MASTER OF PHILOSOPHY

QUB-3005, a bioactive peptide from the defensive skin secretion of Rana amurensis

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QUB-3005, a bioactive peptide from the defensive skin secretion of *Rana amurensis*

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Contents

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Declaration

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

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Abstract

Many biologically active compounds exist in amphibian skin secretions, such as biogenic amines, steroids, complex alkaloids, and peptides. In the latter class of molecules, a large number of peptide antibiotics has been isolated and characterised from different amphibian species. Particularly, antimicrobial peptides (AMPs) are considered as excellent candidates for development of novel drugs for antibiotic therapy, as they normally have a wide range of activities, for example, by disrupting the phospholipid bilayer of the target cell membrane to kill bacteria or inhibit their growth.

In this thesis, a bioactive peptide, named QUB-3005, was isolated using "shotgun" cloning of its biosynthetic precursor-encoding cDNA from a skin secretion-derived cDNA library of *Rana amurensis.* The primary structure of this peptide was confirmed based on experiments of cloning and online BLAST alignment analysis. Then, solid-phase synthesis methodology was used to chemically-synthesise the peptide, and the molecular mass of this peptide was identified by MALDI-TOF mass spectrometry. After that, RP-HPLC was employed for purification purposes and then the bioactivities of this peptide were examined by antimicrobial assays, haemolytic assays and anticancer cell assays.

The data indicated that the QUB-3005 inhibited the growth of *E. coli, P. aeruginosa, S. aureus* and *C. albicans* in antimicrobial assays but showed a relatively high haemolytic activity. In addition, the peptide was found to have ability to inhibit the proliferation of H157 human cancer cells in MTT anticancer assays.

Chapter 1

Introduction

1.1 Background to *Rana amurensis*

Rana amurensis (Khabarovsk frog, Siberian wood frog, Heilongjiang brown frog or Amur brown frog) belongs to the *Ranidae* family and the genus *Rana* (Figure 1.1). This true frog family is widely distributed throughout Northern Asia, including Sakhalin, Northern Korean Peninsula, Northeast China, Northeast Mongolia and West Siberia. Their habitats are in the plains and open areas of reservoirs, puddles, swamps, ditches, paddy fields and other nearby water, often 50-650 m above the sea level. In some places, as a result of urbanisation and other related reasons, their populations have declined while their overall development seems stable. From 1990 to 1999, because of the degradation of the environment, coupled with a large number of people preying on these frogs, there has been a sharp decline in their populations leading to a lowered distribution (Figure1.2).

Figure 1.1: *Rana amurensis*

date: 02/10/2016 (From http://www.balatsky.ru/NSO/Amphibii/Rana_amurensis.htm)

Figure1.2: The distribution of *Rana amurensis* (The Circular Marker area)

date: 03/10/2016 (From: https://www.google.co.uk/search?sa=G&hl=en-GB&q=blue+ringed+octopus+habitat+map&tbm)

1.2 Normal functions of amphibian skin

1.2.1 Structure and function

The amphibian skin is a morphologically, biochemically and physiologically complex organ, having a wide range of functions which are necessary for survival. Their skins play a major role in survival and in their ability to exploit many different ecological niches. The frogs' skins contain two parts: the epidermis (outer skin) and the dermis (inner skin). Due to the functional diversity of the skin, all the functions must be coordinated in order not to interfere with each other. The functions of their skins include anti-predator, anti-microbial, anti-fungal defence, respiration, temperature control and reproduction. These unimpaired simultaneous functions require different controlling factors for their roles and various chemical compounds which are necessary for regulating the complexities of amphibian skin.

The primary functions of the amphibian's skins are for respiration, water regulation, and defence against predators. The potential disadvantage is that the requirements of

the organ means it must be kept moist to enable it to work normally. In addition, amphibians cannot accept sudden environmental change even if they have the ability to deal with a difference in temperature. The permeability of their naked skin makes them easy to encounter sudden water loss. When facing environment changes, the skins of frogs can produce many reactions to adapt such to changes. The amphibians are rarely infected by their pathogen-rich environment which includes many microbes, unless their skin surface is broken. An essential requirement for survival is an efficient anti-microbial and anti-fungal defence mechanism operating on the skin surface. Although the immune systems of amphibians are well-developed, their defence systems cannot prevent fungal infection effectively. Amphibians that hibernate during the winter present an increased sensitivity to bacterial and fungal infection which reveals that these defence systems are efficient when their functions are being used even at a minimal level (1).

1.2.2 Glandular secretions

In general, there are two main types of adult amphibian skin glands: mucus and granular (Figure 1.3). A small group of frogs has the third skin gland type, tubulosaccular or alveolar. Mucus glands are spread throughout the body secreting watery fluid. This secretion is non-toxic and protects the amphibians' skin by slime, enabling them to swim in the water and breathe on land. The glands' function is to prevent mechanical lesions to their skins. Besides, it protects the skin when staying in the water for a long time, prevents evaporation of water loss, and has a bacteriostatic effect. The granular gland secretions of active peptides can be divided into two categories: antibacterial and pressure activated. The granular glands have toxic secretions and they are used to secrete these toxic substances to protect themselves from infection and harm from predators. The granular gland cells in the

toads can form discrete, compact glands as they are spread over the surface of their skins or distributed in enlarged groups (Figure 1.4).

The number of compounds produced by the amphibians in their granular glands is surprisingly high, even within a single species. This is the main reason to treat amphibians as appropriate objects for biochemical prospecting. The compounds serve as a defence and their pharmacological effects can adversely affect a potential predator, through effects such as myotoxicity, cardiotoxicity, neurotoxicity, vasoconstriction and hypotension and even through hallucinogenic effects. The granular gland secretions are chemically stimulated and can be used in the animals' courtship and mating behaviours (2). The different species use various ways to achieve their mating processes but some use pheromones derived from their skin granular glands secretions.

