MASTER OF PHILOSOPHY

Identification and bioactivity evaluation of a novel peptide from the skin secretion of Pelophylax kl. esculentus

Li, Yao

Award date:
2017

Awarding institution:
Queen's University Belfast

Identification and bioactivity evaluation of a novel peptide from the skin secretion of *Pelophylax kl. esculentus*

Yao Li B. A

School of Pharmacy, Faculty of Medicine, Health and Life Science

Queen's University, Belfast

A thesis submitted to Queen's University Belfast for the degree of Master of Philosophy (MPhil)

2017

Declaration

I declare that the research reported in this thesis is my own work except where acknowledgement has been made. All work was carried out in Molecular Therapeutics Research, School of Pharmacy, Faculty of Medicine, Health and Life Science, Queen's University, Belfast.

I hereby declare that for 2 years following the date on which the thesis is deposited in the Library of Queen's University, Belfast, the thesis shall remain confidential with access or copying prohibited. Following expiry of this period I permit the librarian of the University to allow the thesis to be copied in whole or in part without reference to me on the understanding that such authority applies to the provision of single copies made for study purposes or for inclusion within the stock of another library. This restriction does not apply to the British Library Thesis Service. IT IS A CONDITION OF USE OF THIS THESIS THAT ANYONE WHO CONSULTS IT MUST RECOGNISE THAT THE COPYRIGHT RESTS WITH THE AUTHOR AND THAT NO QUOTATION FROM THE THESIS AND NO INFORMATION DERIVED FROM IT MAY BE PUBLISHED UNLESS THE SOURCE IS PROPERLY ACKNOWLEDGED.

Acknowledgements

First and foremost, I give deep appreciation to my respected supervisors, Prof. Chris Shaw, Dr. Tianbao Chen and Dr. Lei Wang. Thanks to them for giving me the opportunity to study in this research area. Their enthusiasm for the research and precious ideas made a strong impression on me and will be to my lifelong benefit. Not only did they inspire me in the research area but also they gave me a lot of care in my life. Without their expert guidance, encouragement and support, I would not have completed these studies and dissertation successfully.

I would also like to show great gratitude to Dr. Chengbang Ma, Dr. Xinping Xi, Dr Mei Zhou, Dr. Lei Li and Dr. Yuxin Wu. In every step throughout the process, they gave me much assistance in my experimentation and gave much valuable advice in writing my dissertation.

I really want to thank my family and friends for their consistent support and encouragement. This dissertation stands as a testament to my family's unconditional love and dedication to me. Finally, I would like to thank the China Scholarship Council and Nanjing University of Chinese Medicine for their financial support.

Abstract

Species of frogs and toads have proven to be a rich source of drugs and contain various components with potential biological activities, especially in their skin or skin secretions. Among these, peptides are found with broad-spectrum antibacterial and antifungal activities, as well as anticancer properties. It is generally assumed that these cationic, amphipathic and α-helical peptides constitute a part of the innate immune system that protects the animal from invasion by pathogenic microorganisms.

In this thesis, a novel peptide (QUB-1342) was discovered in the skin secretion of the European edible frog, *Pelophylax kl. esculentus*. The precursor structure was found through applying a "shotgun" cloning method, and subsequently translating the cDNA to deduce the mature peptide sequence: FLPIVTGLLSGLL-NH_2. From comparison with the sequences of known AMPs, QUB-1342 was classified as a temporin family member. The peptide was chemically-synthesised using solid phase chemistry and afterwards, its molecular mass and purity were confirmed by MALDI-TOF mass spectrometry and reverse phase HPLC.

In pharmacological assays, QUB-1342 displayed a weak activity against *S. aureus*, with an MIC=64μM. It did not show any haemolytic effects at the concentrations employed. Further investigations are underway to seek more functions of the peptide and to examine the relationship between its structure and activity, and also to develop novel candidate drugs for the clinic.

Contents

Chapter 1. Introduction

1.1 Introduction to Anura

1.1.1 Overview

Anura, an order of Amphibia comprised of frogs and toads are widely distributed from the tropical to subarctic areas, but the species diversity is most concentrated in tropical rainforests.

The value of frogs has been discovered in various countries throughout time. For example, the exudate of the skin of *Phyllobates terribilis*, known as the golden poison arrow frog, is the traditional source of poison applied to arrows by natives hunting in Colombia's rainforest. Secretion from the skin glands and parotid glands of toads is also one of the traditional medicines in China, where it is widely-known for its common use as a medicinal treatment. The gland secretions have been proven to cure skin infections in the past, and more recently proven to function like antimicrobials, central nervous system stimulants and cardiotonics (Neerati et al., 2014), insulin-releasers (Marenah et al., 2004), and local anaesthetics, to name but a few. As part of the natural pharmacy, frog toxins are so diverse that they have attracted numerous biochemists to make earnest efforts to reveal other useful scientific applications.

1.1.2 *Pelophylax kl. esculentus*

Pelophylax kl. esculentus, also known as the edible frog, belongs to the family Ranidae, which are commonly referred to as “true frogs”. *Pelophylax kl. esculentus* is the fertile hybrid of *Pelophylax lessonae*, the pool frog and *Pelophylax ridibundus*, the marsh frog, and it can be reproduced by hybridogenesis. The literal meaning of the name *Pelophylax esculentus* means both "mud" and "guardian,” which reflects their living habits; they will seldom deviate far from water, like the conscientious guardians of the muddy banks around lakes, ponds and marshes. Adult edible frogs are mainly green, with pale brown patches scattered on their backs varying in size and number, with yellow eyes and a white underside, usually covered with a few dark spots[1] .

Figure 1.1 *Pelophylax kl. Esculentus*[1]

1.2 Antimicrobial peptides (AMPs)

Among the wide range of compounds secreted from frog skin are peptides with abundant bioactivities. Neuroactive and cytolytic peptides are the two major types of

[1] http://amphibiaweb.org/cgi/amphib_query?where-genus=Pelophylax&where-species=esculentus

peptides in the skin secretion of Anura. Cytolytic peptide is a more accurate term than simply antimicrobial peptides (AMPs), because in addition to fighting against microorganisms, they have also demonstrated effectiveness in anti-cancer, anti-viral, immunomodulatory, and anti-diabetic areas (Conlon et al., 2014). According to their biological functions and structural features, theses peptides are further classified into different families. Neuropeptides are classified as tachykinins, bradykinins, caeruleins, bombesins, opioids, tryptophyllins and miscellaneous peptides. Overall, most peptides of these six classes are often widely distributed across the investigated anuran species. By comparison, families of AMPs are usually found in several closely related frog species (König et al., 2015).

AMPs, also called host defense peptides (HDPs), belong to the innate immune response of life activities found among all living beings. There are six major AMPs sources: bacteria, fungi, amphibians, insects, plants, and mammals. The number of peptides with antimicrobial properties is reported to be significantly higher than that of all previously discussed bioactive peptides from numerous frog species. AMPs have been proven to have the ability of eliminating or inhibiting Gram negative and Gram positive bacteria, enveloped viruses, fungi and even transformed or cancerous cells. These peptides are strong, broad-spectrum antibiotics that show potential as novel therapeutic drugs (Conlon et al., 2014).

1.2.1 Structure-activity relationship of AMPs

The distinctive activities of AMPs are created by the uniqueness and diversity of their constitution and structure, which is generally sequences and folding. The number of constitutive amino acids in one peptide generally ranges from 12 to 50. To be cationic, the AMPs usually consist of multiple positively charged amino acids: arginine, lysine or histidine. Up till now, more than 500 cationic AMPs have been found in the Antimicrobial Sequences Database. Additionally, they are composed of a large proportion of hydrophobic amino acid residues, usually above 50% or more (Papagianni, 2003). There are four types of the secondary structures of most AMPs reported to date: β-sheet, α-helix, extended, and loop (Dhople et al., 2006). α-helical and β-sheet AMPs are the most commonly found in Nature and the most studied so far. The α-helix is a right-handed-coil with the intra-chain hydrogen bond formed between the amide hydrogen and the carbonyl oxygen on all peptide bonds. The best-known examples of such AMPs are protegrin, magainin, cyclic indolicin, and coiled indolicin (Huang et al., 2010). β-sheet peptides consist of at least two β-strands, connected by disulphide bonds (Bulet et al., 2004). Many peptides are not structured in free solution until distributed into a biological environment and then they form their final conformations. These features help AMPs to partition into the biological membranes. The ability to bind to the lipid bilayer membrane is also one of the definitive characteristics of AMPs.

Structure-activity relationships have been studied through analysis of certain amino acids in the sequence, combinational library studies, and connections between interactions with membranes. Currently it is believed that characteristics such as helicity, intrinsic hydrophobicity, hydrophobic moment and the size of the polar/hydrophobic domain and peptide charge, are associated with the antimicrobial ability as well as the side effect, haemolysis (Dathe and Wieprecht, 1999).

Helicity is influenced by several factors. Proline and glycine are known to block the formation of helical structure. The helicity of melittin is enhanced by deleting glycine from the N-terminal or replacing with leucine, and at the same time the antimicrobial and haemolytic activities are increased (Blondelle, et al., 1991). The same effect is present in the substitution by alanine. Inversely, substitution with proline preventing folding will reduce both effects by interrupting the helicity (Thennarasu and Nagaraj, 1996). Also through modification, haemolytic effects can be weakened. The change of L-amino acids to their corresponding D-amino acids will disturb helix formation, but with no influence on the original sequence, hydrophobicity and charge (Powers and Hancock, 2003). Replacement of two neighbouring residues in magainin further resulted in decreased helicity. However, the ability of permeability in negatively charged membranes is not affected, suggesting that helicity is more related to the interaction with membranes of neutral charge than to negatively charged bilayers (Dathe and Wieprecht, 1999).

The intrinsic hydrophobicity plays another key role in the peptide-membrane interaction. A moderate hydrophobicity is required. First, it should be soluble in aqueous solution in sufficient concentration to approach the target. Second, it should have affinity for the phospholipid bilayer to develop disruption. Therefore, if the hydrophobicity is too high or too low, it will decrease these activities. The higher may lead to self-association or precipitation, while the lower could result in weak antimicrobial effects. It seems that the hydrophobicity is more directly related to the haemolytic activity. Blondelle and Houghten (1992) reported that the haemolytic effect is significantly reduced through decreasing the hydrophobicity by replacement of lysine for leucine in the hydrophobic region. On the other hand, substitution of leucine for lysine in the hydrophilic helix region helps increase the hydrophobicity and enhances the haemolytic effect.

When the helix forms, some of the residues distribute regularly on the two sides of the helix, with one side hydrophilic and the other hydrophobic. The hydrophobic moment is introduced to measure this character, and is proportional to the size of and distance between the hydrophobic and hydrophilic domain. The hydrophobic moment is thought to be a more relevant factor than hydrophobicity (Pathak, et al, 1995).

The modification of introducing positive charge is more conducive to targeting the Gram-positive and Gram-negative bacteria, with less influence on haemolytic activity. Charge plays an important role in the antimicrobial activities on identification of the

bacterial membranes and aggregation of peptide near the target membrane. However, Dathe (1999) reported through studies of magainin analogues that highly cationic peptides with high haemolytic effects may still not exhibit antibacterial activity. The larger number of cationic residues brings high affinity to negatively charged membranes, but disturbs the permeability process.

In general, the peptide-membrane interaction is supported by a sensitive balance determined by many factors such as helicity, hydrophobicity, hydrophobic moment, and charge. By modulating some of these factors, it could enhance the antimicrobial effect or minimise the haemolytic effect.

1.2.2 Mechanism of action of AMPs

The mechanisms of action of all AMPs share some common features. The antibacterial mechanisms mainly include destruction of membrane integrity and interaction with certain intracellular targets. The mechanisms of these can be classified into two modes: actions on the membrane and actions on intracellular structures.

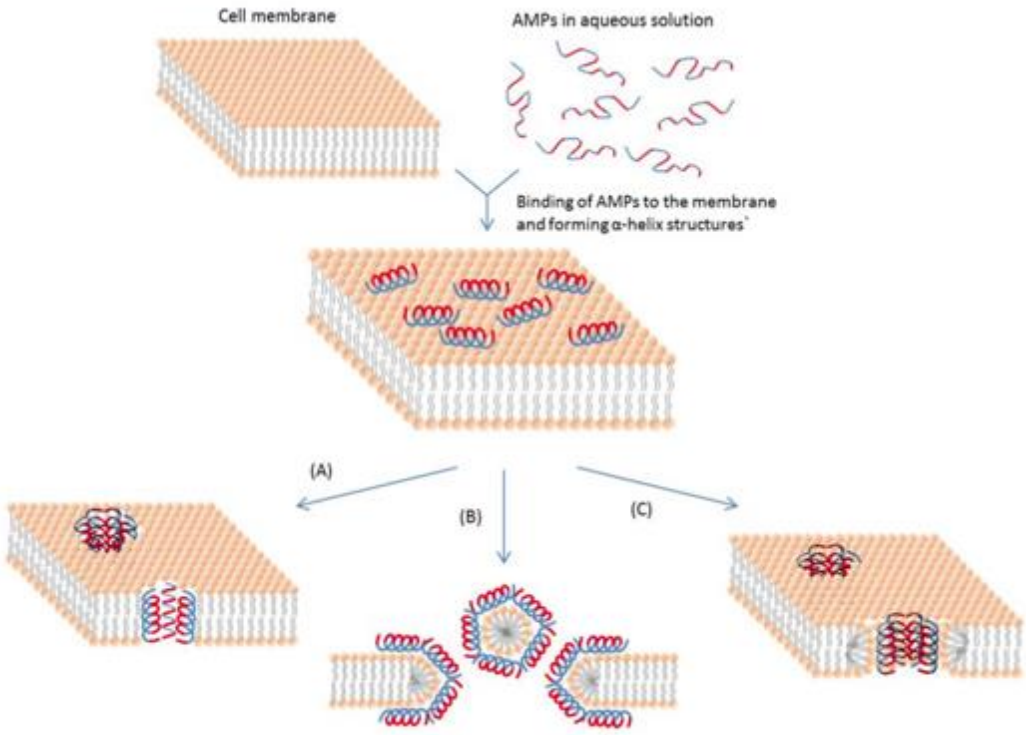
The membrane-permeabilising interaction is involved in the first stage. Amphipathicity is a feature containing both positive charged and hydrophobic faces, which most AMPs acting on membranes share. The cell membrane of bacteria is rich in acidic phospholipids, including phosphatidylglycerol and cardiolipin, the

headgroups of which contains more negative charges than other phospholipids (Matsuzaki, 2009). As a result, the phospholipid bilayer in the outer layer of the bacterial cell membrane is more likely to attract the positively charged AMPs. There are exceptions found by Brogden, Ackermann, and Huttner (1997) that show the existence of the negatively charged AMPs, changing the concept that peptides must be cationic to kill bacteria. For example, maximin-H5 (Lai et al., 2002) from frog skin belongs to the anionic peptides. Then the hydrophobic face of an AMP helps the AMP molecule penetrate into the plasma membrane (Madani et al., 2011). During insertion, pores form on the membrane bilayers by 'barrel-stave', 'carpet' or 'toroidal-pore' mechanisms, which are summarised in Table 1.1 and Figure 1.2 (Bahar and Ren, 2013).

