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In vitro activities of a novel antimicrobial peptide isolated from *phyllomedusa tomopterna*

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Abstract:

Because of the abuse of antibiotics, clinical strains began to become more drug-resistant. Their evolution has long surpassed the speed of us looking for a new generation of antibacterial drugs. Therefore, it is urgent to discover a new antimicrobial substance to alleviate the pressure on conventional antibiotics. Antimicrobial peptides (AMP) are known for their significant activity towards a broad spectrum of bacteria, protozoa, yeasts, filamentous fungi. Here, we report a novel AMP named Dermaseptin-TO. Results demonstrate that Dermaseptin-TO can quickly exhibit antimicrobial activity to bacteria and yeast in a dose-related way. The highest minimum inhibitory concentration (MIC) was observed in the *E.faecalis* group (128 μ M). Also, haemolytic outcomes showed no more than 10.65 % of red blood cells were affected when in the same concentrations or below. Besides, Dermaseptin-TO also showed anticancer activity at a higher concentration. From the above, evidence proved that Phyllomedusine frog skin secretion is still a rich source that contains novel AMP and Dermaseptin-TO is competent to become an antimicrobial agent, its anticancer activity may broaden the way in basic cancer research. Also, following the same templates in molecular cloning may acquire new AMP classes with potent antimicrobial effects that could widen drug design in new anti-infective drugs.

Keywords:

Amphibian; Dermaseptins; Antimicrobial peptides; Anticancer peptides; Molecular cloning

Introduction

A severe problem in public health is the decreasing drug effect, which has led to bacterial resistance and heavy losses in human health and the agriculture industry [1]. Besides, discovering a new antibiotic drug is no longer as easy as it was in past decades, and neither is their efficacy [2,3]. According to previous researches, the skin secretions of anurans are a great source of AMPs. They are integral compounds that can naturally synthesize, reserve and release to protect once the host was stimulated. AMPs are regarded as one of the most hopeful candidates and innovative treatments of amphibian skin, known not only for their significant activity towards a broad spectrum of Gram-positive and Gram-negative bacteria as well as protozoan parasites [4], yeasts and filamentous fungi [5] but also for multiple pharmacological functions such as anticancer activity, antiviral activity or immunomodulatory activity [6] (shown in AMPs database <http://aps.unmc.edu/AP/main.php>). Previous scholars have illustrated some standard features of structure/activity. Structurally, AMPs are approximately composed of 12-50 amino acids. Most of them carry positive charges provided by arginine (R), lysine (K) and form secondary structures with alpha-helix, beta-sheet or coil [7]. The mechanism of antimicrobial action of AMPs that have been most well-illustrated was the α -helix structure with cations peptides in the barrel-stave model and carpet model. Electrostatic attraction between cationic AMPs and the negatively charged bacterial/cancerous membrane surface is critical for the first interaction [6]. After binding to lipid layers, AMPs will either disrupt the biological integrity of cells membranes or other intracellular targets like mitochondria that cause cell death [6,8]. This step is accomplished by structural transformation rather than gruffly disrupt the membrane. In

addition to the direct interaction of AMP with the membrane, other modes will also influence the macromolecular synthesis, intracellular translocation of the peptide, and DNA/RNA/protein synthesis [9], which are also fatal to microbes [10,11].

Furthermore, classifications of AMPs are more than that based on physicochemical properties, and they also consider original genes of species. Dermaseptins is one of the prototypical members in AMP families, mainly extracted from Agalychnis and Phyllomedusa family frog skin secretion and are characterized by a conserved Tryptophan (Trp) residue at position 3, highly repeated gene duplications ‘AA(A/G)KAAL(G/N)’ in the mid-region, focal hypermutations in mature peptide sequences and central or C-terminal region [12,13,14]. Most Dermaseptins are linear polycationic peptides and 24–34 amino acids in length. They have a two-separated hydrophobic face and a positively-charged electrostatic surface, formed in an alpha-helix structure in the nonpolar solution, connect with lipid bilayers by coil-to-helix transition [4,13]. They can exhibit a rapid and cidal lytic activity *in vitro* towards a broad spectrum of bacterial species and even cancerous cells [4,15]. As such, AMPs have captured much attention in clinical drug design owing to its multiple pharmacological functions [16,17]. Most AMPs under clinical trials are human-derived and specifically designed for topical use based on the antimicrobial activity and the ability to penetrate *stratum corneum*, which can further prevent polymicrobial infection of wounds on skin or tissue, inflammatory acne vulgaris, and others [9,10,17].

