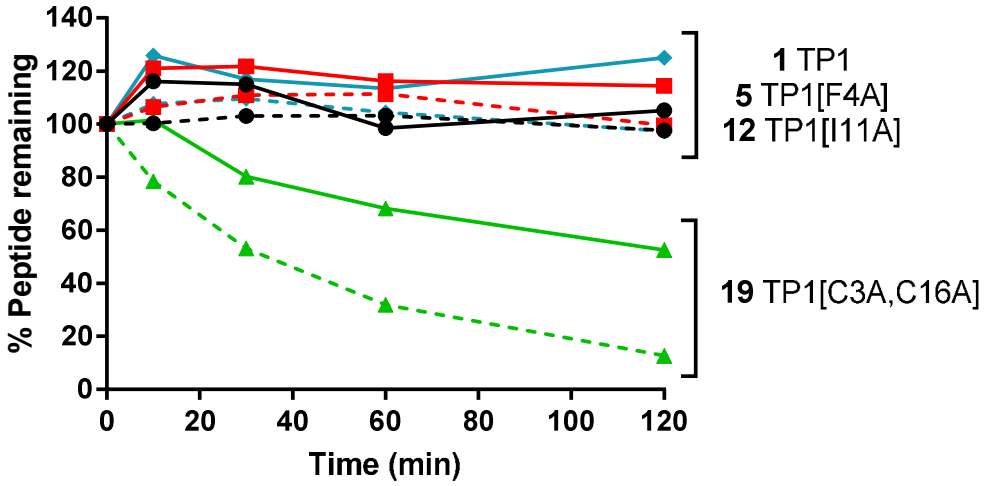

Figure 3 – Plasma stability of tachyplesin-1 and its analogs as monitored by LC-MS. Stability of **1** TP1(black), **5** TP1[F4A] (red), **12** TP1[I11A] (blue) and **19** TP1[C3A,C16A] (green) in mouse (dotted line) and human (full line) plasma over 120 min. Points represent the average of two replicates.

Structural characterization

To better understand the observed variation of peptide stability in plasma, the structural characterization of the four peptides was investigated. NMR spectroscopy was used to confirm the folding of the TP1 analogs and to compare their structures. The NMR assignment of **1** was comparable to the chemical shift assignment reported previously, BMRB 5486.[27] A comparison of the Hα chemical shifts of the four TP1 analogs (Figure 4A) illustrates that the structures are conserved for **1**, **5** and **12**. However, **19** shows very

different Hα chemical shifts as compared to the other three analogs, demonstrating that its secondary structure is not conserved. In Figure 4B, secondary chemical shifts were calculated by comparing the experimentally observed Hα chemical shifts (HA) for each residue with the chemical shifts observed for the corresponding residues in random coil (RC) peptides[28], to compare **1** and its analogs in relation to sheet formation. **1**, **5** and **12** show a similar pattern, with defined β-hairpin structure as compared to **19,** where the β-hairpin motif is lost due to the reduction of length of the first β-sheet region, from Trp2-Cys7 to Arg5-Cys7. It is logical to attribute this to the loss of the disulfide bridge Cys3-Cys16, rendering the N- and C-terminal more flexible and reducing the size of the N-terminal β-sheet, changing the overall β-hairpin secondary structure motif.