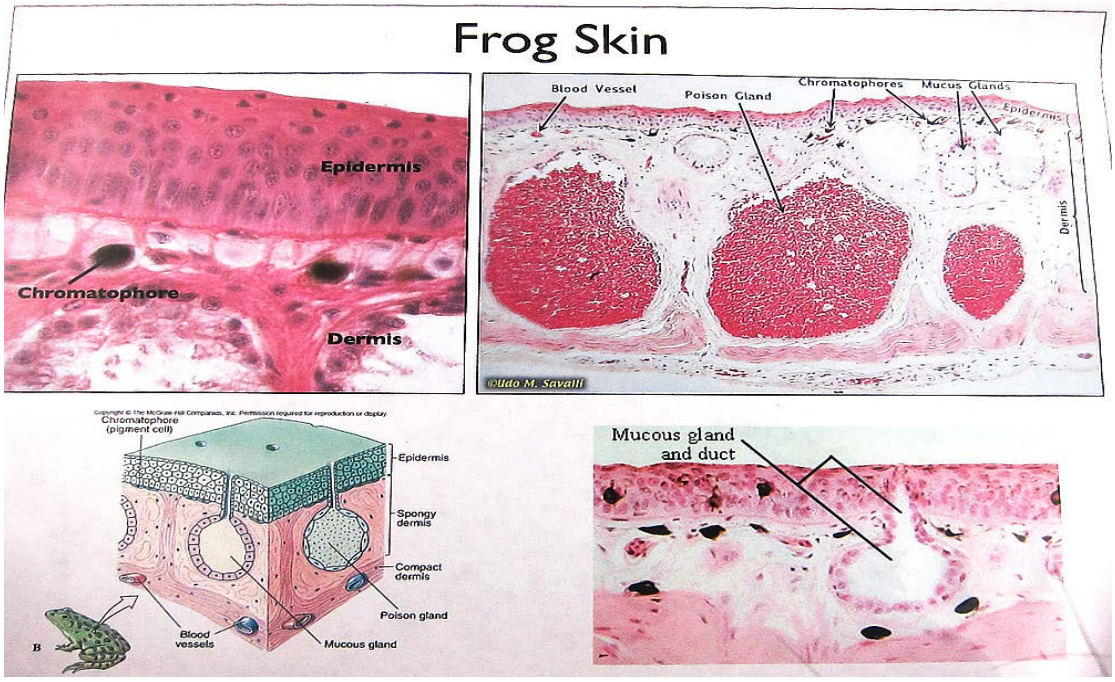

Figure 1.3: Frog skin under the microscope

date: 06/10/2016 (From: https://www.google.co.uk/url?sa=i&rct=j&q=&esrc=s&source=images&cd=&cad=rja&uact=8&ved=0ahUKEwj9mZON5IPWAhVNaFAKHVZxBp8QjRwIBw&url=https%3A%2F%2Fwww.thinglink.com%2Fscene%2F614159033271582720&psig=AFQjCNF7I9YD2K47lAgrSwD-kpy1sgzf5g&ust=1504348128201529)

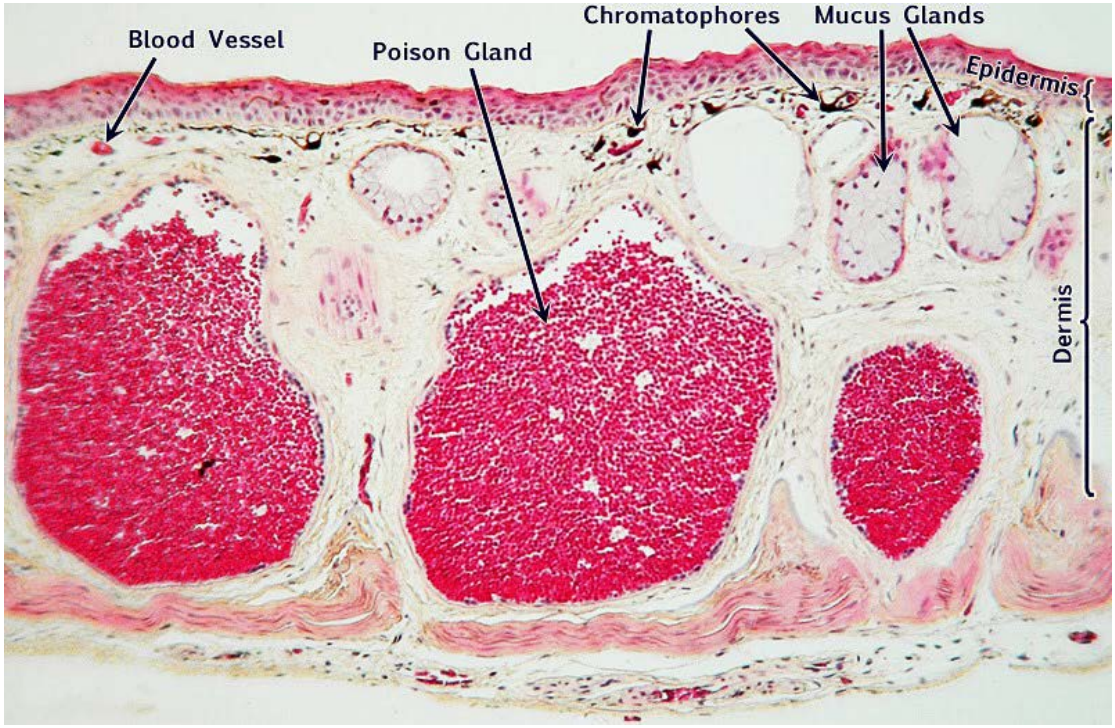

Figure 1.4: Amplification of frog skin glands

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1.3 Antimicrobial peptides (AMPs)