In this study, a novel peptide, named Dermaseptin-TO according to the consistent nomenclature of Mohamed [18], was successfully extracted from a rarely studied frog

Tiger-striped Leaf Frog, *Phyllomedusa tomopterna*, by ‘shotgun cloning’ and synthesized by solid-phase peptide synthesis (SPPS). It displayed potent bacteriostatic and bactericidal activities against Gram-positive, Gram-negative bacteria and yeast immediately after contacting them at the thresholds. The antimicrobial activity is particularly potent *Staphylococcus aureus* (*S.aureus*). Thus, *methicillin-resistant staphylococcus aureus* (*MRSA*) was further used to test the antimicrobial activity, and results indicated that Dermaseptin-TO can inhibit the growth of them within minutes. Besides, Dermaseptin-TO also works on cancer cells without harmful damage to red blood cells. In conclusion, in the in vitro experiments, we found a suitable concentration range in which Dermaseptin-TO could inhibit/kill the bacteria with relatively low haemolysis, and report bioactivities of the novel frog skin-derived antimicrobial peptide which belongs to the Dermaseptins family. Based on its antimicrobial and anticancer properties, it is reasonable to say that Dermaseptin-TO can be a potential antimicrobial drug substitute or strategy in early-stage cancers in the future.

Materials and methods

Acquisition of specimen and skin secretion

Specimens of tiger-striped Leaf Frog, *Phyllomedusa tomopterna* (n=3, both sexes, 4-6 cm), originally from upper Amazon Basin in Colombia, Ecuador, Peru, and Bolivia, were bought from a commercial source in America and were settled in Queens University Belfast Animal Facility. All frogs were adults, maintained in a similar living environment which included 12 h of light 12 h of dark cycles at 18 to 25 °C for at least

4 weeks before extracting skin secretions, and during these time, frogs were fed with crickets three times weekly. After their adaptation, the skin secretion was obtained by mild transdermal electrical stimulation (5V; 3 ms pulses) for 30 s. Secretions were collected by washing from the skin with deionized water (ddH₂O), snap-frozen with liquid nitrogen and lyophilized. After that, the products were stored at -20 °C prior to use. The study was performed according to the guidelines in the UK Animal (Scientific Procedures) Act 1986, project license PPL 2694, issued by the Department of Health, Social Services and Public Safety, Northern Ireland. Procedures had been vetted by the IACUC of Queen's University Belfast, and approved on 1st March, 2011.

“Shotgun” cloning for a novel Dermaseptin-like peptide from *Phyllomedusa tomopterna* skin cDNA library.

Five milligrams of lyophilized *P.tomopterna* skin secretion were dissolved in 1 ml of cell lysis/binding buffer. Magnetic oligo-dT beads were used to isolate polyadenylated mRNA (DynaL Biotech, Liverpool, UK). Then mRNA was subjected to 3'-RACE PCR to obtain the full length of the prepropeptide nucleic acid sequences data by SMART-RACE kit (Clontech, Palo Alto, CA, USA) essentially as described according to the manufacturer. Briefly, the 3'-RACE reactions employed a Nested Universal Primer (NUP) (5'-AAGCAGTGGTATCAACGCAGAGT-3', supplied with the kit) and degenerate sense primer (CCI YTI YTI GCI GGI YT, named PA-S), they were designed based on highly conserved segments of the signal peptides of cDNAs cloned previously from other *Phyllomedusa* frogs within our group. The 3'-RACE

reactions were first purified by gel-electrophoresis, cloned by using a pGEM-T vector system (Promega Corporation, Southampton, UK) and then sequenced by an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). Sequence analysis was carried out with the NCBI-BLAST.

Peptide purification, identification and Structural Analysis of Dermaseptin-TO

Five milligrams of lyophilised skin secretion from *Phyllomedusa tomopterna* were dissolved in 1 ml of trifluoroacetic acid (TFA) /water (0.05/99.95, v/v) and clarified by centrifugation (5000 x g, 10 min). The clear supernatants were injected into an analytical reverse-phase HPLC column (C-18, 300A, 5 µm, 4.6 mm x 250nm; Phenomenex, Cheshire, UK). The elution gradient of the HPLC column was formed from 0.05/99.5 (v/v) TFA/water to 0.05/19.95/80.0 (v/v/v) TFA/water/ acetonitrile in 80 minutes at a flow rate of 4 mL/min and the effluent was detected by UV absorbance at 214 nm. A Cecil CE4200 Adept gradient reverse phase HPLC (Cecil, Cambridge, UK) was used and the collection of every fraction was automatically recorded at the interval. All fractions were interrogated by matrix-assisted laser desorption ionisation time-of-flight (MALDI-TOF) mass spectrometry (Perseptive Biosystems, MA, USA) in positive detection mode and assisted with alpha-cyano-4-hydroxycinnamic acid (CHCA) used as matrix. The MALDI-TOF spectrum was analysed by Thermo Scientific Proteome Discoverer 1.0 software (Thermo Fisher Scientific, SanJose, CA, USA).