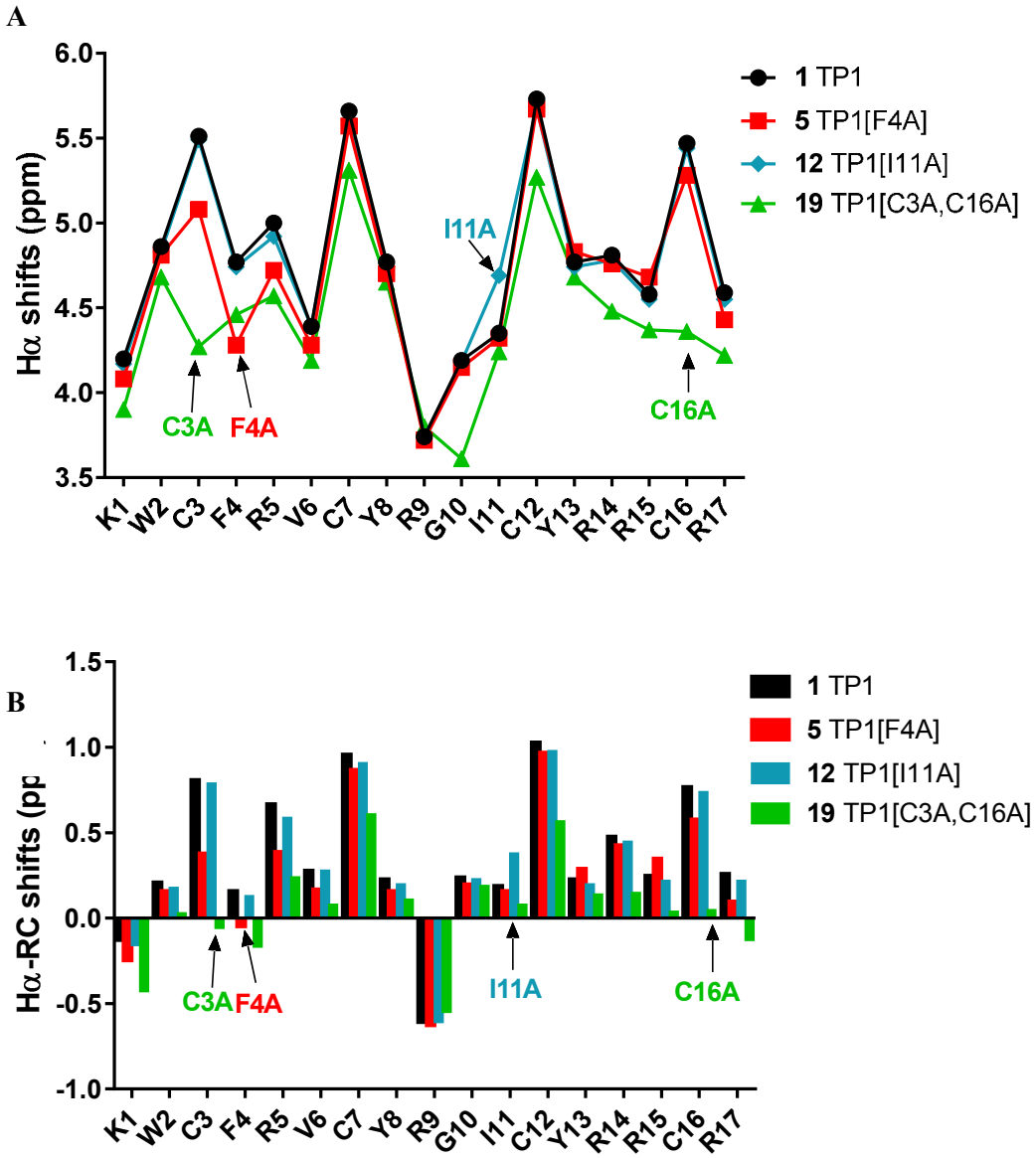

Figure 4 – TP1 and analogs chemical shifts. (A) Hα chemical shifts and (B) Hα secondary chemical shifts of TP1 **1** (black) and analogs **5** (red), **12** (blue) and **19** (green). Secondary chemical shifts were calculated by comparing the experimentally observed Hα chemical shifts (HA) for each residue with the chemical shifts observed for the corresponding residues in random coil (RC) peptides.[28] The sequence mutations are annotated.

CONCLUSION

This work presents a structure-function relationship study of tachyplesin-1 by a systematic amino acid replacement strategy with characterization of the resultant antimicrobial, cytotoxicity and hemolytic activity. We identified three modified peptides: **5** (TP1[F4A]), **12** (TP1[I11A]) and **19** (TP1[C3A,C16A]), that possessed substantially improved therapeutic indexes (26- to 64-fold) over the progenitor. With high potency against broad-spectrum microbes and considerably less hemolytic toxicity, these compounds may be considered as promising hit compounds for further development towards new treatments for infections caused by Gram-negative and Gram-positive bacterial pathogens, as well as potential fungal infections. Structural studies and preliminary ADME testing support further investigations of two of the three promising novel antimicrobial peptides, as **5** and **12** not only conserve the β-hairpin secondary structure motif but are highly stable in mouse and human plasma. Additional studies examining bioavailability, pharmacokinetics, toxicity, mutagenicity and *in vivo* efficacy of **5** and **12** are required to further validate these interesting novel antibiotics.

MATERIALS AND METHODS

Materials

Tachyplesin-1 and tachyplesin-1 analogs were synthesized by Mimotopes Pty Ltd (Clayton, Australia) and WuXi AppTech (Shanghai, China) using Fmoc-based solid phase peptide synthesis and orthogonal Cys protection (Fmoc Cys(Acm)-OH for Cys3, Cys16, and Fmoc Cys(Trt)-OH for Cys7, Cys12); the first disulfide bridge was formed by air oxidation following cleavage from the resin, with iodine treatment generating the fully cyclized peptide that was purified by RP-HPLC to > 90% purity. Identity and purity were confirmed by LC-MS, HRMS and 1H and 2D TOCSY NMR (Figures S1 to S104). Proton chemical shifts of **1** (TP1) were comparable of previously reported tachyplesin-1 (PDB ID 1ma2 and BMRB 5486) and taken as reference for the identity confirmation of **1** analogs.[27] Synthetic lipids palmitoyloleoyl-phosphatidylcholine (POPC), palmitoyloleoyl-phosphatidylglycerol (POPG), palmitoyloleoyl-

phosphatidylethanolamine (POPE) and cardiolipin were purchased from Avanti Polar Lipids (Alabaster, AL). All chemicals were used without further purification. The tachyplesin analogs used in this study do not contain any structures with potential PAINS (Pan Assay Interference Compounds) liabilities.