Antimicrobial peptides (AMPs), also called host defence peptides (HDPs), are an abundant and varied group of molecules, which are present in many tissues and organs in different invertebrates, plants, and animal species. These peptides are prospective novel therapy reagents, because they are potent, broad spectrum antibiotics. They can induce the killing of enveloped viruses, fungi, bacterial and even cancer cells. However, AMPs, unlike most conventional antibiotics, are commonly used as immunomodulators as an effective way to enhance immunity. Also, they are a unique and diverse group of molecules which are classified into many subsets according to their compositions and structures of amino acids (3). Such peptides generally consist of about twelve to fifty amino acids. These peptides contain more than two residues with positively charges, which are mainly due to histidine, lysine, arginine and also they have a large content of hydrophobic residues

(4-6). There are four spatial configurations in the secondary structures of these molecules, including α-helical, β-stranded, β-hairpin or loop and extended (7) (Figure1.5). Many of these peptides which are unstructured in solution, fold into their final configuration in a membrane mimetic environment. They contain two kinds of properties of amino acids, hydrophilic amino acid residues and hydrophobic amino acid residues. In a helical molecule, the positions of these two kinds of amino acid residues are opposed (3). So, AMPs have the ability to penetrate the membrane lipid bilayer due to their amphipathicity (8, 9). These peptides possess different antimicrobial activities ranging from membrane permeabilisation to a wide range of intracellular targets. The skin secretions of the amphibians have many functions and the AMPs are necessary for their survival. For example, these peptides can restrain the growth of microbes such as *Gram-negative* bacteria, *Gram-positive* bacteria, and *Candida albicans*, a yeast.

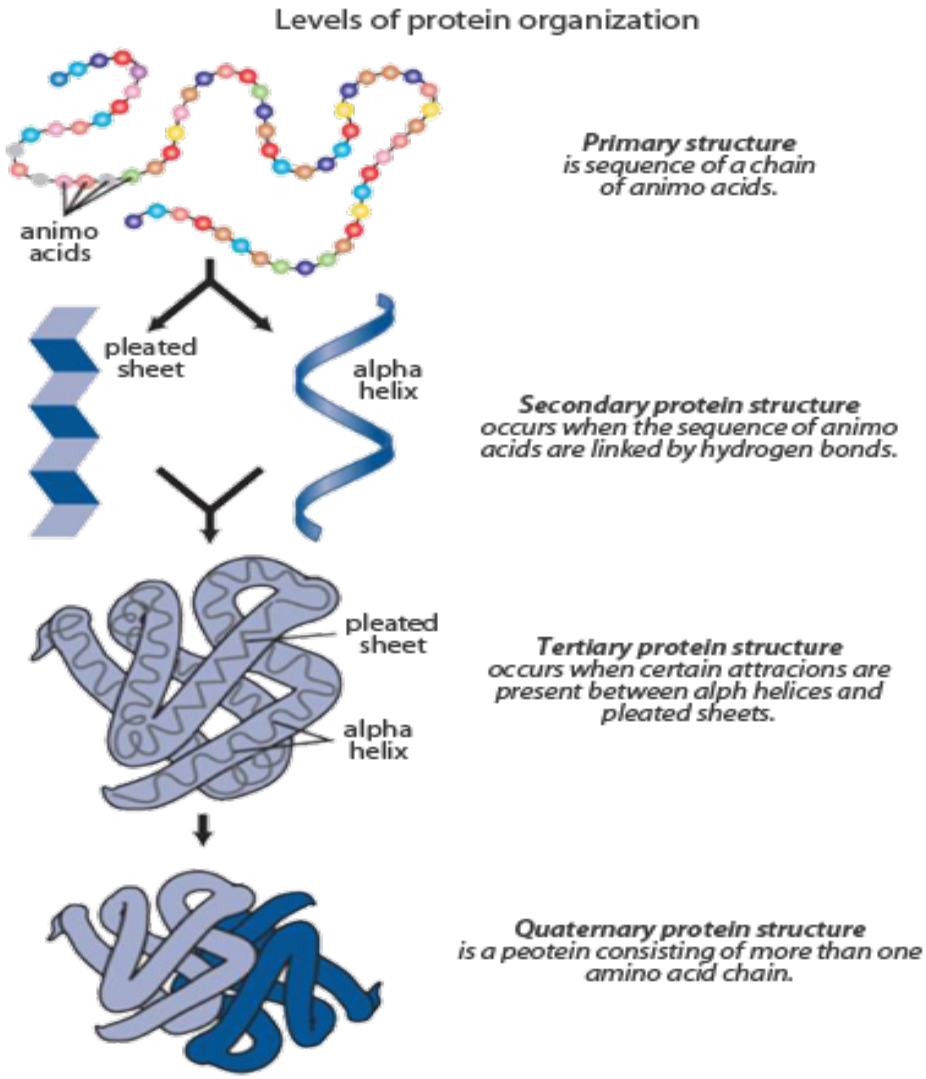

Figure 1.5: Four types of secondary structures of AMPs

date: 11/10/2016 (From: http://reflexions.ulg.ac.be/cms/c_352385/en/revealing-the-3d-structure-of-proteins?portal=j_55&printView=true)

1.3.1 AMPs from *Rana* species

Frogs which are members of the genus *Rana*, are usually known as 'true frogs'. They belong to the family *Ranidae* in the suborder Neobatrachia. There are about two hundred and fifty species of these worldwide. The skin secretions of *ranid* frogs contain peptides with antimicrobial activity, similar to other anuran species. The peptides can be classified into families according to their structural similarities.