Solid-phase peptide synthesis of Dermaseptin-TO

The peptide was chemically synthesized by a Tribute™ 2-channel peptide synthesizer (Protein Technologies, Tucson, AZ, USA). All the amino acids were weighed and mixed with 2-(1h-Benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activator in each reaction vials. Later, the reaction vessel with resin was loaded, and amino acid vials needed to be loaded onto the carousel in the order from C'-terminal to N'-terminal end. Afterwards, the coupling programme for the amino acids, reaction vessel and the start and stop position on the carousel were chosen. At last, the peptide in 'ALWKDLLKNVGIAAGKAALNKVTDMVNQ-NH₂' sequence was synthesized. The confirmation of synthetic peptide replicates was then purified and identified by both reverse-phase HPLC and MALDI-TOF mass spectrometry prior to use.

Antimicrobial Assays

Antimicrobial assays were required when evaluating the antimicrobial effect of the peptide by identifying the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in Gram-positive bacteria *Staphylococcus aureus* (*S.aureus*, NCTC 10788), *Enterococcus faecalis* (*E.faecalis*, NCTC 12697) and *Methicillin-Resistant Staphylococcus Aureus* (MRSA, NCTC 12493). The gram-negative bacterium, *Escherichia coli* (*E.coli*, NCTC 10418) and the yeast, *Candida albicans* (*C.albicans*, NCPF 1467). The peptide was prepared in a gradient concentration from 1 to 512 µM and microorganism was set in Mueller-Hinton broth

(MHB) for 18 h to grow. Then the peptide solutions with microorganism cultures were placed into 96-well microtiter cell culture plates. After an 18 h incubation at 37 °C in the incubator, the MIC determination was confirmed by optical density (OD) by using a Synergy HT plate reader (BioTel, USA) at $\lambda=550$ nm. The minimum concentration with clear wells was considered as the minimum inhibitory concentration and a graph was made and processed by GraphPad Prism 6.0 software.

The viability of each organism was calculated by the following formula:

$$\text{Viability (\%)} = (X - X_0) / (X_v - X_0) \times 100 \%$$

X is the absorbance of each well in sample groups, X_0 is the average absorbance of the blank control group, while X_v is the average absorbance of the vehicle control group.

In this assay, Growth control groups were made by 100 μ l diluted organism suspension; Vehicle control group were made by 1 μ l DMSO + 99 μ l diluted organism suspension; Blank control groups were made by 100 μ l sterile MHB; Positive groups were made by 1 μ l 2 % Norfloxacin + 99 μ l diluted organism suspension (For *E. coli*, *S.aureus*, *MRSA* and *E.faecalis*) / 1 μ l 200 μ M Melittin + 99 μ l diluted organism suspension (For *C.albicans*) and Sample groups were made by 1 μ l peptide solution + 99 μ l diluted organism suspension.

Subsequently, wells with a clear solution were selected. Each well in the same concentration was pipetted before 10 μ l of the solution was dropped onto the MHA plate and dried. After that, the plates were incubated in the incubator for 18 h at 37 °C. Finally, the minimum concentration with no organism growth was the minimum bactericidal concentration.

Kinetic time-killing Assays

Kinetic time-killing assays were performed with peptide solutions in two concentrations, one required MIC value and another needed quadruple MIC value. This assay is set to observe the bactericidal activity of Dermaseptin-TO. At first, 10 µl bacterium was cultured in 100 ml MHB at 37°C and the inoculum was adjusted by optical density. After bacterial suspension reached 5×10^5 CFU/ml, 10 µl peptide concentration series were each added with 990 µl bacterial inoculum to 1.5 ml centrifuge tubes. Test samples were removed from each culture tube and diluted before being dropped onto an MHA plate during the incubation at 0, 5, 10, 15, 30, 60, 90, 120, 180 min, respectively. Then the MHA plates were put in an incubator at 37°C for 24 h for colony counts. For every colony count at different concentrations, they were firstly counted for one dilution that calculated a countable range of 3-100 colonies and the CFU/ml was then obtained.

Haemolysis Assays

Two-millilitre horse red blood cells (TCS Biosciences Ltd, Buckingham, UK) was mixed with sodium phosphate-buffered saline (PBS) and placed on a rotating mixer (Eppendorf, Germany) to wash several times in a new 50 ml test tube until the supernatant became colourless and transparent. Then the blood suspension samples were mixed with a series of peptide gradient concentrations and incubated at 37 °C for 60 min, 120 min. After that, each tube was centrifuged and the supernatant was

transferred to the former 96-well plate and read by an ELISA plate reader (BioLise BioTek EL808, Winooski, VT, USA) at a wavelength of 550 nm and a graph was made and processed by GraphPad Prism 6.0 software, the results were considered to be statistically significant if the *p*-value was < 0.05.