Antimicrobial activity

Both antibacterial and antifungal assays were performed by a broth micro-dilution plate based method as per CLSI guidelines for antimicrobial susceptibility testing.[29, 30] The assay was performed in Mueller Hinton Broth (MHB, Bacto laboratories, 211443) for antibacterial testing and Yeast Extract-Peptone Dextrose (YPD, Sigma-Aldrich, Y1500) for antifungal testing, and the Minimum Inhibitory Concentration (MIC) was determined as the lowest concentration of compound that prevented all visible microorganism growth after 18-24 h. Compounds were prepared at 0.64 mg/mL solution in water:DMSO (4:1).

For antimicrobial assays, the tested strains, Table S1, were cultured either in Luria broth (LB) (In Vitro Technologies, USB75852), Nutrient Broth (NB) (Becton Dickson, 234000) or MHB at 37 °C overnight. A sample of culture was then diluted 40-fold in fresh MHB and incubated at 37 °C for 1.5-2 h. The compounds were serially diluted two-fold across the wells of 96-well plates (Corning 3641, Non-binding Surface [NBS]), with compound concentrations ranging from 0.015 μg/mL to 64 μg/mL, plated in duplicate. The resultant mid-log phase cultures were diluted to the final concentration of 1×10^6 CFU/mL, then 50 μL was added to each well of the compound-containing plates giving a final compound concentration range of 0.008 μg/mL to 32 μg/mL and a cell density of $5x10^5$ CFU/mL. All plates were then covered and incubated at 37 °C for 18 h. Resazurin was added at 0.001% final concentration to each well and incubated for 2 h before MICs were read by eye. Resazurin was added to assist MIC determination as small spindle tissue or precipitate was present in otherwise clear wells.

For the antifungal assay, fungi strains, Table S1, were cultured for 3 days on YPD agar at 30 °C. A yeast suspension of $1x10^6$ to $5x10^6$ CFU/mL was prepared from five colonies. These stock suspensions were

diluted with Yeast Nitrogen Base (YNB) (Becton Dickinson, 233520) broth to a final concentration of 2.5×10^3 CFU/mL. The compounds were serially diluted two-fold across the wells of 96-well plates (Corning 3641, Non-binding Surface [NBS]), with compound concentrations ranging from 0.015 μg/mL to 64 μg/mL and final volumes of 50 μL, plated in duplicate. Then, 50 μL of the fungi suspension that was previously prepared in YNB broth to the final concentration of 2.5×10^3 CFU/mL, was added to each well of the compound-containing plates, giving a final compound concentration range of 0.008 μg/mL to 32 μg/mL. Plates were covered and incubated at 35 °C for 24 h. *C. albicans* MICs were determined by measuring the absorbance at OD_{530}. For *C. neoformans* resazurin was added at 0.006% final concentration to each well and incubated for a further 3 h before MICs were determined by measuring the absorbance at $OD_{570\text{-}600}$.

Hemolytic assay

The ability of the peptides to hemolyse human red blood cells was determined by a previously reported method.[31] Human red blood cells were separated from serum and washed in phosphate-buffered saline (PBS, pH 7.4) with 4–5 times centrifugation at 4000 rpm. Erythrocytes were then resuspended in phosphate-buffered saline as a 0.25% (v/v) solution. Stock solutions (300 μM) of compounds were prepared. Two-fold serial dilutions were prepared yielding eight solutions in total, which were aliquoted (20 μL) into a 96-well plate (NUNC 96 wells polypropylene plates round bottom, Sigma Aldrich P6866). The erythrocyte solution (100 μL) was dispensed into each well and incubated with diluted peptide solution at 37 °C for 1 h. Triton X-100 solution (1%, v/v, 20 μL) was used as a positive control to represent 100% cell lysis, and PBS solution (20 μL) was used as a negative control (spontaneous lysis after 1 h incubation). The 96-well plate was centrifuged to pellet intact red blood cells, and the supernatant of each well was collected in a fresh plate (NUNC 96 wells polypropylene plates flat bottom, Sigma Aldrich P7366) and measured by visual absorption spectroscopy at 415 nm. The percentage of hemolysis was calculated according to the following equation:

$$Hemolysis\ (\%) = \left[\frac{OD_{415}(sample) - OD_{415}(zero\ lysis)}{OD_{415}(100\%\ lysis) - OD_{415}(zero\ lysis)}\right] x100\%$$

All peptide solutions were assayed in triplicate.