Currently, four major types of peptides have been identified in frogs from Eurasian and North America, they are brevinin-1, esculentin-1, esculentin-2, and temporin. However, ranalexin, ranatuerin-1, ranatuerin-2 and palustrin peptides are rarely found in frogs from regions other than North America. Apart from those peptides which are mentioned above, there are some unusual peptides Eurasian frogs, such as brevinin-2, tigerinin and melittin-related peptides. The asymmetric distribution and complexity of these peptides means that some can be used as suitable subjects to help to identify the relatedness of species. Especially in the research of brevinin-1, brevinin-2 and ranatuerin-2, a more comprehensive cognition of the phylogenetic interrelationships among these species can be obtained. Some peptides have the ability to restrain the growth of bacteria and fungi but with a relatively low haemolytic activity. For example, esculentin-1, ranatuerin and ranalexin-1, have properties which make them good alternatives for development of useful therapeutic agents.

1.3.1.1 Brevinin-1

Researchers first isolated brevinin-1 from a skin extract of a pond frog called *R. brevipoda porsa* in Japan, and subsequently discovered many subgroups of the family in ranid species of North America and Eurasia (10). Brevinin-1 peptides can inhibit a wide range of Gram-positive bacteria, Gram-negative bacteria, and pathogenic fungi, but they are also associated with very strong haemolytic activity (11-13). The high antimicrobial and antiviral activities of brevinin-1 are not related to the disulphide bridge, even if it undergoes reduction and carboxamidomethylation, the activity of brevinin-1 is retained (14). Similarly, the antibacterial properties will not be influenced after the C-terminal cyclic hetapeptide region is transposed to the

central domain of the peptide. However, the haemolytic activity will be reduced by this change (15).

1.3.1.2 Ranalexin

Ranalexin was initially discovered in the skin secretion of *R. catesbeiana* tadpoles (16). Ranalexin has a wide range of potency to combat bacteria, for example, it has the strongest ability to inhibit Gram-positives such as *S. aureus.* However, it has a relatively low ability to inhibit some Gram-negatives such as *P. aeruginosa* (17). Also the activity of rananlexin against the intestinal parasite, *Cryptosporidium parvum,* can be increased by using traditional antibiotics such as lasalocid (18).

1.3.1.3 Ranatuerin-1

R. grylio (19) and *R. clamitans* are biologically related (20), and both contain ranatuerin-1. In terms of the four existing members of the family, amino acid substitutions are few and conserved, thus the structure of the primary peptide has not been compromised. Ranatuerin-1 is potent at inhibiting Gram-positive bacteria, Gram-negative bacteria and fungi, such as *S.aureus, E.coli, P. aeruginosa* and *C.albicans*. Ranatuerin-1 might be used in human bodies due to its inherent broad-spectrum antimicrobial activities and of note is that the haemolytic effect corresponding to its MICs, is relatively low.

1.3.1.4 Ranatuerin-2

When scientists first discovered the ranatuerin-2 family in the skin of the bullfrog, *R. catesbeiana,* they only retrieved five invariant amino acids from the severely compromised main structure with a few residue deletions (21). This variance in amino acid sequence is relevant to the antimicrobial function. It can inhibit *E. coli, S.*

aureus, and *C. albicans* at low concentrations and the haemolytic activity of ranatuerin-2 on human erythrocytes, is relatively low as well.

1.3.2 Action modes of AMPs

The modes of action of the AMPs in killing microbes are varied (22), and different peptides may act on specific bacterial species (23). It has been observed that bacteria like *E. coli*, and some filamentous fungi, can be killed by some peptides (24). AMPs can be generally described in two aspects: one is that they are rich in a remarkable number of hydrophobic residues, and the other is their cationic charge. Both anionic charge of AMPs and other biological reactions are critical for the peptides to exert their functions. The activities can be achieved either by using various methods to break down the bacterial cytoplasmic membrane, or by tackling critical cellular targets, such as the inhibition of protein folding and synthesis, cell wall synthesis, and enzymatic activity (25-27). Most of the bacterial surfaces are anionic or hydrophobic. Hence, the initial contact between the peptide and the target organism is via electrostatic connection. Piscidin, for instance, is able to infiltrate the membrane bi-layers, leading to pore formation through “toroidal-pore” “carpet,” and/or “barrel-stave” modes. What’s more, it may infiltrate deeper into the cell and bind to intracellular molecules thereby interfering with the synthesis of cell walls, DNA, RNA, and proteins, transforming the cytoplasmic membrane, stimulating autolysin, and inhibiting certain enzymes, thus threatening the living cells. Nevertheless, researchers tend to adopt the dual polarisation interferometry when conducting studies to illustrate the elimination mechanism of AMPs. Their bactericidal (instead of bacteriostatic) properties are differentiated from other existing antibiotics, which prompts researchers to identify their antimicrobial

activities by examination of the lowest concentrations that are adequate to restrain the bacteria, namely the minimal inhibitory concentration (MIC) (Figure1.6).