Table 1.1 The action mechanisms of membrane-active AMPs.

Interaction model	Mechanism
Carpet like (Detergent-like)	The peptide micelle touches the membrane first and coats a small area of the membrane. Then AMP molecules penetrate the lipid bilayer to let pore formation occur leaving holes behind.
Membrane thinning	AMPs insert themselves into only one side of the lipid bilayer. It can form a gap between lipid molecules at the chain region. This gap creates a force and pulls the neighboring lipid molecules to fill it.
Aggregate	AMPs stick to the membrane parallel to the surface. Then reorientation of AMPs occurs and they insert themselves into the membrane vertically to form sphere-like structures.
Toroidal pore	AMPs align perpendicularly into the bilayer structure with their hydrophobic regions associated with the center part of the lipid bilayer and their hydrophilic regions facing the pore.
Barrel-stave	Staves are formed first parallel to the cell membrane. Then barrels are formed and AMPs are inserted perpendicularly to the plane of the membrane bilayer.

Figure 1.2 Schematic representation of some action mechanisms of membrane-active AMPs. (A) Barrel-Stave model. AMP molecules insert themselves into the membrane perpendicularly.
(B) Carpet model. Small areas of the membrane are coated with AMP molecules withhydrophobic sides facing inward leaving pores behind in the membrane.
(C) Toroidal poremodel. This model resembles the Barrel-stave model, but AMPs are always in contact with phospholipid head groups of the membrane. The blue color represents the hydrophobic portions of AMPs, while the red color represents the hydrophilic parts of the AMPs.



The second mode is the disruption of intracellular molecules. Although most AMPs kill microorganisms by interfering with the integrity of cell membranes, in some cases the microorganisms with broken membranes still survive. Increasing evidence shows that proteins may penetrate through cell membranes to bind cytoplasmic organelles that are vital to cell survival or reproduction. Buforin II does not cause the leakage of the cell membrane of *E. coli*, but penetrates the cell membrane to accumulate in the cytoplasm and bind to DNA and RNA, resulting in bacterial death (Park et al., 1998). Indolicidin at 100 μg/mL completely inhibits the DNA and RNA synthesis of *E. coli*, and significantly inhibits the protein synthesis at concentrations of 150 to 200 μg/mL. Another AMP, thanatin, kills bacteria by inhibiting their respiration. Sarcotoxin II can inhibit the formation of bacterial cell walls, so that bacteria cannot maintain normal cell morphology and it also blocks growth (Bahar, and Ren, 2013). In general, intracellular binding causes the inhibition of the synthesis of cell walls and cell membranes, the activation of autolysins, the inhibition of DNA, RNA and protein synthesis (Nicolas, 2009), and the inhibition of certain enzymes. It is worth noting that despite the two mechanisms, the exact mechanism of killing remains unknown in many cases.

1.3 Classification of AMPs

There are more than 850 species in the frog family Ranidae distributed worldwide. Based on the sequence similarities of amino acids, AMPs from ranid frogs are classified into 14 families: brevinin-1, brevinin-2, esculentin-1, esculentin-2,

japonicin-1, japonicin-2, nigrocin-2, palustrin-1, palustrin-2, ranacyclin, ranalexin, ranateurin-1, ranateurin-2, and temporin (Conlon, 2008).

1.3.1 Brevinin-1

The brevinin-1 family, first discovered from the skin of the Asian frog *R. brevipoda porsa*, now reclassified as *Pelophylax porosus* (Morikawa, Hagiwara and Nakajima, 1992), is widely distributed among North American species. Brevinin-1 peptides often consist of 24 amino acid residues, with a strongly conserved Pro^{14}, which plays an important role in the pore formation on membranes. Another conserved sequence cys-3X-lys/arg-lys-cys forms a C-terminal disulphide bridge, named a Rana-box structure. Studies on circular dichroism have shown that brevinin-1 peptides mainly present as a random coil in aqueous solutions, but in a membrane simulated environment such as 50% trifluoroethanol, they transfer into an extended a-helical conformation (Kwon et al., 1998).

Brevinin-1 peptides share a broad spectrum of antimicrobial effects against Gram-positive bacteria, Gram-negative bacteria, and pathogenic fungi, but at the same time, they possess a very strong haemolytic activity (Conlon et al., 2003). For example, the HC_{50} (the concentration producing 50% haemolysis) values of brevinin-1E from R. *esculenta* are less than 1 μM (Simmaco et al., 1994), which will largely limit its application prospects in treatment. Conlon found when the C-terminally loop of brevinin-1 was substituted by amidation, it could still inhibit the

growth of *E. coli*, and *S.aureus*, suggesting that the Rana-box structure was not necessary for antimicrobial activities. Kwon (1998) also found that the linearisation modification of a brevinin-1 analogue reduced the haemolytic effect, with antiviral effects being retained.

1.3.2 Brevinin-2

Brevinin-2 was first discovered from the skin of the Japanese frog *P. porosus* (Morikawa et al., 1992), and has been found widely distributed in Eurasian species. The primary structures of brevinin-2 peptides are very different among and within species. The only four conserved amino acid residues are Lys^{7}, Cys^{27}, Lys^{28}, and Cys^{33} (Conlon et al., 2004). The brevinin-2 peptides from *R. esculenta* (Ali et al., 2003) and *R. ornativentris* (Sumida et al., 1998) showed strong antibacterial activity against *E. coli* (MIC<10 μM), and also had a bacteriostatic effect on *S. aureus* and *C. albicans*. Peptides of the brevinin-2 family show much lower haemolytic activities compared to brevinin-1 peptides (Simmaco et al., 1994).

1.3.3 Esculentin-1 and esculentin-2

The prototype peptides of esculentin-1 and -2 peptides were first identified in the skin secretion of *Pelophylax lessonae/ridibundus*, previously known as *Rana esculenta* complex (Conlon, 2008) and subsequently discovered in the skins of closely related species. The C-terminals of both esculentin-1 and esculentin-2 are stabilised by an

intramolecular disulphide bridge between two cysteines, developing a cyclic heptapeptide structure.

Esculentin-1 is the longest AMPs found in Anura to date, containing as many as 46 amino acids. The cationic residues and the C-terminal heptapeptide ring are specifically conserved, which occupy more than half of the primary structure. Esculentin-1 has a strong inhibitory effect on various pathogens such as *E. coli*, *S. aureus*, *Pseudomonas aeruginosa* Migula, and *C. albicans*, with MICs <1 μM.

Compared with esculentin-1, esculentin-2 peptides are shorter, consisting of 37 residues. Their cyclic heptapeptide is less conserved, and there are more amino acid substitutions among esculentin-2 family members, but most share similarity in Nature. The positively-charged amino acids and cysteine are conserved. Esculentin-2 has a strong antibacterial activity against *E. coli* and *S. aureus* (MIC<10 μM). At the same time, it also has a certain inhibitory effect on *C. albicans* (MIC = 30~50 μM) (Matutte et al., 2000; Goraya et al., 2000).

1.3.4 Ranalexin

Ranalexin was first discovered in an extract of tadpoles of the American bullfrog, *Rana catesbeiana* and is found only in closely-related species (Conlon et al., 2004). It is expressed during both metamorphosis and maturity. The peptide has 20 amino acid residues. It is α-helical, and possesses a single disulphide bridge that shapes an intramolecular heptapeptide loop (Clark et al. 1994). Ranalexin shows a

broad and strong antimicrobial activity against Gram-positive bacteria, including Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis* Evans and *Streptococcus pneumoniae* Chester. However, against some of the very important Gram negative pathogens in clinical infections, such as *Pseudomonas aeruginosa* and *Proteus mirabilis* Hauser, it has no effect.

1.3.5 Ranatuerin-1 and Ranatuerin-2

Ranatuerin-1 is found only in the skin of the three closely related species, the American bullfrogs *Rana catesbeiana, R. clamitans* and *R. grylio* (Conlon et al., 2004). It contains 25 amino acid residues and a C-terminal heptapeptide ring. Studies have shown that the peptide exhibited growth-inhibition against *E. coli*, *S. aureus*, and *C. albicans*, but the haemolytic activity on human erythrocytes was relatively weak (Goraya et al., 1998). The replacement of the cysteine residues of ranatuerin-1 by serine had little effect on its conformation and bioactivity, indicating that the cyclic structure is not required for the antimicrobial activity of ranatuerin-1.

The ranatuerin-2 family was first isolated from the skin of the American bullfrog *R. catesbeiana* (Goraya et al., 1998), and is widely distributed in North American and Asian ranid frogs. The numbers of amino acids in currently identified ranatuerin-2 family members, ranges from 28 to 31. Its primary structure varies greatly, with only Gly/Ser^{1}, Ala^{15}, Lys^{22}, and the C-terminal hexapeptide formed by intramolecular disulphide bonds (Cys^{27}-Lys^{28}-X-Try^{30}-Gly^{31}-Cys^{32}), being conserved. The variation

of sequence of the ranatuerin-2 family contributes to its broad spectrum of antimicrobial potencies. The family has activity against *E. coli* (MIC=2~30 μM) and *S. aureus* (MIC=2~200 μM), while activity against *C. albicans* is generally low. The haemolytic effect ranges from 35 μM to >200 μM.

1.3.6 Temporin

Temporins are a huge antimicrobial peptide family. Temporins were initially discovered in the skin of the European red frog, *Rana temporaria* in 1996 (Simmaco et al., 1996) and were found in many frog species of Northern American and Eurasian origin, but also in the venoms of wasps (Conlon et al., 2004). Temporins are a large family of more than 40 types. It is one of the smallest amphiphilic α-helical AMPs found in Nature, consisting of 10 to 14 amino acid residues. The properties of the temporin family are as follows:

Among the already found amphipathic a-helical AMPs, temporins contain the smallest number of amino acids, ranging from 10 to 14. Compared with other cationic AMPs, most temporins are mildly cationic peptides, which are usually charged from 0 to +3 in neutral environments. Unlike post-translational modifications in other peptides of Ranidae (Simmaco et al., 1998; Conlon et al., 2004), temporins lack the 'Rana box' modification - the heptapeptide ring of the C terminus. Instead, the C terminals of temporins are amidated (Bradbury and Smyth, 1991).

Temporins are active on an extensive range of pathogenic microorganisms, including bacteria, viruses, fungi and protozoa. Nevertheless, they are non-toxic to human and animal cells. Most are active against Gram-positive bacteria, such as methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*, with minimum inhibitory concentrations in the range of 2.5 to 20 μM (Wade et al., 2000; Giacometti et al., 2005). Some temporins also kill fungi, such as *Batrachochytrium dendrobatidis*, a fungus which resulted in the reduction of global amphibian populations (Rollins-Smith et al., 2003), and *C. albicans* (Simmaco et al., 1996). Temporin L was shown to have broad spectrum antibiotic activity against Gram-positive, Gram-negative bacteria and yeasts (Rinaldi et al., 2002).

The study of the structure/activity relationships of temporin A showed that the hydrophobic N-terminal residues, seventh positive amino acid residue and the hydrophobic residues of the fifth and the twelfth sites, are of great importance to the antibacterial activity (Wade, et al., 2000). The antimicrobial ability is improved by substitution of leucine for isoleucine. In most cases, the antibacterial potency is directly associated with the net positive charge of temporins. However, there is no positive charge in the temporin-1Od molecule and it possesses a strong antibacterial activity with an MIC against *S. aureus* of 13 μM (Kim, Iwamuro, Knoop and Conlon, 2001). Most temporins exhibit no significant haemolytic properties (Mangoni et al., 2004).

1.4 Significance of AMP research and development

Today, bacterial infection and bacteria-related diseases are on the rise. One of the important reasons is attributed to the formation of bacterial resistance, such as drug-resistant *Mycobacterium tuberculosis*, *Escherichia coli*, and the notorious methicillin-resistant *Staphylococcus aureus* (MRSA), known as super bacteria. How to overcome bacterial resistance and develop new antibacterial agents is the hotspot of current research. Among possible solutions, AMPs have gained considerable promising prospects, for their broad spectra of actions, abundant species and highly effective bactericidal activity, and not readily effecting drug resistance.

AMPs directly kill microorganisms through membrane disruption and internal target interference. In addition, they possess antiviral and immune modulation activities among many others. The development of AMPs is underway. Magainin from the African clawed frog had been developed as a treatment for impetigo and diabetic foot ulcers. It was one of the great attempts although it failed to pass the US Food and Drug Administration (FDA) hurdle.

The limitations of AMPs retard the research and development of clinical agents to some extent. The first obvious defect is that peptides are easily broken down by proteases *in vivo*, which reduces their bioavailability as well as efficacy. Some methods have been developed to overcome this such as structural alternation by

D-amino acids and nonpeptidic backbones, or changing of dosage forms. Secondly, peptides come at a high cost. Solid peptide synthesis in laboratory research is too expensive to be applied in large-scale manufacture. Genetic engineering may overcome part of the questions through recombinant DNA and bacteria and fungi fermentation. Another issue exists in the uncertain mechanism of AMPs, causing doubt of potential side effects and toxicity (Hancock and Sahl, 2006).