The haemolysis (%) rate was calculated by the following formula:

$$\text{Haemolysis rate (\%)} = (X - X_0) / (X_P - X_0) \times 100 \%$$

X is the absorbance of each well in sample groups, X_0 is the average absorbance of the vehicle control group, while X_P is the average absorbance of the positive control group.

In this assay, Positive groups contain 200 μ l 2% Triton X-100 + 200 μ l erythrocytes; Vehicle groups contain 200 μ l 1% DMSO in PBS + 200 μ l erythrocytes and Sample groups contain 200 μ l peptide solution + 200 μ l erythrocytes.

Cell lines and cell culture

Human neuropongioma cancer cell line U251-MG (ECACC General Cell Collection: 09063001), human lung cancer cell line H157 (ATCC-CRL-5802) and human prostate cancer cell line PC-3 (ATCC-CRL-1435) were separately cultured in RPMI-1640 culture medium (Invitrogen, Paisley, UK) or Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, St. Louis, MO, USA) in 15 ml medium containing 10 % fetal bovine serum (FBS) (Sigma-Aldrich, USA) and penicillin-streptomycin (PS) (penicillin 100 Units/ml and streptomycin 100 μ g/ml) (Sigma-Aldrich, USA) in advance. Then they were placed into the incubator at 37 °C with 5% CO₂.

Anticancer activity assessment by MTT assays

The peptide was diluted into a concentration gradient from 10^{-4} M to 10^{-8} by 10-fold dilutions with the same medium in culture but with no FBS. Then around five thousand cells were seeded in each well in 100 μ l medium without FBS for starvation for 6 h before dosing. Later, the medium in wells was removed, 100 μ l of peptide solution in different concentrations were loaded and each concentration required five replications in a row. The components of each group were shown in Table 2.3. Then, the plate was incubated at 37 °C with 5 % CO₂ for 24 h. Next, 10 μ l of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added in each well and incubated for another 4 h before measuring the optical density (OD) values by a synergy HT plate reader (BioTek, winnoski, VT, USA) at 570 nm and the viability of cells was calculated according to the viability formula. Graphs were processed by GraphPad Prism 6.0 software.

Cancer cell viability (%) was calculated by the following formula:

$$\text{Viability (\%)} = (X - X_0) / (X_v - X_0) \times 100 \%$$

X is the absorbance of each well in sample groups, X_0 is the average absorbance of the blank control group, while X_v is the average absorbance of the vehicle control group.

In this text, the components of Blank groups were 100 μ l starvation medium; Vehicle group were 1 μ l DMSO + 99 μ l starvation medium; Positive groups were 1 μ l Triton X-100 + 99 μ l starvation medium; Growth groups were 100 μ l starvation medium and Sample groups were 100 μ l peptide solution with starvation medium.

Results

‘Shotgun’ Cloning of Dermaseptin-TO Precursor-Encoding cDNAs from Skin Secretion-Derived cDNA Libraries of *Phyllomedusa tomopterna*

The biosynthetic precursor cDNA encoding peptide Dermaseptin-TO was successfully cloned from the tiger-striped leaf frog skin secretion from the cDNA library of *Phyllomedusa tomopterna* by using a Phyllomedusine-specific degenerate primer according to their highly conserved signal region from previously sequences obtained from other Phyllomedusine frogs. The sequence was obtained by Sanger sequencing, translated by ExPASy and analyzed by the NCBI BLAST program. The open reading frame consisted of three parts: a signal peptide region, a spacer domain, and a mature peptide region, shown in Figure 3.1. A 22-residue putative signal peptide domain was found at the *N*-terminus of the reading frame; after a 23-residue acidic spacer domain following the putative mature peptide sequence, the symbol amino acids ‘KR’ (-Lys-Arg) suggested a typical cleavage site; mature peptide sequence contains of 28 amino acids. An extra Gly (glycine) at the end of the mature peptide sequence served as an amide donor for the COOH-terminal. These characteristics from BLAST analyses showed that Dermaseptin-TO exhibits a high degree of similarity in structural homology to those peptides in the Dermaseptin family (shown in Table 3.1), including Dermaseptin-L, Dermaseptin-2 (Also named Dermaseptin-B4) and Dermaseptin-PS2. Different residues are marked with a dot mark at the bottom, and per cent identities were carried out by comparing with Dermaseptin-TO. The E-value indicates the

probability that other sequences are more similar to the target sequence than the displayed sequence in a random situation, the lower the score, the higher the credibility. The alignments and specific bioactive information of those amino acid sequences were found in the database (<http://splitbioinf.pmfst.hr/dadp/?a=list>).