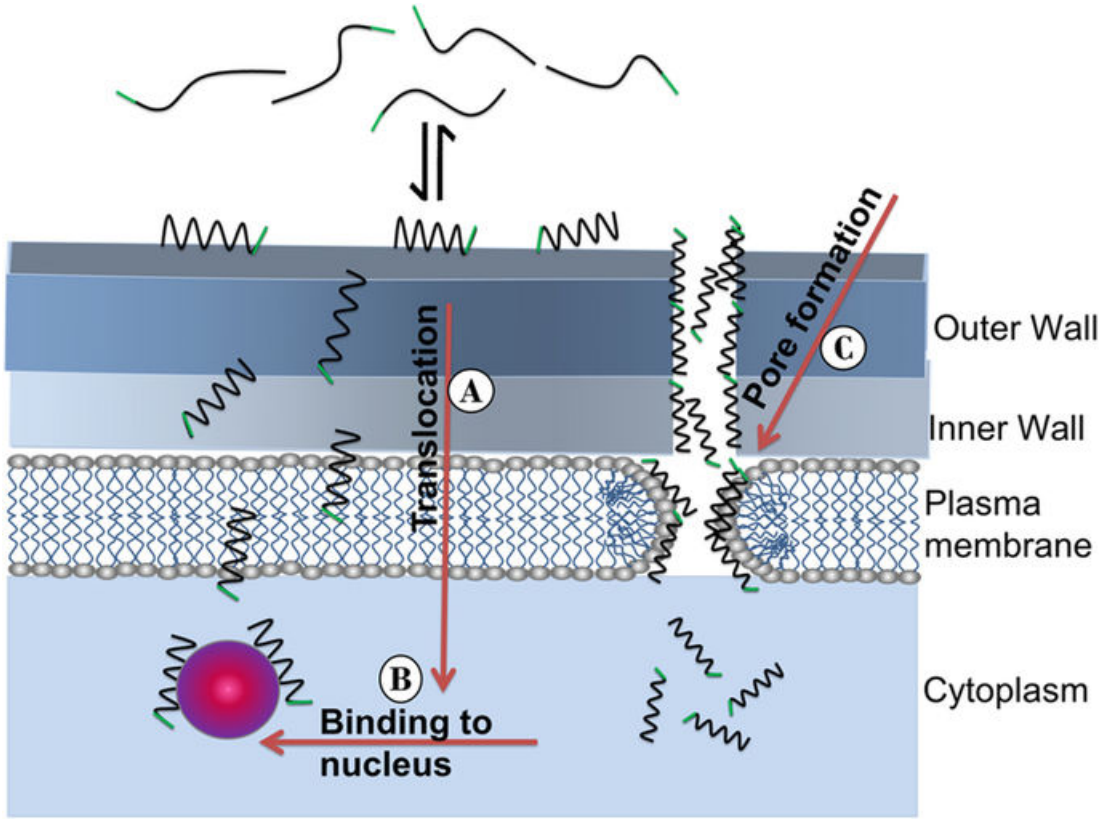

Figure 1.6: The mechanism of AMPs in damaging membranes and targeting intracellular contents. date: 16/10/2016 (From: https://www.nature.com/articles/srep43542?WT.ec_id=SREP-639-20170307&spMailingID=53569466&spUserID=ODkwMTM2NjQzMgS2&spJobID=1121186022&spReportId=MTEyMTE4NjAyMgS2&error=cookies_not_supported)

Model of antimicrobial activity	Examples of peptides
Transmembrane pore-forming mechanisms	
Toroidal pore	Magainin270, protegrin62, melittin55,81, LL-3765 and MSI-7890
Carpet	Dermaseptin S85, cecropin156,157, melittin158, caerin1.1159 and ovispirin64
Barrel stave	Alamethicin61,81
Modes of intracellular killing	
Flocculation of intracellular contents	Anionic peptides30
Alters cytoplasmic membrane septum formation	PR-39109, PR-26109, indolicidin110 and microcin25111
Inhibits cell-wall synthesis	Mersacidin112
Binds nucleic acids	Buforin II113 and tachyplesin114
Inhibits nucleic-acid synthesis	Pleurocidin115, dermaseptin115,PR-3973, HNP-1,-244 and indolicidin110
Inhibits protein synthesis	Pleurocidin115, dermaseptin115,PR-3973, HNP-1,-244 and indolicidin110
Inhibits enzymatic activity	Histatins117, pyrrhocoricin, drosocin and apidaecin118

Table 1.1 The modes of AMP action.

1.3.3 Antibacterial Mechanism of AMPs

AMPs can destroy membranes of cancer cells or bacteria, which causes aqueous solutions to flow from the cytoplasm, resulting in the death of the cancer cells and bacteria. They selectively inhibit bacterial growth or kill the bacteria. The AMPs could eliminate the bacteria through different mechanisms to penetrate cell membranes. The antimicrobial activity modes can be divided into four phases:

i) Initial peptide interaction with the cell membrane.

ii) Threshold concentration, conformational phase transition, and the formation of higher structures.

iii) Membrane permeabilisation.

iv) Cell killing.

The peptides are able to create a lipid bilayer in parallel to the membrane when the peptide/lipid ratios are relatively low (28). The peptides are orientated perpendicularly to the membrane as the ratio increases. As the peptide/lipid ratios rise, the transmembrane pores can be observed, along with peptide molecules veering perpendicularly and inserting into the bilayer. The ratio between the peptide and target lipid changes with their composition, (29) and some models could be employed to identify the membrane permeabilisation.

- In the “barrel-stave” model, the peptide forms a helix coil in the membrane like a central lumen. It is much like a barrel composed of helical peptides as the staves (30, 31). The alamethicin is employed as an inducer to create the unique transmembrane pore. Alamethicin develops into the α-helical configuration, it combines and infiltrates into the oriented bilayers, and this process has been identified by some advanced techniques, such as oriented

circular dichroism (29, 30), neutron scattering (28) as well as synchrotron-based X-ray scattering (32). In this model, the hydrophobic peptide areas, along with the lipid core of the bilayer and the hydrophilic peptide regions, develop the pore's interior region. The composition of the modified bilayer lipid plays a significant role in balancing peptide aggregation and the number of peptides.

- The "carpet model" shows that the AMPs are active on the membrane's surface because there is an increasing number of peptides on the bilayer surface (33). These peptides are attracted to the anionic phospholipid head groups through the electrostatic mode, and they could cover the surface of the membrane like a carpet. At high peptide concentrations, the surface-oriented peptides can prevent the bilayer from creating micelles (34, 35). At a critical threshold concentration, many more peptides could pass through the membrane as a consequence of the formation of toroidal transient holes. After the disruption of the bilayer of cell membrane, some components will be released, such as micelles (36, 37).
- In the "toroidal-pore" model, the AMPs enter the membrane in the form of helical structure and create the cross-membrane channels. They are able to access into the lipid bilayer where the peptides are always combined with the lipid head groups, even if they are perpendicularly inserted, and this is the specific behaviour that used to differentiate other models (30). The polar faces of the peptides are similar to the polar head groups of the lipids (38).