Research on AMPs has a long way to go. It is believed that AMPs will play an important role in agriculture, medicine, bio feed additives, or other fields in the future.

1.5 Aims and objectives of this research

- To construct a cDNA library from *Pelophylax kl. esculentus* skin secretion and interrogate this with degenerate primers in a "shotgun" cloning technique to identify transcripts encoding the biosynthetic precursors of novel peptides.

- To predict the structures of novel peptides encoded by these precursors and to locate mature peptides in HPLC fractions of the same skin secretion. To confirm primary structures through MS/MS fragmentation sequencing.

- Once structures are confirmed, the peptides will be chemically synthesised to generate sufficient quantities for biological screening purposes.

- Biological screening will be performed using a variety of functional assays, both antimicrobial and pharmacological.

Chapter 2. Materials and Methods

2.1. Animals and skin secretion harvesting

Specimens of edible frog, *Pelophylax kl. esculentus* (n = 30) were obtained from a commercial source (Le QuerruyCellier, Notre Dame de Monts, France) and had been captive bred. All frogs were adults and defensive skin secretion was harvested after which frogs were released. They were maintained in the purpose-designed amphibian facility at 18-25℃ under a 12h/12h light/dark cycle. They were fed multivitamin-loaded crickets three times per week in the animal facility in Queen's University Belfast.

Defensive skin secretions of the frogs were obtained by two methods. The first one was a mild electrical stimulation (6V DC; 4 ms pulse-width; 50Hz) (C.F. Palmer, UK). This method was used to stimulate the glands to secrete on the dorsal surface of frogs, each of 20 s duration. Each animal was restrained and a bipolar electrode of 21 G platinum moved along the moistened dorsal skin surface. By this method, the glands in the surface of skin were stimulated to secrete. Distilled deionised water was used to wash the secretion from the surface of the skin and this was collected into a chilled glass beaker. Liquid nitrogen was used to freeze the skin secretion, then this was freeze-dried in an ALPHA 1-2/LD freeze dryer (Germany) and stored at –20 ℃ before use. The other and preferred technique was gently massaging the dorsal skin surface with a latex-gloved finger and this method was found to be equally effective. Secretion washings were treated as described before. Both techniques caused no harm

and minimal stress to the animals. All the procedures were subject to ethical approval and carried out under appropriate UK animal research personal and project licenses.

2.2 Molecular cloning

2.2.1 mRNA isolation

Poly-A mRNA was isolated from the lyophilised skin secretion with a Dynabeads® mRNA DIRECT™ Kit (Dynal Biotech Ltd, UK). Polyadenylated mRNA was isolated from stabilisation buffer with the use of magnetic oligo-dT beads in the kit and this relies on A_T base pairing. At the 3' end of mRNA, the poly-A tails anneal to the oligo-dT forming mRNA-dT polymers. The mRNA can be isolated from the secretion after washing and elution. The operation in detail was as follows:

Preparation of sample lysate

5 mg of lyophilised skin secretion from *Pelophylax esculentus* were dissolved in 1 ml of lysis/binding buffer (Dynal Biotech, UK) in a 1.5mL tube to remove the mRNA from any cells. The tube was shaken for a few seconds and then returned to the ice, alternately for 20min. After 20min extraction, the tube with sample was centrifuged at 18,000 × g for 5 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany).

Prepration of Dynabeads® Oligo $(dT)_{25}$ beads

250 µl of Dynabeads Oligo$(dT)_{25}$ (5 mg/ml) suspension was added to another 1.5ml RNase-free tube and this tube was placed on a Dynal MPC-S magnet for 5 min. The supernatant in the tube was discarded carefully as cleanly as possible.

250 µl of Lysis/Binding buffer was added to the beads tube and the tube was placed on the Dynal MPC-S magnet for a few minutes. The supernatant was then discarded carefully. The tube was kept at the magnetic rack.

Hybridisation between the poly-A tail of mRNA and bead-bound oligo-dT

The supernatant from the lysate solution was transferred into the 1.5 ml tube containing prepared beads which were combined with oligo-dT on the surface by covalent binding. The mixture of lysate and beads were blended by slowly and gently shaken for 15 min at room temperature, which made the poly-A tail of mRNA hybridise to the bead-bound oligo-dT through A-T base pairing. Finally, the supernatant was discarded completely on the magnetic rack. The intact mRNA was isolated from the secretion and retained on the surface of the beads.

Washing

The mRNA/beads complex was washed with 500 µl of Buffer A three times and then washed with 500 µl Buffer B two times. The beads were separated from the washing solution and the washing solution was discarded on the magnetic rack after each washing step. Finally, all the liquid was discarded.

Elution

This used 18 µl of the elution solution (10 mM Tris-HCL) to elute the mRNA from Dynabeads. Then the tube was incubated at 80℃ for 2 min. After this, it was placed on the magnet rack followed by transfer of all the supernatant by pipette to a 0.2ml PCR tube and then placed on ice immediately.

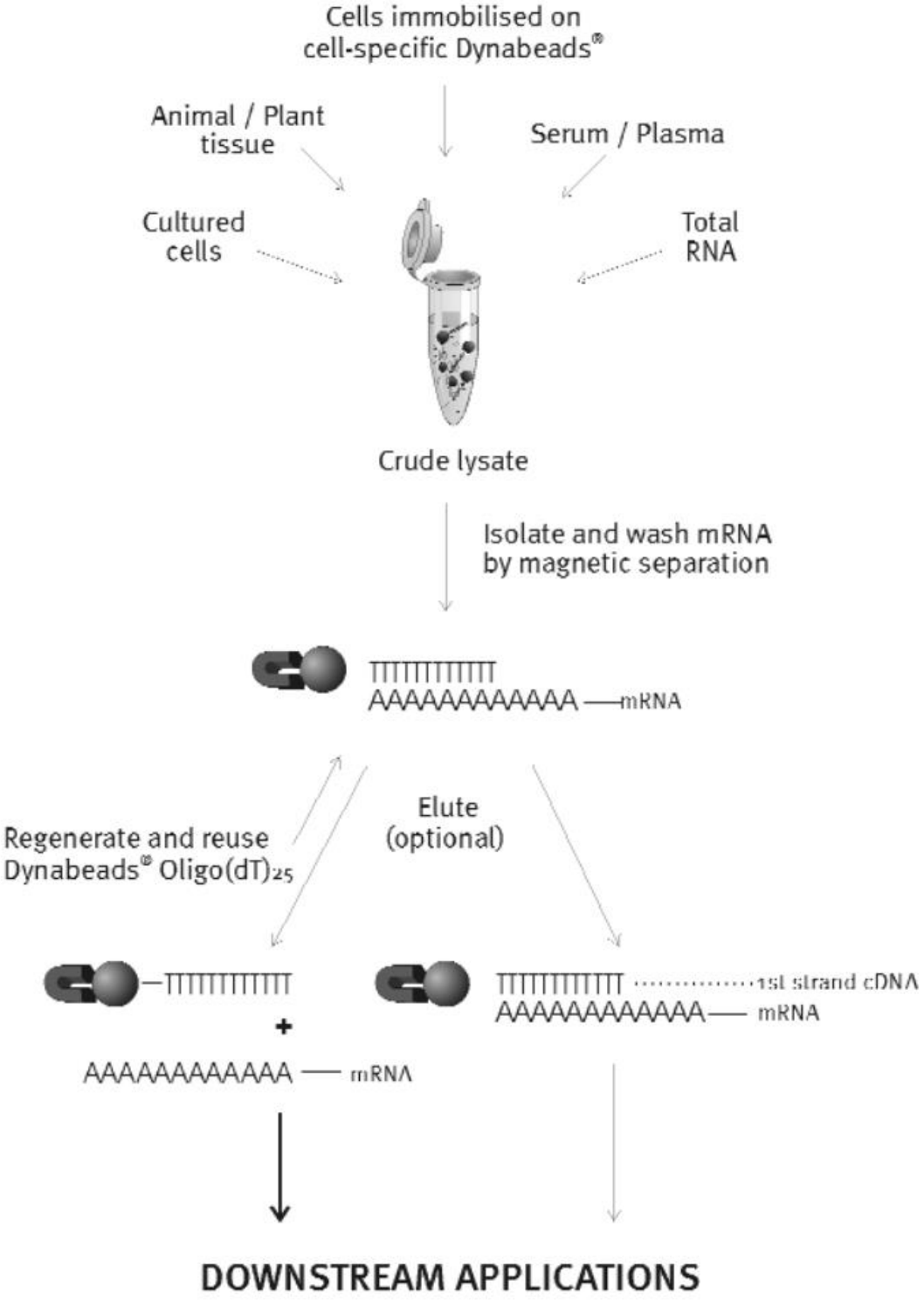


Figure 2.1 Outline of the protocol for isolating mRNA from a crude starting sample using Dynabeads Oligo(dT)25

2.2.2 cDNA library construction

The cDNA library was constructed by using a BD SMART^{TM} RACE cDNA Amplification Kit (BD Bioscience Clontech, UK). First-strand synthesis was primed using a modified oligo (dT) primer. After reverse transcriptase reaches the end of the mRNA template, it adds several dC residues. The BD SMART II A Oligonucleotide anneals to the tail of the cDNA and serves as an extended template for BD PowerScript RT. 5’ RACE Ready cDNA was synthesised using a 5’-RACE CDs Primer and the BD SMART II™ A Oligonucleotide which contained a terminal stretch of G residues to pair dC-rich cDNA tail at the end. 3’-RACE-Ready cDNA was synthesised using 3’-RACE CDs Primer by a reverse transcription reaction.

Table 2.1 The sequence of primer BD SMART II, 3’-CDS Primer A and 5’-CDS Primer

Primer	Sequence
BD SMART II	5'–AAGCAGTGGTATCAACGCAGAGTACGCGGG–3'
3'-CDS Primer A	5'–AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N–3' (N = A, C, G, or T; V = A, G, or C)
5'-CDS Primer	5'–$(T)_{25}$V N–3' (N = A, C, G, or T; V = A, G, or C)

Preparation of mixture for 3’ RACE Ready cDNA synthesis and 5’ RACE Ready cDNA synthesis

The following reagents were combined in three 0.2 ml PCR tubes respectively. One extra volume of reagents was calculated and added to ensure sufficient volume for the RT-PCR reaction.

Table 2.2 The components of the 3’ RACE cDNA reaction

Component	Final Volume	Final Concentration
RNA sample	4 µl	10-1000 ng
3’-RACE CDS Primer	1 µl	12 µM
dNTP Mix	1 µl	4 mM
DTT	1 µl	4 mM
5× First-Strand Buffer,30mM $MgCl_2$	2 µl	1 ×
Reverse Transcriptase	1 µl	20 unit

*The Master Mix includes dNTP Mix, DTT and 5× First-Strand Buffer for 5 reactions.

Table 2.3 The components of the 5’ RACE cDNA reaction

Component	Final Volume	Final Concentration
RNA sample	3 µl	10-1000 ng
5’-RACE CDS Primer	1 µl	12 µM
BD SMART II™ A Oligonucleotide	1 µl	12 µM
dNTP Mix	1 µl	4 mM
DTT	1 µl	4 mM
5× First-Strand Buffer,30mM $MgCl_2$	2 µl	1 ×
Reverse Transcriptase	1 µl	20 units

*The Master Mix includes dNTP Mix, DTT and 5× First-Strand Buffer for 5 reactions.

Reverse transcription polymerase chain reaction (RT-PCR)

After preparing each solution, they were mixed thoroughly and transferred into PCR tubes, then centrifuged briefly. The tubes were incubated at 70 ℃ for 2 min. This step allows the primer to bind to the template. After this, it was cooled on ice for 2 min. The mixture was centrifuged at 5000 × g to concentrate the liquid.

A master mix was made as following in each reaction tube (already containing 5 µl): 2 µl 5× first-strand buffer, 1 µl DTT (2mM), 1 µl dNTP Mix(1mM), 1 µl BD

PowerScript Reverse Transcriptase(10 Unit/μl). The contents of the tubes were mixed by gentle pipetting. The tubes were centrifuged briefly to collect the contents at the bottom. Each tube was incubated at 42℃ for 1.5 h in a thermal cycler in the PCR machine. Then 50μl of PCR grade water were added to each tube and these were returned to the PCR machine at 72℃ for 7 min. The samples were then stored in a -20℃ freezer.

The 3'-RACE and 5'-RACE Ready cDNA amplification products could be stored at-20℃ for up to 3-12 months.

2.2.3 RACE-PCR

Preparation of mixture for RACE-PCR reaction

The following reagents were mixed in a PCR tube. And an extra volume was calculated and added to ensure sufficient volume for the RACE-PCR reaction.

Table 2.4 The components of the RACE-PCR reaction

Reagent	Volume	Final concentration
PCR-Grade Water	3.1 μl	
10×BD Advantage 2 PCR Buffer	1.5 μl	1.5×
dNTP Mix (10 mM)	0.2 μl	0.2 mM
NUP (20 μM)	0.5 μl	1 μM
Sense Primer (20 μM)/Anti-sense primer	0.5 μl	1 μM
50 ×BD Advantage™ 2 Polymerase Mix	0.2 μl	1 ×
cDNA library	5 μl	

The two negative control tubes were added with 5 μl PCR-Grade Water instead.

3' RACE-Ready cDNA amplification

The 3'-RACE reaction employed a nested universal primer (NUP) supplied with the BD SMART^{TM} RACE cDNA Amplification Kit (BD Bioscience Clontech, UK), and

a sense primer (5’-GAWYYAYYHRAGCCYAAADATGTTCA-3’) that was designed to a segment of the 5’-untranslated region of peptide cDNAs previously cloned from the skin of *Pelophylax esculentus.*

The sample and negative control tubes were transferred into a PCR machine and then a programme was initiated to amplify the cDNA. The thermal cycling parameters were as follows:

Table 2.5 3’/5’-RACE PCR programme

Stage	Parameter
Stage 1	initial denaturation at 94 ℃ for 1min
Stage 2	40 cycles (denaturation at 94 ℃ for 30 s, primer annealing at 62 ℃ for 30 s, extension at 72 ℃ for 3 min)
Stage 3	final extension at 72 ℃ for 10 min

The annealing temperature in one group included one 3’ RACE-Ready cDNA template and one negative control at 60 ℃, whereas it was set at 62 ℃ in another group.