Cytotoxicity

Cytotoxicity to HEK293 cells was determined using the resazurin assay.[32,33] In brief, HEK293 cells were seeded as 4000 cells/well in black-wall, clear-bottom 384-well plates (Corning, Australia) and incubated for 24 h at 37 °C, 5% CO_2. Compounds were then added into each well. After 24 h incubation, 5 μM resazurin was added per well and incubated at 37 °C for 2 h. Fluorescence intensity was then read using a Polarstar Omega with excitation/emission 560/590. The data were then analyzed using GraphPad Prism 6 software. CC_{50} values were calculated using the following equation: Percentage Viability = (FI_{TEST} − $FI_{Negative}$/$FI_{UNTREATED}$ −$FI_{Negative}$)*100.

Surface Plasmon Resonance

The binding affinity of **1** and its analogs for model membranes composed of POPC/Cholesterol (4:1) and POPE/POPG/cardiolipin (15:4:1) were compared using SPR. Solutions were freshly prepared and filtered (0.22 μM pore size). L1 sensor chip and BIAcore 3000 system (Biacore, GE Healthcare) were used. Small unilamellar vesicles (diameter, 50 nm) were prepared by freeze-thaw fracturing and sized by extrusion. Vesicle suspensions were deposited on an L1 chip over 40 min at a flow rate of 2 μL/min to allow the vesicles to bind, fuse and form a stable lipid bilayer. The association of the peptide sample to the lipid bilayer was evaluated by injection for 240 s at flow rate of 5 μL/min and the dissociation was followed for 600 s at the same flow rate. The L1 chip was regenerated after each injection cycle, by injection of 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) (5 μL/min, 60 s), followed by an injection of 10 mM sodium hydroxide in 20 % methanol (w/w) (50 μL/min, 60 s) and to finish an injection of 10 mM sodium hydroxide (50 μL/min, 36 s) buffer. All measurements were conducted at 25 °C. 10 mM HEPES buffer pH7.4 containing 150 mM NaCl (to mimic the physiological conditions) was used as running buffer and to prepare samples and lipid vesicles.[34,35] The peptide samples

were prepared at 7 different concentrations, starting from 64 to 1 μg/mL following 2-fold dilution steps. The lipid deposited onto the chip surface is dependent on the lipid mixture, therefore, the response units were converted into peptide to lipid ratio (mol/mol).

Plasma stability

Test compound (final concentration 2 μM) was combined with pre-warmed CD-1 mouse or human plasma at 37 °C (100 μL volume). At each time point (t = 0, 15, 30, 60 and 120 min), 100 μL of 4% H_3PO_4/H_2O was added and the reaction was quenched with 600 μL of cold stop solution (200 ng/mL tolbutamide plus 20 ng/mL buspirone in 50% ACN/MeOH). Upon centrifugation at 4,000 g for 10 min, the clear supernatants (100 μL) were transferred to a 96-well plate and mixed with 100 μL of ultra pure water. The samples were shaken at 800 rpm for 10 min before being analysed by LC-MS/MS. The percentage of remaining parent compound at each time interval relative to the t = 0 min sample is calculated from peak area ratios in relation to the internal standard. All samples were run in duplicate with propantheline bromide as a positive control and tolbutamide as internal standard.

NMR characterization

1H and 2D TOCSY NMR were acquired on a Bruker Avance III spectrometer equipped with a cryogenically cooled triple resonance probed operating system at a normal ^1H frequency of 600 MHz at 298 K. Water suppression was performed via excitation sculpting. Secondary chemical shifts were calculated by comparing the experimentally observed Hα chemical shifts (HA) for each residue with the chemical shifts observed for the corresponding residues in random coil (RC) peptides.[28] The NMR assignment of **1** TP1 was compared to the chemical shifts reported previously, BMRB 5486.[27]

ANCILLARY INFORMATION

Supporting Information. *Bacterial strain descriptions and QC data of each compound by HRMS, LC–MS and, 1H and 2D NMR. This information is available free of charge via the Internet at http://pubs.acs.org/.*

Abbreviations Used: *TP1, tachyplesin-1; ADME, Adsorption Distribution Metabolism Elimination; AMP, Antimicrobial peptide; GRAVY, grand average of hydropathicity index; MIC, Minimum inhibitory concentration; MM, Median of the peptide MICs against all bacteria and fungi tested; MHC, Minimum hemolytic concentration; ATI, activity/toxicity index; ATCC, American Type Culture Collection; G-ve, Gram-negative; G+ve, Gram-positive; HEK293, human embryonic kidney 293; RBC, Red Blood Cell; RC, Random Coil.*

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Author contribution: *IAE, AGE, MAC and MATB conceived the study. IAE, AGE and AMK performed the experiments and analysed the data. IAE wrote the paper with input from all authors. MAC oversaw the research program.*

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