Currently, several approaches are employed to identify the AMPs' mode of actions. Specifically, the solid-state NMR is a useful technique to analyse the disruption of the bacteria's membrane. It is based on the atomic-level resolution to achieve this

purpose. However, each method leads to a slightly different explanation as to how the peptide exerts it function. A single technique is not sufficient to identify the mechanism of action of the peptides comprehensively.

1.4 Research Approaches

1.4.1 Microscopy

Microscopy is a useful approach to observe the activity of AMPs on microbial cells with the naked eye. The analysis through microscopy has shown that the effects of the different AMPs are varied on different cells. This indicates that different peptides can be differentiated by various mechanisms or target sites, and this hypothesis has been confirmed by studying the activities of SMAP29 and CAP18 against *P. aeruginosa* (39).

1.4.2 Fluorescent dyes

The amount of released dextran, which is labelled by fluorescein, can be used to reflect the membrane permeability of cells. Also, the number of immunoglobulins, calcein or other probes, which infiltrate into the membrane vesicles, are able to evaluate the ability of cell permeation (40-44). At a certain period of time, an analysis can be conducted by detecting the release of labelled peptide concentration probes and the membrane composition probe (45). Magainin 2, for example, induces rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. As a result, the flip-flop half-life of the fluorescent lipids is extended to several minutes, and there is no direct link between the flip-flop rate and the initial labelling conditions (46). Therefore, a model can be summarised from these results, that is, the lipids are spread over the membrane by the way of lateral diffusion. All of this occurs on the pore walls which are constituted by peptides and lipids.

1.4.3 Ion channel formation

Another useful approach is to monitor voltage-dependent channels, which are used for evaluating whether an AMP-induced pore is formed and stable in membrane bilayers. The ability of AMPs to infiltrate bi-layers can be evaluated by measuring electrical currents. Using this technique together with electron microscopy, it can be found that there are some small lesions in cecropin-treated *E. coli*. In this situation, the osmotic concentrations are approximately equal to the concentrations required to kill cells *in vitro*, it indicates that there is an intimate relationship between the physiological effects of HNP-1 and NP-1 and their cytotoxic effects (47).

1.4.4 Circular dichroism (CD) and orientated circular dichroism

CD is usually adopted to measure the secondary structure and orientation of an AMP that adheres to the lipid bi-layer. The environment for CD measurement is constructed by researchers, which is humid and with a light incidence similar to the sample surface (29, 48). Results obtained from the measurements indicate that conditions of the outer surface of the bacteria and their membranes play a critical role in conformational shifts of AMPs, which help the peptide to stick to and permeate into the membrane.

1.4.5 Solid-state NMR spectroscopy

Solid-state NMR spectroscopy is one of the useful techniques which is used for measuring the secondary structure. It can also be applied for detecting the orientation of AMPs and the variations of the lipid bilayer (36, 38). The activities of AMPs and the composition of membranes can be deduced from the data.

1.4.6 Neutron and X-ray diffraction

In the neutron in-plate scattering process, the densities of the neutron scattering length are different between two kinds of membranes. One is the membrane that has the alamethicin- and magainin-induced pores, the other is the membrane without any other components (49, 50). It is a simple and efficient method used in the target multilayers or liquids which reflect the diffraction by peptide-induced pores (51, 52). Deuterium can be used as a contrast in measuring the diameters of the inner and outer pores. The data can be precisely obtained by analysing the contrast variations (52). In the in-plane scattering and off-plane scattering, the curves between alamethicin and magainin are varied. The patterns in the in-plane scattering are similar, however, the latter are obviously distinct (51). This result indicates that the pores have different sizes even though both are formed in membranes (52). In the layered x-ray diffraction, there is a significant relationship between the concentration of AMPs and the thickness of the membrane (29, 53).

1.5 Aims and objectives of this thesis

1. Isolation of mRNA from frog skin secretions and construction of a cDNA library by reverse transcription,

2. To perform ‘shotgun’ cloning by using degenerate primers to identify a peptide precursor-encoding cDNA.

3. To use bioinformatic tools to translate DNA sequence and confirm the primary structure of the encoded peptide via online BLAST alignment analysis.

4. To chemically synthesise the identified mature peptide by solid-phase synthesis methodology and purify the crude synthetic peptide using RP-HPLC.

5. Assessment of the bioactivities of this peptide by use of antimicrobial assays, haemolytic assays and anti-cancer cell assays.

6. To become familiar with the approaches for the development of novel drugs at an early stage.

7. To become capable of operating equipmemt and conducting experimental design and performance independently.

Chapter 2

The determination of QUB-3005 peptide sequence using molecular cloning

2.1 Materials and Methods

2.1.1 Skin secretion collection from *Rana amurensis*

Skins of the Heilongjiang brown frog (*R. amurensis*) were purchased in dry form from a number of Traditional Chinese Medicine pharmacies throughout Northern China in Heilongjiang, Jilin and Liaoning Provinces. The dried skins were cellophane-wrapped and had been kept at ambient shop temperatures (20–35 °C) for many months.

Samples from the skins of four frogs (dry weight 202 mg in total) were chopped into small (1–2 mm^2) pieces and placed directly into 10 ml of boiling deionised water for 10 min. Following this, the extract was removed from the heat source and permitted to cool to room temperature. Particulates were removed by centrifugation at 8000 x g for 10 min after which the resultant clear supernatant was decanted and lyophilised. The lyophilised skin secretion was stored at -20 °C before use.