The PCR cycles were stored at 4 ℃, making preparation for the agarose gel analysis and purification.

5’ RACE-Ready cDNA amplification

5’-RACE PCR was almost the same process as 3’ RACE PCR, except for adding 5ul 5’-cDNA library in the reaction mixture.

2.2.4 Agarose gel electrophoresis for RACE-PCR product

Gel electrophoresis is a method that can separate DNA fragments on the basis of their different sizes. Through comparing the sample band on the gel with those of the DNA calibration ladder, the size of sample DNA fragments can be determined. Different gel pore sizes will have different formulae.

Preparation of 1.5% agarose gel

0.45g agarose (Invitrogen, UK) was weighed and dissolved in 35ml of 1×TBE buffer (Invitrogen, UK) in a conical flask. A microwave oven was used to heat the suspension until all the agarose had dissolved. The flask was then cooled to around 60 ℃. 3.5μl of ethidium bromide (EtBr, 10 mg/mL) (Invitrogen, UK) were added to the flask and shaken gently to mix. Two combs were inserted in the gel-forming block which was placed in a dry Consort E122 gel electrophoresis tank (Consort, Belgium). The melted agarose was then poured into the gel tank, ensuring that all bubbles were eliminated. After approximately 40 min the liquid gel solidified. The combs were removed carefully and the wells were generated. 1×TBE Buffer was poured into the gel electrophoresis tank. The buffer was added to just above the surface of gel.

Gel electrophoresis analysis

1.5μl PCR product was mixed with 0.5μl loading dye (0.25% bromophenol blue, 15% Ficoll 400 in TAE) by pipetting. Then the mixture was transferred to the gel wells by pipette. 2.5μl of DNA ladder (Invitrogen, UK) was loaded into the first well of the gel. 90 V were used to perform the electrophoresis for 30 min until the yellow colour

indicator reached two-thirds down the gel. Then the gel was removed from the gel tank for later detection.

Detection of bands and gel analysis

The electrophoresis gel was placed under the UV trans-illuminator BioDoc-It® Imaging System (NVP, Cambridge, UK) and a photographic image was recorded as the result. The DNA bands of the samples were compared with those of the ladder to determine whether the DNA amplification was successful or not. The 1× TBE Buffer was recycled and the samples were stored at -20 ℃ in the freezer.

2.2.5 Purification of RACE-PCR product

A Rapid PCR purification system was used to perform the PCR product purification (Marligen Biosciences Inc, USA). The DNA amplification products from the previous steps were miexed and merged into one tube. Then, 4 to 5 times of volume of Buffer CP were added into the 1.5 ml tube. A purification column was used here. The purification column was placed into a 1.5 ml tube. The solution was transferred drop by drop onto the centre of a filter cartridge into the purification column and centrifuged at 5000 × g for 1 min. Then the liquid at the bottom of the tube was discarded and the purification column was retained. The purification column was placed into a new tube, and 700μl of washing buffer were added to the column, then centrifuged for 1 min at 8000 × g. Subsequently, the filtrate was discarded and the collection tube was placed back in the bottom of the cartridge. Similarly, 500 μl DNA Washing Buffer were also added into the cartridge and centrifuged again at 8000 × g for 1 min. Then, the filtrate was discarded and the collection tube was placed back

into the cartridge. Finally, the empty cartridge with collection tube was centrifuged at the maximum speed of 8000 × g for 2 min to dry the column matrix and remove ethanol completely as this could interfere with the downstream applications.

The purification column was placed into a new tube and 30μl of PCR grade water was added to the column. The column was left for 2 min so that the sample could dissolve in the water and when centrifuged, the sample DNA would permeate through the filter at the bottom of the column with the water. Then the tube was centrifuged for 1 min and the flow-through liquid, containing the PCR product DNA, was retained.

Finally, the DNA purification products were placed for 50 min in a concentrator (Eppendorf, Hamburg, Germany) to dry the DNA sample and drive the ethanol away thoroughly. After evaporation, the DNA sample was sealed with parafilm and stored at -20 ℃ in the freezer.

2.2.6 Ligation

A pGEM®-T and pGEM®-T Easy Vector (Promega, USA) kit was used for ligation, transformation, blue and white colony screening and isolation of recombinant DNA reactions. The DNA with A at both ends of the strand could bind to and insert into the site of the pGEM®-T Easy Vector (50 ng/μl) with T through A-T based pairing.

Reagent preparation

Ten μl of deionised water were added into the 1.5 ml tube containing 7 μl DNA purification products to dissolve the DNA and then tube was vortexed completely by tapping and micro-centrifuged briefly to collect all contents at the bottom and then placed on ice to cool. This step was repeated five times for DNA preparation. 2×

Rapid Ligation Buffer was vortexed vigorously without centrifuging as the Buffer was so heavy. Also, the pGEM®-T Easy Vectors were micro-centrifuged briefly without pipetting to avoid damaging the fragile vectors. The T4 DNA Ligase was also micro-centrifuged briefly without pipetting as the enzyme was susceptible to inactivation under the changes of environment.

Ligation between DNA and vector

The following prepared reagents were combined and mixed without pipetting in a DNase-free PCR tube.

Table 2.6 The components of the ligation reaction

Component	Volume	Final Concentration
2 × Rapid Ligation Buffer,20mM $MgCl_2$	2.5 μl	1 ×
pGEM®-T Easy Vectors	0.5 μl	50 ng/μl
PCR Products	1.5 μl	10-1000 ng
T4 DNA Ligase	0.5 μl	3 Weiss units

A 0.2 ml PCR tube with ligation reaction product was incubated for 1 h at room temperature and then incubated at 4℃ overnight (16-24 h) in the thermal cycler. The vectors with a 3' single terminal thymidine (T) in the insertion site were re-cyclised with a single guanosine (A)-ending DNA sequence derived from Taq DNA Polymerase through A-T base pairing.

2.2.7 Transformation

The recombinant vectors were transformed into the competent cell and selected by ampicillin, IPTG and X-Gal using the pGEM®-T and pGEM®-T Easy Vector kit (Promega, USA).

Preparation of LB/ampicillin/IPTG/X-Gal plates

6.4 g of LB Agar (Invitrogen, UK) were added into a glass flask, then 200 ml of deionised water were added. The solution was autoclaved for sterilisation. When the solution was ready to use, 550 μl of ampicillin were added into the bottle and shaken to mix. 11 ml of agar mixture were placed into standard Petri dishes and these were left to solidify. The LB agar plates could be stored at 4 ℃. After solidification, 100 μl of IPTG (100mM, Promega, USA) were spread slowly and gently over the surface of each LB-ampicillin plate with a spreader, which induced the expression of lacZ gene. Subsequently, 20 μl X-Gal as a chromogenic substrate, was added and spread over the surface completely in a dark environment and this reacted with β-galactosidase and was detected by a colour change. All the plates were incubated upside down keeping the water inside the agar for 45 min at room temperature, and then the plates were ready for bacterial culture.

Transformation

The DNA ligation product was placed on ice from the PCR Amplifier. The sample were transferred into a 1.5 ml tube. The competent JM109 *E.coli* bacteria stored in -80 ℃ was defrost on ice for about 4 min. Then 50μl of bacteria suspension were added into the sample tube. Both were mixed by gently flicking and tapping at the bottom of the tube three times and then returned back to ice for 20 min. The mixture was heat-shocked in an incubator at 42 ℃ for 47 s, then transferred to ice immediately for 2 min. Finally, 950μl of SOC medium were added into the mixture tube which was placed into a shaking incubator at 150 rpm and 37 ℃ for 2.5 h.

Plating and culture for amplification

When incubation had finished, 100 μl of transformation suspensions were transferred and spread over the surface of LB/ampicillin/IPTG/X-Gal plates. All plates were incubated upside down at 37℃ overnight (16-24 h) for bacterial culture and DNA amplification.

2.2.8 Blue and white colony screening

Before the bacteria were transferred, three plates were prepared. Each plate was separated into 18 individual areas. There were three kinds of colonies found growing on the LB/ampicillin/IPTG/X-Gal plates from the previous procedures including white colonies, blue colonies and white colonies with a blue dot. The white bacterial colonies were readily differentiated from the blue colonies and were marked. The white bacteria were the targets because they had been successfully transfected with the vectors containing the ligated DNA products. The blue colonies were from the bacteria without vectors. The white bacterial colonies were then selected and subcultured by an inoculating loop onto the 18 areas that were already prepared on the plates under a sterile environment. All the plates were then incubated upside down at 37℃ overnight (16-24 h) for subculture and further selection.

2.2.9 Isolation of recombinant DNA by cloning PCR

A single DNA was isolated and amplified by the cloning PCR reaction, in which the M13 Forward Primer (5'-GTAACGCCAGGGTTTTCCCAG-3') and M13 Reverse Primer (5'-TGTGAGCGGATAACAATTTCAC-3') bound to the 5' and 3' ends of inserted DNA, respectively by use of an Advantage® 2 PCR Kit (Clontech Inc, USA).

Bacterial harvesting

White colonies of bacteria from the second incubation were picked up and transferred to the tubes with 20 μl deionised water for dispersion. Then, 14 samples of bacteria were obtained for recombinant DNA isolation.

Vector release

All the tubes were vortexed briefly and placed to a heating block to incubate at 100 ℃ for 5 min, then cooling down on ice for 5 min in order to make the cells fragile. Then, each tube was vortexed for 30 s. After vortexing, the tubes were centrifuged at 8000 × g for 5 min to ensure the recombinant plasmid DNA was released to the supernatant.

Preparation of reagent mixture for cloning PCR

The following components were combined and mixed completely by pipetting. One extra volume of reagents was calculated and added.

Table 2.7 The components in each cloning PCR reaction

Component	Final Volume	Final Concentration
dNTP Mix	1 μl	0.2mM
PCR-Grade water	31 μl	-
Cloning PCR Buffer	10 μl	1 ×
M13 Forward Primer	2.5 μl	5 μM
M13 Reverse Primer	2. 5 μl	5 μM
Taq Polymerase enzyme	0.25 μl	units
DNA template	2.5 μl	10-1000 ng

*The Master Mix includes PCR-Grade water, dNTP Mix, Cloning PCR Buffer, M13 Forward Primer and M13 Reverse Primer for 14 reactions.

The Master Mix was prepared in a 1.5 ml tube. 47μl of Master Mix were transferred to each new 0.2 ml PCR tube. Then, 0.25 μl Taq Polymerase enzyme and 2.5 μl

supernatants containing recombinant DNA were added into each 0.2 ml PCR tube in proper order and pipetted completely and evenly. All of the fourteen 0.2 ml PCR tubes were micro-centrifuged briefly to collect all contents at the bottom without bubbles. The isolation of target DNA from the vector and target DNA amplification relied on a cloning PCR reaction in which the M13 Forward Primer and M13 Reverse Primer could bind to the 5' and 3' ends of inserted DNA, respectively. Finally, the cloning PCR reaction was set and commenced using the following programme: each cycle in the thermal cycler is shown in Table 2.8. After this cloning PCR reaction, the 14 samples tubes were stored at -20 ℃ in the freezer.

Table 2.8 PCR programme of cloning

Stage	Parameter
Stage 1	initial denaturation at 94 ℃ for 1min
Stage 2	31 cycles (denaturation at 94 ℃ for 30 s, primer annealing at 55 ℃ for 30 s, extension at 72 ℃ for 3 min)
Stage 3	final extension at 72 ℃ for 10 min

2.2.10 Agarose gel electrophoresis analysis of cloned PCR product

The process was almost same as in section 2.2.4, except that the 1.5μl coloured-samples from fourteen tubes were loaded respectively without additional loading dye.

The electrophoresis gel was removed to a UV trans-illuminator BioDoc-It® Imaging System (NVP, Cambridge, UK) and the a image was recorded as a result. According

to the principle that the molecular weight was 200 bp higher than the RACE-PCR result, the DNA band of the samples was compared with the ladder to determine whether the target DNA was of expected size or not. In addition, the TBE Buffer was recycled and all samples were stored at -20 ℃ in the freezer.

2.2.11 Selected PCR product purification

An E.Z.N.A.® Tissue DNA Kit (Omega, Norcross, UK) was employed in PCR product purification, in which DNA was bound to silica-based filter membranes during washing steps and eluted for collection.

This process was the same as that described in section 2.2.5.

Finally, the fourteen DNA samples were stored at -20 ℃ in the freezer.

2.2.12 DNA sequencing

The sequencing reaction was performed using A BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA) in which the sequence was detected by fluorescence during DNA extension and termination process.

Preparation of mixture for sequencing PCR reaction

Some of the DNA samples were selected for the sequencing reaction. The components of master mix in this step are shown below, and an extra volume was calculated and added to ensure sufficient volume for the sequencing PCR reaction.

Table 2.9 The components of a sequencing PCR reaction

Component	Final Volume	Final Concentration
PCR-Grade water	12.4 μl	-
5× Sequencing Buffer	3.57 μl	1 ×
M13 Forward Primer	1.14 μl	0.8 μM

Terminator Ready Reaction Mix	2.86 µl	13.68%
DNA template	2.5 µl	10-1000 ng

*The Master Mix includes PCR-Grade water, 5X Sequencing Buffer, M13 Forward Primer and Terminator Ready Reaction Mix for 4 reactions.

DNA sequencing reaction

Master Mix and 2.5 µl sample were added into 0.2 ml PCR tubes Then the tubes were centrifuged briefly and placed into the PCR Amplifier. The programme was set to "sequencing". The thermal cycling parameters were as shown in Table 2.10. Finally, the four sequencing products were stored at -20 ℃ in the freezer.