Table 3.1 Alignment of cDNA deduced mature Dermaseptin-TO sequence with similar Dermaseptin family peptides.

Sequence	Name	Species	Percent Identity	E-value
ALWKTLLKNVGKAAGKAA LNAVTDMMVNQ-NH ₂	Dermaseptin-L	<i>Phyllomedusa distincta</i>	89.29%	7e-17
ALWKDILKNVGKAAGKAV LNTVTDMVNQ-NH ₂	Dermaseptin-2	<i>Phyllomedusa tarsius</i>	88.89%	2e-17
ALWKTLLKNVGKAAGKAV LNAVTDMMVNQ-NH ₂	Dermaseptin-PS2	<i>Phyllomedusa sauvagii</i>	86.00%	7e-12
ALWKDLLKNVGIAAGKAAL NKVTDMVNQ-NH ₂	Dermaseptin-TO	<i>Phyllomedusa tomopterna</i>		

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      M A F L K K S L F I V L F L G L V
1  ATGGCGTTCC TGAAGAAATC TCTTTTCATT GTACTATTCC TTGGACTTGT
   TACCGCAAGG ACTTCTTTAG AGAAAAGTAA CATGATAAGG AACCTGAACA
   S L S I C E E E K R E T E D E E K
51 CTCTCTTTCT ATCTGTGAAG AAGAGAAAAG AGAGACTGAA GATGAGGAGA
   GAGAGAAAAG TAGACACTTC TTCTCTTTTC TCTCTGACTT CTACTCCTCT
   Q E D D E Q S E E K R A L W K D
101 AACAGAAGA TGATGAGCAA AGTGAAGAGA AGAGAGCTCT GTGGAAAGAC
   TTGTTCTTCT ACTACTCGTT TCACTTCTCT TCTCTCGAGA CACCTTTCTG
   L L K N V G I A A G K A A L N A V
151 TTATTAAAAA ATGTAGGGAT AGCTGCAGGA AAAGCGGCGT TAAATGCAGT
   AATAATTTTT TACATCCCTA TCGACGTCCT TTTCGCCGCA ATTTACGTCA
   T D M V N Q G E Q *
201 TACTGATATG GTAAATCAAG GAGAGCAATA AAGTTAAGAA AATGGTAAAT
   ATGACTATAC CATTTAGTTC CTCTCGTTAT TTCAATTCTT TTACCATTTA
251 CAAATTGCTC CAAGGAGTGC AATTATCCAA TAATTGTACA AACTTATATT
   GTTTAACGAG GTTCCTCACG TTAATAGGTT ATTAACATGT TTGAATATAA
301 AAAGCATATT GAACATAAAA AAAAAAAGAA AAAAAAAGAA
   TTTCGTATAA CTTGTATTTT TTTTTCCTT TTTTTTTTTT

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Figure 3.1 Nucleotide and translated open-reading frame amino acid sequence of cloned cDNA encoding the precursor of the novel peptide, Dermaseptin-TO. The putative signal peptide sequence is double underlined, the mature peptide sequence is

single underlined, and an asterisk marks the translation stop codon.

Secondary structure prediction

HeliQuest was used to obtain further information, including the data from Figure 3.2 (a), which could provide peptide physicochemical properties and polar, nonpolar residues analysis; the helical wheel plot in Figure 3.3 shows the putative distribution of amino acids in secondary structure. Peptide2 online analysis tool was used to get a specific hydrophobicity percentage in Figure 3.2 (b). Moreover, the Zhanglab online website, which could give a predicted structural characteristic of secondary structure, is shown in Figure 3.2 (c). Through these tools, the results showed that Dermaseptin-TO carried +2 net charge and the main proportion of putative secondary structure exhibited in α -helix.

Helical wheel diagrams of Dermaseptin-TO and its similar peptides Dermaseptin-L (DRS-L), Dermaseptin-2 (DRS-2) and Dermaseptin-PS2 (DRS-PS2), showed the same direction of hydrophobic moment. Comparing these AMPs to Dermaseptin-TO, one significant difference is the presence of two hydrophilic residues Threonine (Thr) in the out surface, resulting in a higher amphiphilicity; and the other is the Lysine (Lys) in position 12 creating a more pronounced hydrophilic and hydrophobic separation surface. In addition, the absence of an Aspartic acid (Asp) amino acid in DRS-L and DRS-PS2 increases their charge to +3.

Figure 3.2 Structural parameters of Dermaseptin-TO by the online analysis tool HeliQuest, Peptide2, and Zhanglab.