2.1.2 Molecular Cloning

2.1.2.1 mRNA isolation from the skin secretion of *Rana amurensis*

The Dynabeads® mRNA DIRECT™ Kit is designed for simple and rapid isolation of poly-A mRNA. Short sequences of oligo-dT are covalently bound to the Dynabeads oligo$(dT)_{25}$, which will hybridise to the poly(A) tail of mRNA. The isolated mRNA obtained in this way can be directly used in all downstream applications in molecular biology. Importantly, all the reaction tubes, tips and solutions used to handle mRNA should be RNAse-free.

5.1 mg of skin secretion of *Rana amurensis* was placed in a 1.5 ml tube and then was dissolved in 1 ml Lysis/Binding Buffer. The tube was vortexed and placed on ice for

3 min and these steps were repeated five times. Afterwards, the tube was centrifuged briefly and stored on ice.

The Dynabeads were resuspended thoroughly to obtain a uniform suspension before use. The beads were transferred to a 1.5 ml tube and placed on a magnet for 30 s. Following that, 250 µl lysis/binding Buffer was added into the tube to wash the beads.

The sample was transferred to the Dynabeads tube slowly to avoid physical impact on the beads. The tube was shaken gently for 3 min and then placed on ice for 2 min, which was repeated for 4 times without touching the tube. After about 20 min, the tube was placed on the magnet bar, and then the supernatant was discarded when the liquid turned transparent. Then washing buffer was added for mixing for 3 min of gentle shaking then 2 min on ice. This was repeated 4 times.

After these washing steps, 18 µl of Tris-HCl was added to the tube to elute the mRNA from the Dynabeads. The tube was incubated in a heating block at 80 ℃ for 2 min and placed immediately on the magnet bar to remove the supernatant to a new RNase-free tube, which was placed immediately on ice because of the instability of mRNA.

2.1.2.2 cDNA library construction

First-strand cDNA synthesis was accomplished by use of a SMART^{TM} RACE cDNA Amplification Kit (BD Clontech, UK).

The mRNA solution was separated into five tubes. Three of these contained 4 µl of isolated mRNA for each 3'RACE-ready cDNA and the other two contained 3 µl of mRNA for each 5'RACE-ready cDNA. Different reagent mixtures were prepared as shown in Table 2.1.

Table 2.1 The components of the 3’ RACE and 5’ RACE PCR

5’-RACE-Ready cDNA	3’-RACE-Ready cDNA
3 μl sample	4 μl sample
1 μl 5’ CDS primer (12 μM)	1 μl 3’ CDS primer (12 μM)
1 μl SMART Ⅱ oligo (12 μM)	

All the tubes were centrifuged briefly to collect all the solution at the bottom. The tubes were incubated at 70℃ for 2 min and then cooled on ice for another 2 min. The components of each reaction were prepared as follows:

Table 2.2 The components for each reverse transcription reaction.

Component	Final volume	Final concentration
5X First-Strand Buffer	2 μl	1 X
DTT	1 μl	2 mM
dNTP Mix	1 μl	1 mM
BD PowerScript Reverse Transcriptase	1 μl	10 U
mRNA template	5 μl	200 ng

Furthermore, all the tubes were briefly centrifuged and incubated in the PCR machine at 42℃ for 1.5 h. Subsequently, 50 μl of PCR-grade water was added to each tube, incubated at 72℃ for 7 min and stored at -20℃.

The PCR cycling procedure was set as follows. First of all, initial denaturation contained 1 cycle at 94 °C for 60 s. Then there were 40 cycles in the next stage. It mainly contained 3 steps in this stage. The first step was denaturation, it lasted for 20 s at 96 °C. The second step was annealing, in this step, primer annealing for 50 s at 53 °C and 55 °C. The last step was extension, it would last for 4 min at 60 °C. Then tubes were kept at 4 °C.

2.1.2.3 Polymerase Chain Reaction (PCR) of cDNA

To obtain full-length prepropeptide nucleic acid sequence data, a SMART-RACE kit (Clontech UK) was used in this reaction. The cDNA was subjected to 3'- RACE procedures as described by the manufacturer. The 3'-RACE reactions employed a NUP primer (supplied with the kit) and degenerate sense primer pool (S1; 5'-GAWYYAYYHRAGCCYAAADATG-3') that was designed according to a highly-conserved domain of the 5'-untranslated region of previously-characterised antimicrobial peptide cDNAs from *Rana* frog species.

Reagents (Table 2.3) were taken out of the freezer. After thawing, all the reagents were centrifuged and prepared to be used (except PCR-Grade Water). Then 12.4µl PCR-Grade Water, 6µl 10 × BD Advantage 2 PCR Buffer, 0.8 µl 50 × BD advantage 2 Polymerase mix (Taq DNA polymerase) (10 mM), 2 µl sense primer (20 mM), 2 µl NUP (20 mM) and 0.8 µl Polymerase mix were added into a tube. After mixed, 12 µl master mix was separated to 2 tubes. To make the control group, 10 µl PCR-grade water was added into one of the tubes which contains 12 µl master mix. After a thorough mix, the 22 µl solution was separated into two tubes. These two tubes were used as control group.

On the other hand, in order to set up the sample group, 10 µl cDNA sample was added into the other tube which had 12 µl master mix. After mixing thoroughly, the 22 µl solution was separated into two tubes. These two tubes were used as a sample group.

Then one tube of the control group and one tube of the sample group were put in temperature A, the other one in control group and sample group were put in temperature B in PCR machine (Temperature A and B are primer annealing

temperatures. Primer annealing temperatures are controllable, generally different kinds of primer is variably efficient at different temperatures. In this experiment, they were 53 °C and 55 °C).

Table 2.3 Reagents and volume of the Master Mix in PCR.