Table 2.10 PCR programme for sequencing reaction

Stage	Parameter
Stage 1	initial denaturation at 96 ℃ for 1min
Stage 2	26 cycles (denaturation at 96 ℃ for 20 s, primer annealing at 55 ℃ for 10 s, extension at 60 ℃ for 4 min)
Stage 3	Cooling at 4 ℃ for 7 min

Extension product purification by ethanol

Before the extension product purification step, 95% ethanol and 70% ethanol were prepared by mixing 100% ethanol and PCR-grade water in appropriate percentages. 72 µl of 95% ethanol were added to each sequencing reaction product and mixed by pipetting gently before transferring quickly to the 1.5ml tubes with 10 µl of PCR-grade water. All the tubes were vortexed for 30 s and kept at room temperature for 20 min and then centrifuged at the maximum speed of 10000 × g for 20 min.

Immediately after this, the supernatants were discarded as cleanly as possible. Similarly, 260 μl 70% ethanol were added into each 1.5 ml tube with sequencing reaction products and mixed, vortexed for 30 s and centrifuged again as before. Then the supernatants were discarded quickly. A one-min cooling step and a one-min heating step at 95℃ were repeated 3 times. Finally, the contents of the 1.5ml tubes were concentrated for 3 h to dry the DNA and to evaporate the ethanol. Finally, the samples were stored at -20℃ in the freezer.

Sample preparation for sequencing

All the tubes with ethanol-purified products were placed in the concentrator for 1 h with the lids of the tubes open. 10 μl of highly-purified HiDi formamide were added to each tube with DNA sample. All the tubes were vortexed and then centrifuged briefly as before. Then the tubes were placed in a heating block to incubate at 95℃ for exactly 4 min before being transferred to ice and cooled for at least 3 min. After briefly centrifugation, 9 μl of well-prepared mixture sample was loaded into the 96-well plate in odd or even rows. The sequencing results were obtained using an ABI 3100 automated sequencer (Applied Biosystems, Foster City, CA, USA). The elongation of DNA strands in the solution was terminated by the modified ddNTPs randomly and detected by fluorescence.

Sequence analysis

After the sequencing, the nucleotide sequences of the DNA strands were obtained. Then the Expert Protein Analysis System (ExPASy) translate tool (http://web.expasy.org/translate) was applied to translate base sequences into protein

sequence while the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to search for proteins similar to the sequenced product. At this point, the putative nucleotide and translated open reading frame amino acid sequence of cloned cDNA encoding precursor were obtained and subjected to further mass spectrometry identification.

2.3 Solid phase peptide synthesis

The novel mature peptide was chemically-synthesised by solid phase Fluorenylmethoxycarbonyl (Fomc) chemistry in a PS4 automated solid-phase synthesiser (Protein Technologies, Inc, Tucson, AZ, USA). The translated primary structure of the novel peptide was as follows: FLPIVTGLLSGLL-NH_2.

2.3.1 Peptide synthesis

All of the amino acids except the last one in the sequence, were weighed and mixed with 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) activator in equal proportion and in three-fold molar excess and added to the vials. The vials were sealed with a cap and spanner. Then, 0.3 mmol rink amide resin which contained an amide group for the C-terminus was also weighed into the reaction vessel. The bottles placed on the machine were filled with reagents as follows: DMF (Dimethylformamide) in bottle 1 and bottle 2, piperidine: DMF= 1:4 in bottle 3, NMM(N-Methylmorpholine):DMF=11:89 in bottle 4, DCM (Dichloromethane) in bottle 5. Vials which contained amino acids were placed in the slot position of the SPPS machine in an inverted order of the sequence. A nitrogen environment was

needed. The glass flask containing the resin, was placed on the machine as well. Then the synthesis programme was initiated.

During the peptide synthesis reaction, the reaction vessel and pipeline were firstly washed by DMF. Then the Fmoc protecting groups were deprotected by DMF with piperidine. Each amino acid residue was activated and coupled using NMM and DMF combined with activator HBTU. During these processes, the peptide was synthesised from C-terminal to N-terminal. Finally, DCM was employed for washing the peptide/resin complex after the synthesis reaction. After the synthesis, the product was left to dry in the glass flask.

2.3.2 Peptide cleavage

The resin linked with synthesised peptide was weighed and transferred into a 50 ml round-bottomed flask with a magnetic rotor. The volume of cleavage cocktail was calculated according to the weight of the product. Cleavage cocktail containing 94% (v/v) trifluoroacetic acid (TFA), 2% (v/v) Thioanisole (TIS), 2% (v/v) water and 2% (v/v) 1,2-Ethanedithiol (EDT) were added into the 50 ml round-bottomed flask to deprotect the protecting groups of side chains. The cleavage and deprotection reaction was allowed to proceed at room temperature for 4 h, with continuous stirring. After that, the mixture was suction filtered using the Buchner funnel to remove the resin in the upper layer. The solution in the lower layer was transferred into a 50 ml universal tube and the ether was supplemented up to 50 ml for the peptide precipitation in the freezer overnight.

2.3.3 Peptide washing

The 50 ml universal tube was centrifuged at a speed of 5000 × g for 5 min to collect the peptide precipitate at the bottom and then the supernatants were discarded as cleanly as possible. Then, 45 ml ether was refilled and the washing step was repeated three times. After the last step of discarding the supernatant, the product was dried at room temperature.

2.3.4 Peptide lyophilisation

The dry peptide was dissolved in TFA/water/acetonitrile (0.5/19.95/80, v/v) (Buffer B) and TFA/water (0.5/99.95, v/v) (Buffer A). The peptide solution was lyophilised using the Alpha 1-2 freeze-drying system (Martinchrist, Germany). Finally, the lyophilised peptide was weighed and stored at -20 ℃ in the freezer.

2.3.5 Peptide calculation

The molecular weight of the peptide was calculated by an online tool called Peptide Property Calculator (www.pepcalc.com). The yield of the peptide was then calculated to assess the success of the peptide synthesis.

2.3.6 Reversed-phase high performance liquid chromatography (HPLC)

1 mg of crude lyophilised peptide was weighed and dissolved in a 1.5 ml tube with Buffer A and Buffer B in certain ratio according to the polarity. After fully vortexed and 15 min centrifugation at the maximum, the clear supernatants were transferred into another 1.5 ml tube. An analytical reverse phase HPLC Jupiter C5 column (250nm × 4.6 mm, Phenomenex, UK) was washed with Buffer B for 30 min and equilibrated in Buffer A for 30 min before use. During operation, 1 ml of clear

supernatant was injected into the Jupiter C5 column on a Cecil Adept CE4200 HPLC system (Cecil, Cambridge, UK) with 214nm wavelength detection. The peptide was eluted from the column using a linear gradient from 100% Buffer A to 100% Buffer B over 80 min at a flow rate of 1 ml/min. The fractions were collected in polypropylene tubes (Sarstedt, Germany) at every peak and utilised for identification.

2.3.7 Peptide analysis by matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS)

MALDI-TOF mass spectrometry was used for peptide identification by mass analysis using a linear time-of-flight mass spectrometer (Voyager DE, PerSeptive Biosystems, Framingham, MA, USA) in positive detection mode. 2 μl of samples were loaded and spotted onto the MALDI ground-steel target plate and left to dry. Then, 1 μl of matrix solution (10 mg/ml) which contained alpha-cyano-4-hydroxycinnamic acid (CHCA) diluted in acetonitrile/TFA/water (70/0.02/30, v/v) was also loaded and spotted onto the same spots and left to dry. The plate was loaded into the mass spectrometer. The results were recorded as a mass/charge ratio (m/z) against abundance and the masses observed were compared with the theoretical mass values that had been calculated earlier.

2.4 Antimicrobial assays

Three types of model microorganisms were used in these assays including the Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*, NCTC 10788), the Gram-negative bacterium *Escherichia coli* (*E.coli*, NCTC 10418) and the pathogenic

yeast *Candida albicans* (*C. albicans*, NCPF 1467) to assess the peptide antimicrobial activity.

2.4.1 Microorganism inoculation

A bead each of S.aureus, E.coli and C.albicans was transferred from frozen stock to flasks with 100ml MHB (Muellar Hinton Broth). The flasks were incubated overnight (16-20h) in a vibrating incubator (Stuart, UK) at a speed of 150 rpm at 37 ℃. For each organism, at least two McCartney bottles of 20ml MHB were placed in the 37℃ incubator to warm up overnight.

2.4.2 Peptide preparation

Lyophilised peptide was weighed and dissolved in dimethyl sulphoxide (DMSO) to make the stock solution at a concentration of 512×10^2 μM. Then the stock solution was double-diluted with DMSO to achieve a series of gradient stock concentrations: 512, 256, 128, 64, 32, 16, 8, 4, 2, 1×10^2 μM.

2.4.3 Subculture

To subculture, 500 μl of bacteria suspension from each flask, which had been incubated overnight, were transferred to McCartney bottles of pre-warm MHB and replaced in the vibrating incubator, to continue growth until the log phase. The optical density (OD) value of the subcultured bacteria was measured at 550 nm wavelength by a UV spectrophotometer. In table 2.11, the appropriate OD values of the three kinds of microorganism cultures and their corresponding concentrations are given.

Table 2.11 The appropriate OD values for the three microorganisms used.

Organism	Subculture incubation	OD	Concentration (cfu/ml)

	time		
S. aureus	1.5 h	0.23	1×10^{8}
E. coli	1.0 h	0.41	1×10^{8}
C. albicans	0.5 h	0.15	5×10^{6}

2.4.4 Minimum inhibitory concentration (MIC) measurements

The MIC measurements were performed by using a 96-well plate. The cultures of *S.aureus, E.coli* and *C.albicans* were diluted to final concentrations of 5×10^{5} cfu/ml when they had grown to the log phase and and dispersed completely in the Petri dish. One μl of peptide solution at each concentration and 99 μl diluted bacterial suspension were added to the wells in 5 replicates of the plate to form a series of final concentration of peptide at 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 μM. The positive control was set with 100 μl of diluted bacterial suspension which indicated the growth of the organisms. And 100 μl of MHB medium were added as negative controls (blank control). In addition, 1 μl DMSO and 99 μl diluted bacterial suspensions in 5 replicates were set as vehicle controls to observe the impact of 1% DMSO on the growth of bacteria. The 96-well plates were placed in a vibrating incubator to make the mixture well-distributed at 37 ℃ for 5 min and then were transferred to an incubator at 37 ℃ overnight. After overnight incubation, the growth of samples and controls were determined by using a Synergy HT plate reader (BioTek, USA) to detect the absorbance of each well at 550nm. MICs were defined as the lowest concentration at which no growth was detected.

2.4.5 Minimum bactericidal concentration (MBC) measurements

The clear solution in each well from the MIC value to largest concentration was chosen for the assessment of minimum bactericidal concentration (MBC). 20 μl clear solution in 5 replicates were spotted onto a new MHA plate and then incubated at 37℃ overnight (16-20 h). Finally, the MBC value was obtained as that in which no colonies grew at the lower concentration.

The antimicrobial assays should be repeated at least three times and the standard error of the mean (SEM) of three experiments was calculated to show the variability and repeatability.

2.5 Haemolysis assay

2.5.1 Peptide and control preparation

The lpeptide was weighed and dissolved in PBS to prepare a stock solution with the concentration of 1024 μM. Then, the stock solution was 2-fold diluted in the ratio of 1:1 in the PBS solution to achieve a series of gradient concentrations including 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2 μM. A positive control was prepared with Triton X-100 (Sigma Aldrich, St. Louis, MO, USA) diluted in PBS solution in which 1% (v/v) non-ionic detergent Triton X-100 (Sigma-Aldrich) can produce a 70% haemolytic effect. Meanwhile, PBS solution was regarded as a negative control (blank control) for the comparison of non-haemolytic effects.

2.5.2 Horse blood preparation

The fresh horse blood (TCS Biosciences Ltd, Buckingham, UK) was gently mixed by shaking. Two ml of horse blood were transferred into a 50 ml universal tube. After that 30 ml of PBS solution were added into the universal tube to mix with the horse blood. The PBS and blood were mixed by a rotating mixer. Then the tube was centrifuged at 500 × g for 5 min. The supernatant was aspirated carefully and discarded. Then 30 ml of PBS solution were added into the universal tube again to wash the horse blood. This step was repeated until the supernatant was clear. When the supernatant was clear and discarded, 50 ml of PBS solution were added into the tube to disperse the red blood cells. The concentration of blood cells in the suspension was 4% (v/v).

2.5.3 Haemolysis assay

1.5 ml tubes were prepared and filled with 200 µl prepared peptide solutions, positive control, negative control and vehicle control with five replicates of each. Then, 200 µl of erythrocyte suspension was added slowly into each 1.5 ml tube. Therefore, the final concentration of peptide in each tube was same as concentrations in the MIC assay. All the samples and controls were incubated in the incubator (Genlab Limited, UK) at 37 ℃ for 2 h. Afterwards, all the 1.5 ml tubes were centrifuged at the speed of 500 × g for 5 min. 100 µl of supernatants from each tube were transferred into the wells of a 96-well plate. The optical density (OD) values were measured at 550nm by using a Synergy HT plate reader (BioTek, USA). The percentage of haemolysis was calculated using the following formula and a graph was drawn.

Haemolysis% = (A-AO) / (AX-AO) × 100%

Where A represents the OD (λ550) of peptide/ erythrocyte mixture, AX the OD (λ550) of the positive control and AO the OD (λ550) of the negative control.

2.6 MTT cell viability assay

This process was performed in a microbiological safety cabinet at an appropriate containment level (Class 2).

The cells used in this study were PC-3 (Human Prostate Carcinoma (GIV) cell line), NCI-H157 (non-small cell lung cancer), MCF-7 (breast cancer non-tumourigenic mammary gland) and U251MG (Human Neuronal Gliblastoma(Astrocytoma)). The PC3 and the NCI-H157 were cultured in RPMI-1640 medium (Invitrogen, Paisley, UK), whereas the U251MG and MCF-7 were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose (25 mM) (Sigma, St. Louis, MO, USA) in a humidified environment containing 5% CO_2 at 37 ℃. Importantly, both the 10% foetal bovine serum (FBS) (Sigma, UK) which provided nutrition for cells growing and 1% penicillin streptomycin solution (Sigma, UK) which inhibited the growth of bacteria were also added into the medium.