HeliQuest (<http://heliquet.ipmc.cnrs.fr/>) for (a).

Peptide2(https://www.peptide2.com/N_peptide_hydrophobicity_hydrophilicity.php) for (b).

Zhanglab (<https://zhanglab.ccmb.med.umich.edu/>) for (c).

Figure 3.3 Helical wheel diagrams of Dermaseptin-L (a), Dermaseptin-2 (b), Dermaseptin-PS2 (c), Dermaseptin-TO (d). Blue circles represent the positively-charged amino acids and red circles represent the negatively-charged amino acids. Neutral hydrophobic residues are in yellow, the hydrophilic are in purple, the amide are in pink and other small residues are in grey. Red circle represents the position 12. Arrows in the figure indicate the direction of the hydrophobic moments.

Isolation and structural characterization of Dermaseptin-TO

The lyophilized crude synthetic Dermaseptin-TO was purified by RP-HPLC, as shown in the upper part of Figure 3.4. Fractions containing different natural components were collected every minute. MALDI-TOF MS analyzed each of them, and the fraction with a mass coincident with the approximate predicted molecular mass of Dermaseptin-TO was detected among all fractions. The arrow in the figure indicates

the retention time of Dermaseptin-TO, and the MALDI-TOF MS analysis of this position is shown in the lower part of Figure 3.4. The observed molecular weight of Dermaseptin-TO was 2981.4 Da, which was consistent with the predicted molecular weight of 2981.52 Da.

Figure 3.4 (a) RP-HPLC chromatogram of crude synthetic Dermaseptin-TO. The abscissa was ‘retention time (min)’, and the ordinate was ‘Absorbance (mA)’. The peak with the arrow indicates the position containing the purified Dermaseptin-TO. The retention time of Dermaseptin-TO was at 57 min. The detection wavelength was 214 nm with a flow rate of 4 ml/min in 80 min.

(b) MALDI-TOF spectrum of synthetic peptide Dermaseptin-TO after RP- HPLC. The highest peak had the mass-to-charge (m/z) ratio of Dermaseptin-TO.

Antimicrobial activity and haemolysis of Dermaseptin-TO

The effects of Dermaseptin-TO on the inhibition of microorganisms are illustrated

in Table 3.2 and Figure 3.5. The gram-positive bacterium, *S. aureus*, *MRSA*, *E. faecalis*, Gram-negative bacterium, *E. coli*, and the pathogenic yeast, *C. albicans*, were participated. The results showed Dermaseptin-TO exhibited MIC at 4, 64, 128, 8 and 32 μ M respectively, and minimum bactericidal concentration (MBC) at 8, >512, 256, 8, >512 μ M respectively, and indicated the relative antimicrobial potency of Dermaseptin-TO, Gram-positive bacteria > Gram-negative bacteria > yeast, is a typical pattern for many amphibian skin AMPs. Besides, Dermaseptin-TO was specifically effective to *S. aureus* as it provided the lowest inhibit concentration.

As for haemolytic activity, red blood cells were used to test the haemolysis. Dermaseptin-TO possessed a relatively low level of haemolytic activity, which can be considered as a low cytotoxicity peptide. From Table 3.2, the highest MIC value was showed in the *E. faecalis* group (128 μ M), and there were only 10.65 % at most of the red blood cells were influenced when at the same concentration.

Table 3.2 The MICs, MBCs and HC₅₀ of Dermaseptin-TO and its similar peptides.

Peptides	DRS-TO	DRS-L	DRS-2	DRS-PS2
	MIC/MBC (μM)			
Gram-positive				
<i>S. aureus</i>	4/8	1.3/NG ¹	3.0/NG ¹	8/16
<i>MRSA</i>	64/>512	NG ¹	NG ¹	16/32
<i>E. faecalis</i>	128/256	10/NG ¹	NG ¹	NG ¹
Gram-negative				
<i>E.coli</i>	8/8	2.5/NG ¹	5.0/NG ¹	8/16

Yeast

<i>C.albicans</i>	32/>512	NG ¹	NG ¹	16/32
<hr/>				
	HC ₅₀ ² (μM)			
Red blood cells	436.7	NG ¹	NG ¹	210.7

¹NG, NOT GIVEN. The article or research did not provide the corresponding data of the peptide.

²The peptides concentration causing 50% haemolysis of the red bloods cells.