2.6.1 Resuscitation of frozen cell lines

The tubes containing the frozen cells were transferred from the -80 ℃ freezer storage to a water bath which was set at 37 ℃. The tubes were gently flicked to increase the thawing of the cells. Then, all the cells were transferred into an 75 cm^2 culture flask (Nunc, Denmark) with pre-warmed 15 ml medium drop by drop and swayed gently

and slowly to dilute the cryoprotectant DMSO. The culture flask was then incubated in a CO_2 incubator (37℃, 5% CO_2) overnight. The next day (after 24 h), when the cells had become adherent, the spent medium with DMSO was discarded and fresh medium was added to the culture flask. The medium with FBS (Foetal Bovine Serum) and PS (Penicillin Streptomycin Solution) was used in this process.

2.6.2 Cell subculture and passage

In order to prevent the dying of cells, they should be sub-cultured regularly as soon as they reached confluence. The flask was placed vertically and the spent culture medium was removed with a transfer pipette carefully. Then 10 ml of PBS were added into the flask followed by gentle swaying to wash the cells. The PBS was removed by the same method with a transfer pipette and 1000 μl 1X Trypsin/EDTA (Invitrogen, UK) was added into the culture flask in order to strip the cells from the surface of the flask. Then the flask was incubated at 37℃ for 2-5min until the cell suspension became turbid. A microscope was used to observe the state of the cells in the flask to ensure that all the cells were detached and floating. Subsequently, 10 ml pre-warmed medium was added into the culture flask to cease the digestion, followed by cell aspiration. The suspension in the flask was transferred into a 15ml universal tube and was centrifuged at 350 × g for 5 min. The supernatants were then discarded and 5 ml pre-warmed medium were added into the 15 ml universal tube. The cells in the tube were mixed thoroughly. Finally, the cell suspension was transferred into a new flask and diluted by medium to a volume of 10ml then incubated in a humidified atmosphere at 37℃ under 5% CO_2. The medium in the flask should be changed every

2 days or changed when the medium colour changed. The second generation of cells could be used for the MTT cell viability assay or continuing passage when they reached confluency.

2.6.3 Cell counting

Cell counting is important in this assay because of the need of comparison between parallel experiments and the repeatability of experiments. The cell counting was carried out using an AS1000 Improved Neubauer haemocytometer (Hawksley,UK). The haemocytometer slide and coverslip were washed by water and dried. The counting area on the haemocytometer slide was covered by the coverslip. The previous procedures including cell washing, digestion, transfer, centrifugation and discarding of media were used with all subsequent cell passages. Five ml of pre-warmed medium was added into a 15 ml universal tube with cells and then the tube contents were mixed evenly by vortexing. After that, 50 μl of cell suspension and an equivalent volume of 0.4% (w/v) trypan blue (Invitrogen, UK) were mixed together. The mixture was transferred to the haemocytometer slide on both counting area notches on the top and the bottom. A microscope was used to count the cells. The counting area of the haemocytometer slide was divided into nine areas. The dead cells could be dyed by Trypan Blue while the live cells remained transparent. Three random areas were chosen and the numbers of live cells were counted. The cell concentration was determined by use of the following formula:

Cells per ml = average number of the cells per 0.1 mm^2 square $\times 10^4 \times 2$

2 represented the dilution factor. Finally, the volume of cells and medium were calculated to achieve the desired concentration of 5×10^4 cells per ml.

2.6.4 Cell seeding

An appropriate volume of cell suspension and pre-warmed medium were mixed and diluted to the final concentration of 5000 cells/100µl in the tray. Then, 100 µl samples of cell suspension were seeded in each well of the 96-well plate for cell attachment. After that, the 96-well plate was incubated at 37 ℃ under 5% CO_2 in a humidified atmosphere for 24 h.

2.6.5 Cell starvation

All media from the wells was removed and discarded as cleanly as possible and 100 µl of pre-warmed FBS-free medium was added into each well. Subsequently, the 96-well plate was incubated at 37 ℃ under 5% CO_2 in humidified surroundings for 6-12 h in order to starve the cells which can eliminate the impact of FBS in the parallel assay.

2.6.6 Peptide preparation

The lyophilised peptide was weighed and dissolved in DMSO to make a stock solution with a final concentration of 10^{-2} M. For preliminary screening of the anticancer functions, the peptide was diluted in FBS-free medium to make a concentration of 10^{-4} M. For gradient experiment, 70 µl of the stock solution was 10-fold diluted in 630 µl of pre-warmed FBS-free medium to achieve a range of concentrations of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9} M.

2.6.7 Peptide loading

The 96-well plate was taken from the incubator and the FBS-free medium was discarded carefully by pipette. Then 100µl of each prepared peptide solution were transferred into the wells with 5 replicates. Fresh FBS-free medium was added as the control group and an equal volume of 1% DMSO solution was also added as a vehicle control which reflected the impact of 1%DMOS on the cell growth. Lastly, the 96-well plate was incubated at 37℃ in a humidified environment containing 5% CO_2 for 24 h.

2.6.8 MTT assay

After 24 h of incubation, the plate was removed and 10µl of 5mM yellow-coloured MTT solution (Sigma, UK) were added into each well in a dark environment. The plate was knocked on the table gently to make sure the MTT sank down upon the cells that were attached to the bottom of the plate. Then the plate was incubated for 4-6 h. After this, the supernatants were discarded completely by use of a syringe. Then 100 µl of DMSO were added into each well quickly followed by gentle agitation in the orbital incubator (Stuart, UK) for 10 min in order to dissolve the insoluble purple formazan crystals. Then the plate was placed in a shaking incubator with a shaking rate of 250 rpm. Finally, Synergy HT plate reader (BioTek, Winooski, VT, USA) was used to measure the absorbance of the coloured solution at 570 nm. The statistical analysis of data was performed using GraphPad prism 5.0 software.

Chapter 3. Results

3.1 Animals and skin secretion harvesting

The skin secretion of *Pelophylax esculentus* was successfully extracted and lyophilised to store.

3.2 Molecular cloning

A cDNA encoding the precursor of a novel peptide was successfully cloned from the library during RACE-PCR. The nucleotide and translated open-reading frame amino acid sequence of the cDNA is shown in Figure 3.1. The predicted novel peptide is comprised of 13 amino acid residues and was named QUB-1342.

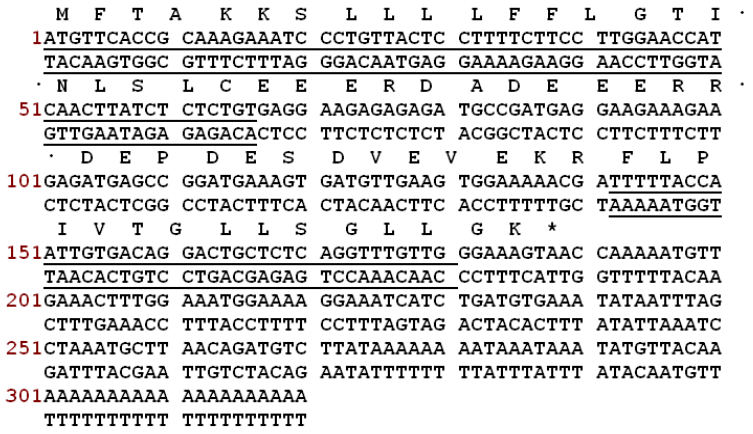


Figure 3.1: Nucleotide and translated open-reading frame amino acid sequence of cDNA encoding prepro-QUB-1342 cloned from *Pelophylax esculentus* skin secretion-derived library. The putative signal peptide and the mature peptide sequences are both single-underlined and the stop codon is indicated by an asterisk.

3.3 Structural characterisation of peptide QUB-1342

3.3.1 RP-HPLC chromatography of synthetic peptide QUB-1342

After solid-phase peptide synthesis, the crude product QUB-1342 was analysed by RP-HPLC and the result is shown in Figure 3.2. The major peaks 1, 2, 3 and 4 were further subjected to MALDI-TOF MS analysis. Among these, peaks 3 and 4 were proven to be representative of the expected product.

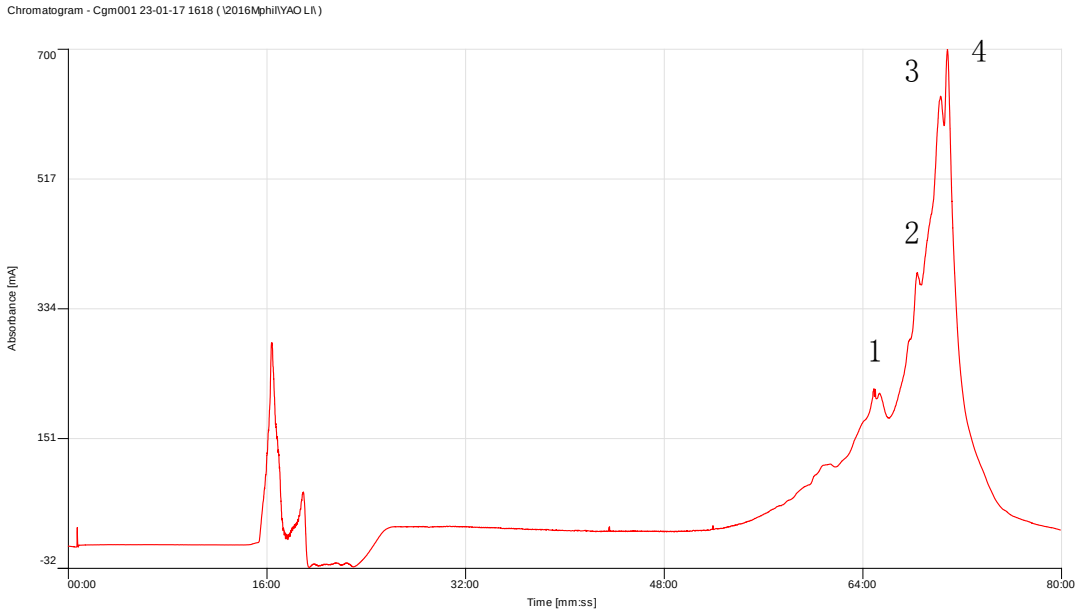


Figure 3.2 RP-HPLC chromatogram of synthetic QUB-1342.

3.3.2 MALDI-TOF MS of QUB-1342

The crude solid-phase synthesis product of QUB-1342was analysed by MALDI-TOF MS, and the spectrum obtained is shown in Figure 3.3. Its theoretical molecular mass is 1341.68 Da. The observed mass is 1342.03 Da, with a peak bound with a sodium ion as 1364.03 Da and a potassium ion as 1381.00 Da.

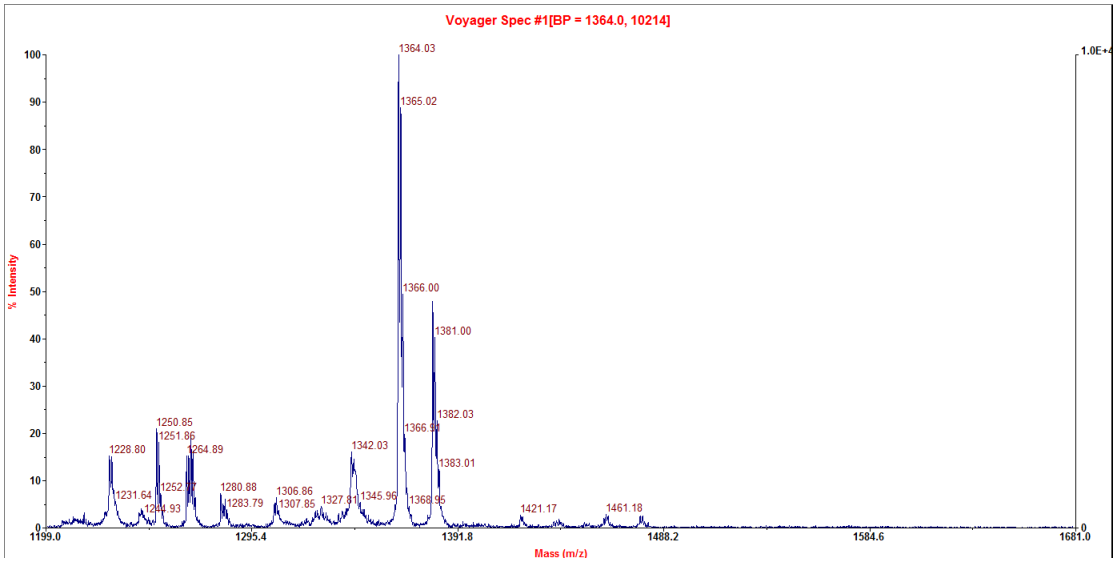
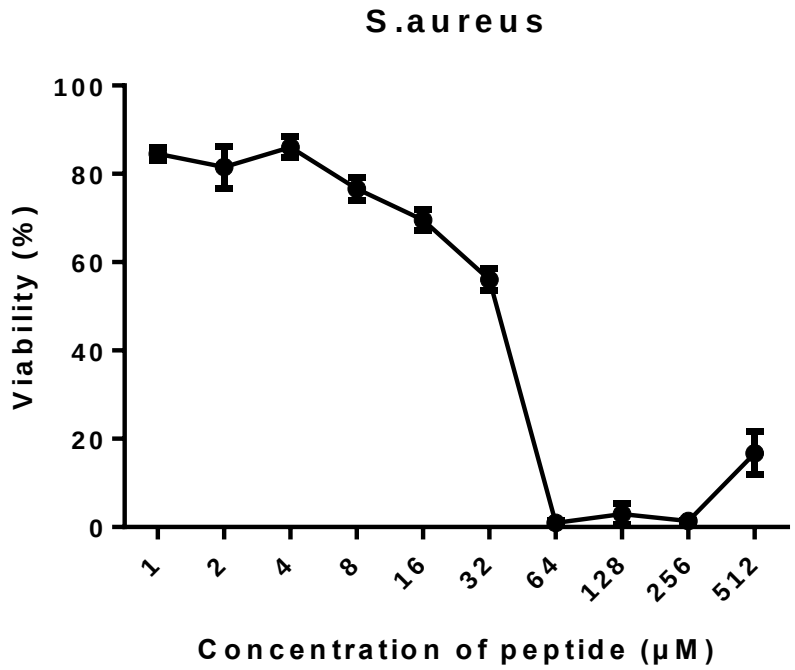


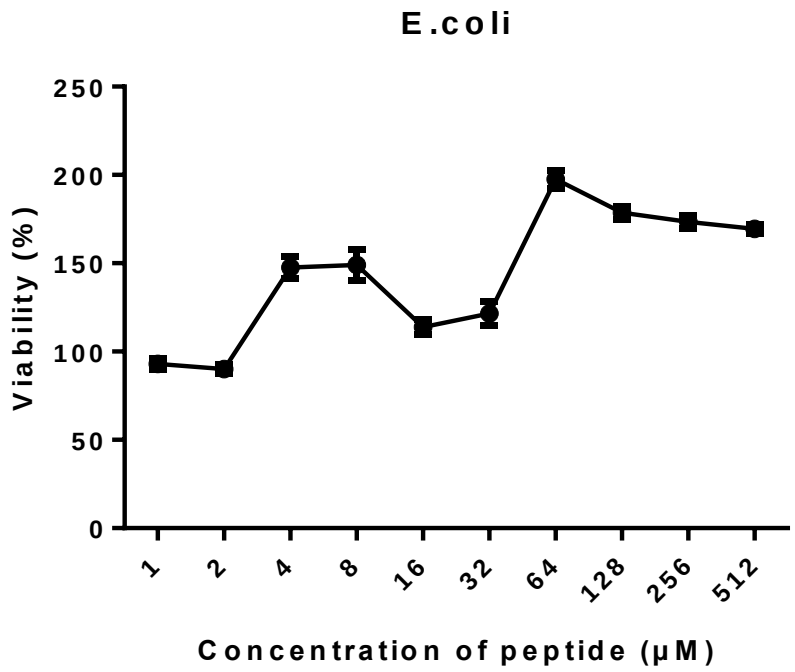
Figure 3.3 MALDI-TOF mass spectrum of synthetic peptide QUB-1342

3.4 MIC assay and MBC assay

The MIC assay of QUB-1342 was performed on *S. aureus*, *E.coli*, and *C.albicans*. The results are shown in Figure 3.4. In S.aureus, QUB-1342 started to inhibit the bacteria growth at 64μM. The rise of the curve when the concentration approaches 512μM was considered to be caused by precipitation of the peptide. However, this did not produce any effect on *E.coli* or *C.albicans* in the concentration range employed that is standard for all AMPs tested in the lab. The experiment was repeated three times.

The MBC assays showed that *S.aureus* was able to grow at the all the tested concentration of QUB-1342, indicating that it did not possess bactericidal activity at the concentration up to 512 μM.


S.aureus
Viability (%)
100
80
60
40
20
0
1
2
4
8
16
32
64
128
256
512
Concentration of peptide (μM)


E.coli
Viability (%)
250
200
150
100
50
0
1
2
4
8
16
32
64
128
256
512
Concentration of peptide (μM)

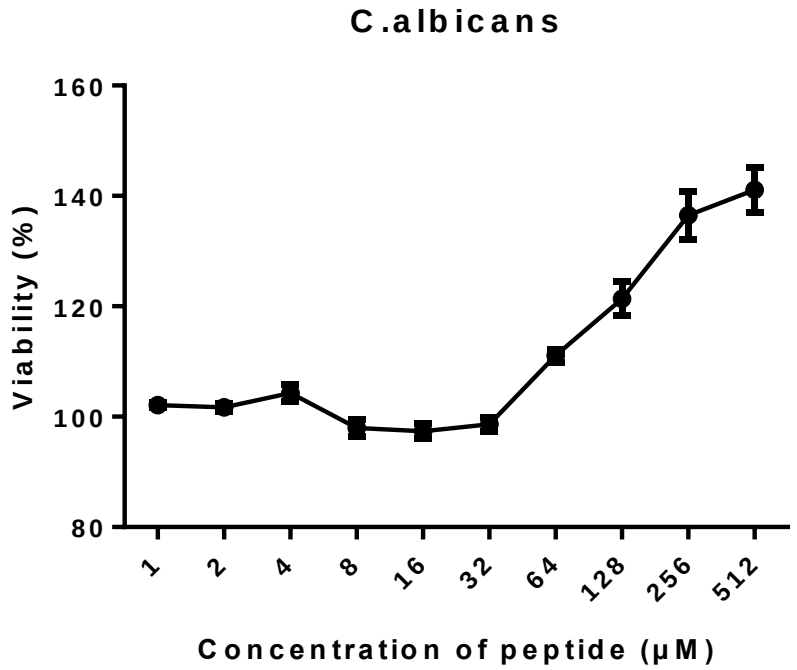


Figure 3.4 MIC of QUB-1342 against *S.aureus. E.coli* and *C.albicans*. Each experiment was performed as 15 replicates.

3.5 Haemolysis assay

The results of the haemolysis assay are shown in Figure 3.5. The peptide did not produce a haemolytic effect at concentrations between 1 and 256μM. The average haemolytic ratio when the concentration reached 512μM was 6.47%. The peptide is thought to produce little haemolytic effect.

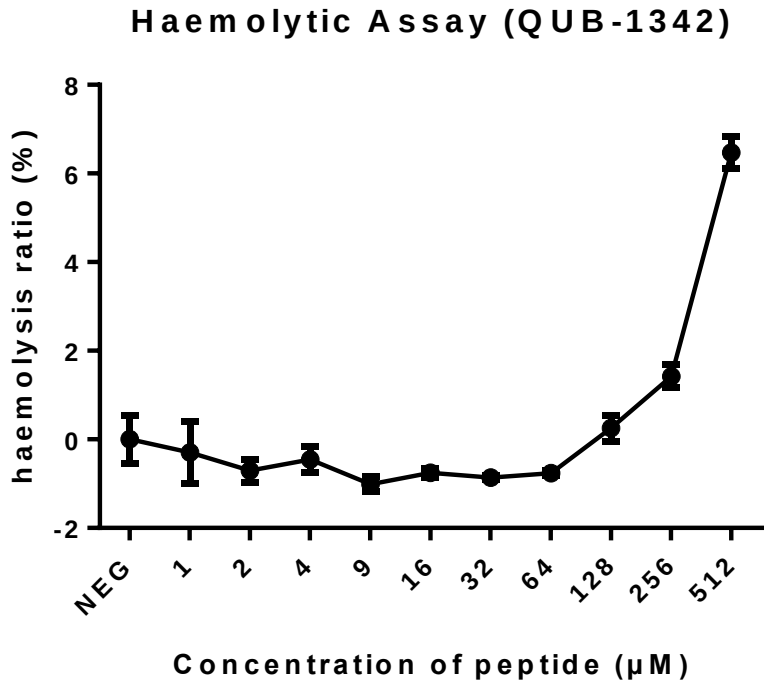


Figure 3.5 The haemolytic effect of QUB-1342. In total 5 replicates were subjected to the assay.

3.6 MTT assay

The QUB-1342 was prepared at concentration of 10^{-4} M for preliminary screening and the results were shown in Table 3.1. For all of the viability was above 50%, indicating that QUB-1342 did not show obvious growth inhibition on the cancer cell lines tested including cell lines, U251-MG, H157, PC3, and MCF-7.

Table 3.1 The cell viability of cancer cell lines U251-MG, H157, PC3, and MCF-7 at peptide concentration of 10^{-4} M.

	Cancer cell lines			
	U251-MG	H157	PC-3	MCF-7
Viability	52%	54%	64%	57%

Chapter 4. Discussion

Amphibians are widely distributed on the Earth. In order to avoid external pathogen invasion and adapt to the needs of diverse environments, they rely on secretions from skin glands, which contain a wide variety of complex functional peptides. These peptides have an amazing advantage in species and quantity compared with those in mammals. Ranidae, the third family of Anura, with 50 genera and 650 species, is the most widely distributed in almost every continent. Most of the frogs can synthesise and secrete a variety of active components. For their molecular diversity, unique mechanism of action on bacteria and mild side effects, the AMPs from Ranidae are worth of study and expected to be novel clinical drugs as antibiotics, antivirals and antineoplastics.

Activities of AMPs are closely related to amino acid form and structure, including length, charge, hydrophobicity, hydrophobic moment, helicity, and so on (Dathe and Wieprecht, 1999). The peptide-membrane interaction is supported by a sensitive balance determined by those factors. Helicity is more related to the interaction to membranes with neutral charge than to negatively charged bilayers, therefore is one of the influencing factors of haemolysis. On the contrary, the modification of introducing positive charge is more conducive to targeting the Gram-positive and Gram-negative bacteria, with less influences on haemolytic activity.

In these experiments, the peptide originated from the skin secretion of *Pelophylax esculentus* and was defined as a typical peptide of the temporin family through analysis of net charge, length and consensus sequence（FLPLIASLLSKLL-NH2) derived by Wade (2000). Temporins are a vast group of peptides containing 10-14 amino acid residues with C-terminal amidation, containing net charges from 0 to +3. In most cases, naturally produced temporins contain a single basic amino acid, which mediates their effect against Gram positive bacteria, such as clinically isolated methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* and *Enterococcus faecalis*. In temporins, the length and net charge are two significant factors that are correlated to the pharmacological effects. For example, peptides with zero basic amino acids that contain net charges of 0 to +1, only possess weak or absent antimicrobial effects, such as temporin-1Ja, C, D, or E (Simmaco, et.al, 1996). One of the most active peptides, temporin-L, with a net charge +3, not only exhibits a strong effect against Gram positive, Gram negative bacteria and fungi, but is also active against parasites, cancer cells, and mammalian cells (Rinaldi, et al., 2002). There is an exception to this rule, in that temporin-1Od does not contain positive amino acids, but still has a strong antibacterial activity, with an MIC against *S. aureus* of 13μM (Kim, Iwamuro, Knoop and Conlon, 2001). Furthermore, when the number of residues reduces to 10, the activities will also vanish, such as in temporin H and K (Mangoni, 2006). However, exceptions exist again in temporin-SHf, a peptide with an ultrashort length of 8 amino acid residues, which is active against Gram positive, Gram negative bacteria and fungi (Abbassi, et al, 2010).

QUB-1342 shows a weak bacteriostatic effect against the Gram positive bacterium *S.aureus*, with an MIC=64μM. Only a small amount of haemolysis occurs compared to the positive control Triton X-100 at high concentrations. The peptide scarcely exhibited any haemolysis towards horse RBC. Although it is highly hydrophobic according to the retention time in RP-HPLC (Figure 3.2), its disruption of phospholipids in the membranes of RBCs is very weak. In addition, the MIC value is not in the range of the haemolytic concentration.

To better understand its structure-function relationships, QUB-1342 was analysed by the Peptide Property Calculator (www.pepcalc.com). The hydrophobic amino acid residues occupy most sites, including one aromatic, seven aliphatic amino acid residues, one proline and two glycines. QUB-1342 has no basic amino acid residue, and only possesses one net charge at pH 7. According to the characteristics of temporins, the lack of positive charge is the main cause of the weak antimicrobial activity.

By comparison through NCBI-BLAST and the Antimicrobial peptide database, QUB-1342 was found to be 92.3% identical to two AMPs, temporin-SHb and temporin-1CSc.

Table 4.1 Comparison of amino acid sequence, net charge and antimicrobial activity of QUB-1342, temporin-SHb (Abbassi, et al, 2008), temporin-1CSc (Conlon, et al.,

2007), temporin-1P (temporin-1CSa) (Conlon, et al, 2007), temporin-LF1 (Guo and Hu, 2014), and temporin-CSb (Conlon, et al., 2007)

Peptide	Source	Sequence	Net charge	Activity	
				microbials	MIC (μM)
QUB-1342	*Pelophylax esculentus*	FLPIVTGLLSGLL-NH2	+1	*S. aureus*	64
				E. coli	ND
				C. albicans	ND
temporin-SHb	*Pelophylax saharica*	FLPIVTNLLSGLL-NH2	+1	*S. aureus*	58
				E. coli	>116
				C. albicans	>116
Temporin-1CSc	*Rana cascadae*	FLPLVTGLLSGLL-NH2	+1	*S. aureus*	64
				E.coli	>128
temporin-1P×/temporin-1CSa	*Rana cascadae*	FLPIVGKLLSGLL-NH2	+2	*S. aureus*	8
				E. coli	128
temporin-LF1	*Hylarana taipehensis*	FLPFVGKLLSGLL-NH2	+2	*S. aureus*	12.5
temporin-CSb	*Rana cascadae*	FLPIIGKLLSGLL-NH2	+2	*S. aureus*	8
				E. coli	128

ND: no detected activity
The basic amino acid residue is marked in red.
*Temporin-1P was reported against *S.aureus* (MIC=110 μM) (Goraya, Wang and Li, 2000). The same sequence named temporin-1CSa was reported by Conlon (2007) with MIC=8μM against *S.aureus*.

From Table 3.1, the three homologues, QUB-1342, temporin-SHb, and temporin-1CSc, with one amino acid difference, respectively, share similar properties. All of the three peptides have no basic amino acid residues, and produce weak bacteriostatic effects on Gram positive bacteria only. The distinctions in leucine and isoluecine at position 4, and asparagine and glycine at position 7 do not cause much change in activities. Investigation on the structure-activity relationship of temporin A and its synthetic homologues illustrate several vital positions that influence the

antimicrobial effect (Wade, et al, 2000): the N-terminal hydrophobic group, basic amino residue at position 7, large side chain of hydrophobic residues of position 5 and 12. In addition, the replacement of leucine from isoleucine at amino acid position 5 or 12 will enhance its antibacterial ability. In comparison, the 4th-position distinction between Temporin-1Ef and Temporin-CSc seems to not work, suggesting that it is not a crucial site.

However, the activity changed dramatically in temporin-1P (temporin-1CSa), with only 2 residues altered. The MIC against *S.aureus* reduced from 64μM to 8μM, as the Thr^{6} and Gly^{7} change to Gly^{6} and $Lysine^{7}$. Obviously, the key role is the substitution of the basic amino acid at 7^{th} position as mentioned in temporin A study. The higher the net charge, the stronger antibacterial ability is promoted. It is also proven in temporin-LF1 and temporin-CSb. As for QUB-1342 and the two homologues, other requirements of hydrophobic residues are matched except a basic amino acid, but the activity is weak. Therefore, it is assumed that the 7^{th} basic amino acid residue is a more crucial factor in temporin properties than others.