Figure 3.5 Antimicrobial function of Dermaseptin-TO against the growth of *S. aureus* (a), *E. faecalis* (b), *E. coli* (c), *C. albicans* (d), *MRSA* (e) in different peptide concentrations. The horizontal axis is peptide concentration (μM) while the ordinate axis is the viability of each bacterium (%). The graph was made according to the repetitive times (represent by letter n) on the right top side. While, in the haemolysis activity of Dermaseptin-TO on horse red blood cells (f), the horizontal axis is peptide concentration (μM) and the ordinate axis is haemolysis rate (%). The positive control was 1% Triton X-100 lysis buffer which shows the haemolysis and defined as 100 % haemolysis. Data represent the mean and the standard deviation of nine replicate

experiments.

Kinetic time-killing assays of Dermaseptin-TO towards *S.aureus*

In the time-killing assay, the most sensitive strain was used to perform a precise result. Thus, the *S.aureus* group chosen to observe the potency of the antimicrobial activity of Dermaseptin-TO as it gave the minimum inhibit concentration value. From Figure 3.6, Dermaseptin-TO mainly exerted its killing effect at around first 10 min; at the beginning, the bactericidal potency of the 16 μM concentration group was almost four times that of the 4 μM concentration group; after 60 min, compared to the 4 μM group, few bacteria were alive in the peptide concentration of 16 μM . Based on outcomes, the antimicrobial activity of Dermaseptin-TO took effect immediately, and its potency was positively related to the concentration.

Figure 3.6 Antimicrobial function of Dermaseptin-TO against the growth of *S.aureus*. The horizontal axis is the time that peptide interacted with the bacterium (min) while the ordinate axis is the approximate amount of bacterium alive (CFU). The graph was made according to the repetitive times (represent by letter n) on the right top side.

Anticancer activity of Dermaseptin-TO

In vitro antiproliferative studies on human cancer cell lines were tested by U251MG, H157 and PC-3 cancer cells. Results suggested that at the concentration of 10^{-4} M and above, Dermaseptin-TO could inhibit the growth of these three types of cancer cells (Figure 3.7), and their viability was significantly decreased. The results were expressed with cancer cell viability (%) and different peptide solution concentrations from 10^{-4} M to 10^{-8} M. Each column was based on three independent assays. In each assay, there were five separate wells for each peptide group. Data in different concentrations were compared with the vehicle control group. The column with asterisks suggested a significant difference after comparison.

Figure 3.7 The effect of Dermaseptin-TO against U251MG (a), H157 (b), PC-3 (c) cancer cells in different concentrations. The horizontal axis is log peptide concentration (M) while the ordinate axis is the viability of each cancer cell (%). The graph was made according to the repetitive times (represent by letter n) on the right top side. The positive control was 1 % Triton X-100 lysis buffer and the vehicle control was 1 % DMSO dissolved in the medium. Data represent the mean and the standard deviation of fifteen replicate experiments. The asterisks on the bars represent a significant difference compared to the Growth Control group (**** means $p < 0.0001$, * means $p < 0.1$).

Discussion

Dissemination of resistant microbes has led to significant morbidity and mortality that have placed pressure on health systems and medical costs, reflecting the lack of progress in developing new antimicrobials. According to previous studies,

phyllomesusine frog skin secretions are rich in bioactive peptides, molecule compounds. The relative diversity may be due to various isomeric forms of AMP, which shows high identity in primary structure and then constructed a structurally divergent AMP families. AMP is the most representative substance in frog skin secretions found with remarkable antimicrobial activity towards a broad spectrum of bacteria. Their unique membrane disruptive effects are unlikely to stimulate bacterial resistance and thus are considered an alternative.

Here, we described the identification and characterisation of a novel AMP, named Dermaseptin-TO from frog *phyllomedusa tomopterna*. In classification, this 28 amino acid residues belonging to Dermaseptins. Compared to other peptides in the Dermaseptin family, it showed a high similarity to others in the C-terminal amidation, repeatable amino acid sequence region, and identical motif, ALWKXX(L/I)L [19] and AA(A/G)KAAL(G/N). Although peptides in Dermaseptins showed a high homology level, they still differ in biological properties [20]. After careful screening, DRS-L [21], DRS-2 [22] and DRS-PS2 [23] are similar peptides to Dermaseptin-TO based on their reported bio-activity studies. They all have antimicrobial activity against *E.coli* and *S.aureus*; The stronger bactericidal activity of DRS-L and DRS-PS2 towards *E.faecalis*, *C.albicans* and *MRSA* which may be most relevant to an additional positive charge. Unfortunately, the more substantial antimicrobial power also results in a more robust increase in haemolysis of erythrocytes. In addition, DRS-2 is an insulin-releasing-peptide while Dermaseptin-TO was not. Therefore, it is unsurprising that Dermaseptin-TO can inhibit the growth of *S.aureus* and *E.coli*. Nevertheless, in this test, its tested