Temporins are peptides with low haemolytic side effects and so does QUB-1342. Studies on the helix structure of temporin L and its synthetic homologues, indicated that the Pro^{3} residue helps decrease haemolytic activity (Saviello , et al, 2010). Since the high haemolytic effect is induced by the high and complete helicity, the introduction of Pro^{3} is good for forming a turning structure at the N-terminus,

therefore cutting down on the unexpected effects. In addition, the replacement of native Pro^3 in temporin A increased the helical region at the N-terminus, but had little antimicrobial activity promotion effects, confirming that the helix is more involved in haemolysis than in antimicrobial activity.

Leucine and phenylalanine are known to help in membrane-anchoring interactions (Meijer, Spruijt, Wolfs, and Hemminga, 2001). Temporin-1Od, though without any basic amino acid residues, is relatively potent towards *S.aureus* with an MIC=13μM. It may attribute to the special structure or function leucine and phenylalanine displayed, suggesting that these two amino acids help to enhance the potency of AMPs.

However, most mechanism of temporins still remains unclear. Due to the small charge and the short length, every residue change can produce great functional changes. Through the existing literature on the speculation of functions of residues and structures, several hypotheses to improving the antimicrobial effects of temporins have been made:

1. Substitute with basic amino acid residues. As the natural peptide contains Lys^7, it is assumed that replacement of arginine and histidine will provide similar properties. From the consensus sequence, we can know that the 11^{th} residue is also a typical site for a basic amino acid, which could be tested.

2. Substitute with leucine. This may be restricted by position differences in the chain.

3. Maintain or introduce proline at position 3 to promote lower haemolysis. As for the already discovered temporins with broad spectra of action and high potency but high haemolytic activity, it may be of great benefit to lower the side effects and accelerate the development of clinical use.

In summary, the study of QUB-1342 has implications for detecting pharmacological functions, and speculating on the structure-activity relationships of the temporin family. The temporins and their homologues are expected to be novel candidate drugs for the clinic, for small design alternations in amino acid sequence can result in dramatic improvements in antimicrobial activity but maintain a weak haemolytic activity. These factors are significant for solving obstacles provided through antibiotic resistant pathogens and in developing new anti-infective agents.

References

Abbassi, F., Lequin, O., Piesse, C., Goasdoue, N., Foulon, T., Nicolas, P. and Ladram, A. (2010) Temporin-SHf, a new type of phe-rich and hydrophobic ultrashort antimicrobial peptide. ***J Biol Chem***. 285(22), 16880-92.

Abbassi, F., Oury, B., Blasco, T., Sereno, D., Bolbach, G., Nicolas, P., Hani, K., Amiche, M. and Ladram, A. (2008) Isolation, characterization and molecular cloning of new temporins from the skin of the North African ranid *Pelophylax saharica*. ***Peptides***. 29(9), 1526-1533.

Ali, M., Knoop, F., Vaudry, H. and Conlon, J. (2003) Characterization of novel antimicrobial peptides from the skins of frogs of the *Rana esculenta* complex. ***Peptides***, 24(7), 955-961.

Bahar, A. and Ren, D. (2013) Antimicrobial Peptides. ***Pharmaceuticals***, 6(12), 1543-1575.

Blondelle, S. E., Forood, B., Houghten, R. A., Perez Paya, E. (1997) Secondary structure induction in aqueous vs membrane‐like environments. ***Biopolymers***, 42, 489-498.

Blondelle, S. E., Houghten, R. A. (1992) Design of model amphipathic peptides having potent antimicrobial activities. ***Biochemistry***, 31, 12688-12694.

Bradbury, A. and Smyth, D. (1991) Peptide amidation. ***Trends in Biochemical Sciences***, 16, 112-115.

Brogden, K.A., Ackermann, M. and Huttner, K.M. (1997) Small, anionic, and charge-neutralizing propeptide fragments of zymogens are antimicrobial. ***Antimicrob. Agents Chemother***. 41, 1615-1617.

Bulet, P., Stocklin, R. and Menin, L. (2004) Anti-microbial peptides: from invertebrates to vertebrates. ***Immunological Reviews***, 198(1), 169-184.

Clark, D. P., Durell, S., Maloy, W.L. and Zasloff, M. (1994) Ranalexin. A novel antimicrobial peptide from bullfrog (*Rana catesbeiana*) skin, structurally related to the bacterial antibiotic, polymyxin. ***J Biol Chem***, 269, 10849-10855.

Conlon, J. (2008). Reflections on a systematic nomenclature for antimicrobial peptides from the skins of frogs of the family Ranidae. ***Peptides***, 29(10), 1815-1819.

Conlon, J., Kolodziejek, J. and Nowotny, N. (2004) Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. ***Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics***, 1696(1), 1-14.

Conlon, J., Mechkarska, M., Lukic, M. and Flatt, P. (2014) Potential therapeutic applications of multifunctional host-defense peptides from frog skin as anti-cancer, anti-viral, immunomodulatory, and anti-diabetic agents. ***Peptides***, 57, 67-77.

Conlon, J., Sonnevend, A., Patel, M., Davidson, C., Nielsen, P., Pal, T. and Rollins-Smith, L. (2003) Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog *Rana boylii*. ***Journal of Peptide Research***, 62(5), 207-213.

Dathe, M. and Wieprecht, T. (1999) Structural features of helical antimicrobial peptides: their potential to modulate activity on model membranes and biological cells. ***Biochimica et Biophysica Acta***. 1462, 71-87.

Dathe, M., Wieprecht, T., Nikolenko, H., Handel, L., Maloy, W.L., MacDonald, D.L., Beyermann, M. and Bienert, M. (1997) Hydrophobicity, hydrophobic moment and angle subtended by charged residues modulate antibacterial and haemolytic activity of amphipathic helical peptides. ***FEBS Lett***, 403(2), 208-212.

Dhople, V., Krukemeyer, A. and Ramamoorthy, A. (2006) The human beta-defensin-3, an antibacterial peptide with multiple biological functions. ***Biochimica et Biophysica Acta (BBA) - Biomembranes***, 1758(9), 1499-1512.

Giacometti, A., Cirioni, O., Kamysz, W., D'Amato, G., Silvestri, C., Del Prete, M. S., Licci, A. Łukasiak, J. and Scalise, G (2005) In vitro activity and killing effect of temporin A on nosocomial isolates of *Enterococcus faecalis* and interactions with clinically used antibiotics. ***J. Antimicrob. Chemother***, 55, 272-274

Goraya, J., Knoop, F. and Conlon, J. (1998) Ranatuerins: Antimicrobial Peptides Isolated from the Skin of the American Bullfrog, *Rana catesbeiana*. ***Biochemical and Biophysical Research Communications***, 250(3), 589-592.

Goraya, J., Wang, Y., Li, Z., O'Flaherty, M., Knoop, F., Platz, J. and Conlon, J. (2000) Peptides with antimicrobial activity from four different families isolated from the skins of the North American frogs *Rana luteiventris*, *Rana berlandieri* and *Rana pipiens*. ***European Journal of Biochemistry***, 267(3), 894-900.

Hancock, R. E. W. and Sahl H. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies, ***Nature biotechnology***, 24(12), 1551-1557.

Huang, Y., Huang, J. and Chen, Y. (2010) Alpha-helical cationic antimicrobial peptides: relationships of structure and function. ***Protein & Cell***, 1(2), 143-152.

Kim, J. B., Iwamuro, S., Knoop, F. C., Conlon, J.M. (2001) Antimicrobial peptides from the skin of the Japanese mountain brown frog, *Rana ornativentris*. ***J. Peptide Res***, 58, 349-356.

Kwon, M., Hong, S. and Lee, K. (1998) Structure-activity analysis of brevinin 1E amide, an antimicrobial peptide from *Rana esculenta*. ***Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology***, 1387(1-2), 239-248.

König, E., Bininda-Emonds, O. R. P. and Shaw C. (2015) The diversity and evolution of anuran skin peptides. ***Peptides***, 63, 96-117.

Lai, R., Liu, H., Hui Lee, W. and Zhang, Y. (2002) An anionic antimicrobial peptide from toad *Bombina maxima*. ***Biochemical and Biophysical Research Communications***, 295(4), 796-799.

Madani, F., Lindberg, S., Langel, Ü., Futaki, S. and Gräslund, A. (2011) Mechanisms of Cellular Uptake of Cell-Penetrating Peptides. ***Journal of Biophysics***, 2011, 1-10.

Mangoni, M. L. (2006) Temporins, anti-infective peptides with expending properties. ***Cell. Mol.Life Sci***, 63, 1060-1069.

Mangoni, M., Saugar, J., Dellisanti, M., Barra, D., Simmaco, M. and Rivas, L. (2004) Temporins, Small Antimicrobial Peptides with Leishmanicidal Activity. ***Journal of Biological Chemistry***, 280(2), 984-990.

Marenah, L., Flatt, P. R., Orr, D. F., Mcclean, S., Shaw, C., and Abdel-Wahab, Y. H. (2004) Skin secretion of the toad *Bombina variegata* contains multiple insulin-releasing peptides including bombesin and entirely novel insulinotropic structures. ***Biological chemistry***, 385(4), 315-321.

Matsuzaki, K. (2009). Control of cell selectivity of antimicrobial peptides. ***Biochimica et Biophysica Acta (BBA) - Biomembranes***, 1788(8), 1687-1692.

Matutte, B., Storey, K., Knoop, F. and Conlon, J. (2000) Induction of synthesis of an antimicrobial peptide in the skin of the freeze-tolerant frog, *Rana sylvatica*, in response to environmental stimuli. ***FEBS Letters***, 483(2-3), 135-138.

Meijer, A. B., Spruijt, R. B., Wolfs, C. J., and Hemminga, M. A. (2001) Membrane-anchoring interactions of M13 major coat protein. ***Biochemistry,*** 40, 8815-8820.

Morikawa, N., Hagiwara, K. and Nakajima, T. (1992) Brevinin-1 and -2, unique antimicrobial peptides from the skin of the frog, *Rana brevipoda porsa*. ***Biochemical and Biophysical Research Communications***, 189(1), 184-190.

Neerati, R., Meesala, A., Talari, S., Neerati, P., and Yanamala, V. (2014) Cardiotonic activity of parotoid gland secretion of common indian toad *Bufo melanostictus* on isolated heart of frog. ***Journal of Pharmacy***, 4(5), 61-67.

Nicolas, P. (2009) Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. ***FEBS Journal***, 276(22), 6483-6496.

Papagianni, M. (2003) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. ***Biotechnology Advances***, 21(6), 465-499.

Pathak, N., Salas Auvert, R., Ruche, G., Janna, M.H., McCarthy, D. and Harrison, R.G. (1995) Comparison of the effects of hydrophobicity, amphiphilicity, and α - helicity on the activities of antimicrobial peptides. ***Proteins***, 22(2), 182-186.

Park, C. B., Kim, H.S. and Kim S.C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. ***Biochemical and Biophysical Research Communications***, 244(1), 253-257

Powers, J. S. and Hancock, R. E. W. (2003) The relationship between peptide structure and antibacterial activity. ***Peptides***, 24, 1681-1691.

Rinaldi, A., Mangoni, M., Rufo, A., Luzi, C., Barra, D., Zhao, H., Kinnunen, P., Bozzi, A., Giulio, A. and Simmaco, M. (2002) Temporin L: antimicrobial, haemolytic and cytotoxic activities, and effects on membrane permeabilization in lipid vesicles. ***Biochemical Journal***, 368(1), 91-100.

Rollins-Smith, L., Carey, C., Conlon, J., Reinert, L., Doersam, J., Bergman, T., Silberring, J., Lankinen, H. and Wade, D. (2003) Activities of Temporin Family Peptides against the Chytrid Fungus (*Batrachochytrium dendrobatidis*) Associated with Global Amphibian Declines. ***Antimicrobial Agents and Chemotherapy***, 47(3), 1157-1160.

Saviello, M. R., Malfi, S., Campiglia, P., Cavalli, A., Grieco, P., Novellino, E., Carotenuto, A. (2010) New Insight into the Mechanism of Action of the Antimicrobial peptides temporins. ***Biochemistry***, 49(7), 1477-1485.

Simmaco, M., Mignogna, G. and Barra, D. (1998) Antimicrobial peptides from amphibian skin: what do they tell us? ***Biopolymers***, 47, 435-450.

Simmaco, M., Mignogna, G., Barra, D., and Bossa, F. (1994) Antimicrobial peptides from skin secretions of *Rana esculenta*. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides. ***Journal of Biological Chemistry***, 269(16), 11956-11961.

Simmaco, M., Mignogna, G., Canofeni, S., Miele, R., Mangoni, M. and Barra, D. (1996) Temporins, Antimicrobial Peptides from the European Red Frog *Rana temporaria*. ***European Journal of Biochemistry***, 242(3), 788-792.

Sumida, M., Ogata, M., Kaneda, H. and Yonekawa, H. (1998). Evolutionary relationships among Japanese pond frogs inferred from mitochondrial DNA sequences of cytochrome b and 12S ribosomal RNA genes. ***Genes & Genetic Systems***, 73(2), 121-133.

Thennarasu, S. and Nagaraj, R. (1996) Solution conformations of peptides representing the sequence of the toxin pardaxin and analogues in trifluoroethanol - water mixtures: Analysis of CD spectra. ***Biopolymers***, 41, 635-645.

Wade, D., Silberring, J., Soliymani, R., Heikkinen, S., Kilpeläinen, I., Lankinen, H. and Kuusela, P. (2000) Antibacterial activities of temporin A analogs. ***FEBS Letters***, 479(1-2), 6-9.

Amphibiaweb.org. (2017). AmphibiaWeb - Pelophylax esculentus. [online] Available at: http://amphibiaweb.org/cgi/amphib_query?where-genus=Pelophylax&where-species=esculentus [Accessed 10 Mar. 2017].