spectrum is broader than those and showed effects against drug-resistant strain as well as cancerous cells with less harm to mammalian cells. The intensity of the antimicrobial activity of Dermaseptin-TO was proved by the time-killing assay on *S.aureus*, which is the most straightforward bacterial strain for Dermaseptin-TO to inhibit and eradicate. After quadrupling the MIC to 16 μ M, the number of bacteria at the start point decreased significantly, suggested the antimicrobial activity of Dermaseptin-TO is quick and powerful. According to the helical wheel projection, the variability of results may be attributable to slight changes in the kind of amino acid which might regulate the degree of biological activity; charge seems to be one of the decisive parameters that control the antimicrobial potency. Position 12, which we circled in red in Figure 3.3, seems to be a decisive point, managing the degree of bioactivity that bridging the charges from two parts. In Dermaseptin-TO, Lys (12) was replaced by Ile (12), separate the charge and hydrophilic/hydrophobic surface, which marginally weaker the antimicrobial activity. It also proves that Dermaseptins with a less distinct separation between hydrophobic and hydrophilic surfaces still have their family bioactive characteristics. Another hypothesis is based on the membrane targeting mechanism of AMPs. Also, because Dermaseptins usually form the helical structure at the *N*-terminal domain when interacting with lipids. Therefore, it is reasonable that charges aggregate on the same side and the amphipathcity and terminal helix may together influence the membrane-binding ability.

AMP works in multiple mechanisms; most researchers attribute the function to the membrane permeabilization, and infer AMPs are membrane-targeted. So, it is

reasonable to say that AMPs are more specifically useful towards bacteria unless their mutations are membrane related. Some researchers also hold that the interaction with intracellular compartments is equally important; that is why AMPs cannot exhibit the same bactericidal potent on drug-resistant bacteria. Second structure transformation was another essential factor that determines the activity of AMPs. According to results, Dermaseptin-TO has a typical antimicrobial structure; most of its secondary structure is the helix, and the coil structure can only be found at the beginning and the end. 53.57% hydrophobicity and two positive charges in a neutral environment. Consequently, cationic Dermaseptin-TO will bind the negatively-charged bacterial/cancer cell membrane through electrostatic interaction. After attachment, it will perforate/disruptive the cell wall/membrane via secondary structure transformation from coil to helix [4] and then affect intracellular synthesis by targeting cytoplasm/enzymes. This action may last a short time (within minutes). These instincts all guarantee Dermaseptin-TO a bioactive peptide, and preliminary observations may also provide fundamental data for inspiring more in-depth research in Dermaseptin-TO in terms of drug design. AMPs are not invincible and irresistible; some bacteria like *MRSA*, are obstinate because Dermaseptin-TO cannot fully eradicate them; *E.faecalis* is another intractable bacterium, to control them, the peptide solution was configured to the highest concentration (128 μ M) among others. *MRSA* evolved from *S. aureus*, and their virulence genes are mobile, leading to distinct differences between follows that are hard for traditional antibiotics to target at a specific channel [24,25]. Besides, *MRSA* is prone to different responses when faced with different stressful conditions.

Thus, the absence of bactericidal activity of Dermaseptin-TO to *MRSA* still needs further studies to confirm. *E.faecalis* are resilient as they can survive under hostile conditions, which requires microbes a similar metabolism to adapt to the surroundings. We supposed it is the high adaptability with hosts that help them escape from AMPs because these peptides are initially innate immune effectors [26]. Just like most cationic AMPs that preferably form large hollow polymers on the membrane. In turn, *E.faecalis* also evolve particular mutations for self-protection [27]. Firstly, charges on their surfaces are neutralised (moderate resistance) or even become cationic (higher resistance); secondly, the composition of fatty acids in the lipid bilayer are alternated. Also, their branched-chain level decreased. Both of them can reduce the probability of penetration and prevent further damage like pore-formation from AMPs. Haemolysis is regarded as a side effect when AMPs are applied in clinical use, and it is a commonness for many amphibian skin-derived AMPs. However, this can be solved by designing structural analogues, like this Dermaseptin-TO, at the concentration of 4 to 128 μ M, the haemolysis rate was under 10.65 % but still exhibited antimicrobial activity.

Although these results may endow Dermaseptin-TO a light future application, strategies that may lead to mutation under selective pressure should be more critically treated for drug design and application *in vivo*. Nowadays, some bacteria are vancomycin-resistant, which means they have evolved one step ahead of us and may cause more severe diseases/losses that we cannot deal. Therefore, we report the Dermaseptin-TO, not only for its potential to become a new candidate for bacterial diseases owing to its unique membrane-destructive activity but also to determine a

relative safety concentration range for instruction. With the help of Dermaseptin-TO, we believe it may improve clinical treatment effects and be used in other relating therapies.

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