Reagent	**Volume for one portion of the Master mix (μl)**	**Final concentration**
PCR-Grade Water	2.6	
dNTP Mix (10 mM)	0.2	0.2 mM
sense primer (20 μM)	0.5	1 μM
NUP (20 mM)	0.5	1 μM
10×BD Advantage 2PCR Buffer	1	
50 × BD advantage 2 Polymerase mix	0.2	

The PCR cycling procedure was set as follows. First of all, initial denaturation contained 1 cycle at 94 °C for 60 s. Then there were 40 cycles in the next stage. It mainly contained 3 steps in this stage. The first step was denaturation, it lasted for 20 s at 96 °C. The second step was annealing, in this step, primer annealing for 50 s at 53 °C and 55 °C. The last step was extension, it would last for 4 min at 60 °C. Then tubes were kept at 4 °C.

2.1.2.4 Gel analysis for RACE PCR products

In molecular cloning, agarose gel electrophoresis can be used to separate DNA and RNA fragments amplified by PCR from mixed solutions by their relative sizes. DNA

fragments of a larger size move at a slower rate than those of a smaller size in an electrical field due to the pores in the gel matrix, which play a sieving or sorting role. The sizes of the DNA or RNA sequences can be estimated by comparing with standard DNA bands on the solid gel or the distances that DNA fragments have travelled in the agarose gel. The percentage of agarose in the gel decides the positions of DNA fragments of different sizes after separation. The more agarose in the gel, the slower the DNA fragments move in the electrical field.

0.45 g of agarose was dissolved in 35 ml of 1 x Tris-borate-EDTA (TBE) buffer and ethidium bromide (EB), was added into this mixed solution as a fluorescent marker for DNA band visualisation in a UV transilluminator. The mixed solution was poured into a casting tray and an 8-toothed comb was inserted. Solidified agarose gel with pores was formed in the casting tray after 30 min. Then the solid gel was placed in the correct orientation and was soaked in 1 ×TBE buffer. Subsequently, 3.5 µl of DNA Ladder was loaded into one of the wells and 2.5 µl of PCR products were mixed with 0.7 µl of loading dye for other wells individually. A 95 V electrical current was applied and ran until the smallest sized dye band reached the border of the gel.

2.1.2.5 Purification of RACE PCR products

The product generated from the PCR step contains some impurities, such as redundant dNTPs, enzymes, non-specific PCR products and inaccurate or unused primers. Purification of PCR product is essential for the following transcription step and one must avoid side reactions caused by impurities. A silica-based membrane is used to rapidly isolate target PCR product based on the size of DNA fragment.

The samples in Tube 1 and Tube 2, were put together in a 1.5 ml tube (total 17µl). 100µl CP Buffer was added (CP Buffer: Sample = 5:1(v/v)). All the liquid was

removed to the purification column, centrifuged for 60 s and the flow-through was discarded. The samples were washed in the same way by 500 µl and 700 µl washing buffer respectively. After centrifugation for 2 min, the flow-through was discarded and the column was transferred to a new 1.5ml tube. 30 µl water was added to the column, and the column was kept for 2 min, centrifuged for 2 min, and the column was discarded finally. Those tubes were placed in the Eppendorf concentrator plus for 1 h to dry.

2.1.2.6 Ligation

The ligation was performed using the pGEM®-T Easy Vector system (Promega). The sequences of promoter and multiple cloning sites of the pGEM®-T Easy Vectors are shown in Figure 2.1.

To achieve the optimised concentration, 10 µl of deionised water were added into the dried DNA sample and mixed by vortexing. Then the volume and final concentration of the following components were added into the ligation reaction.

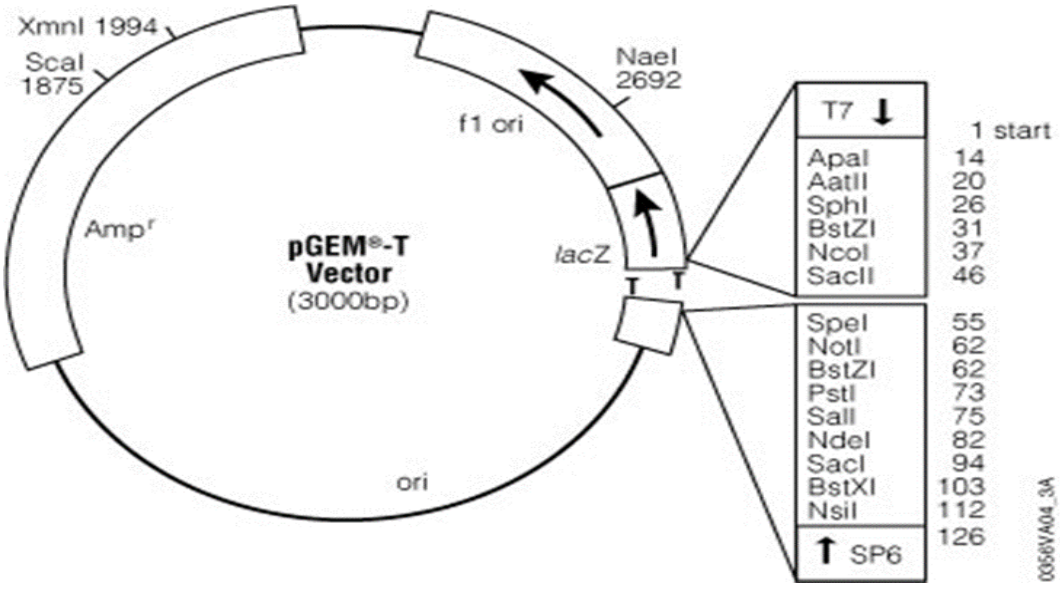

Figure 2.1: pGEM-T Easy Vector System

(From: https://www.promega.co.uk/resources/product-guides-and-selectors/protocols-and-applications-guide/cloning/)

Table 2.4 The volume and final concentration of the components in the ligation reaction.

Reagents	Volume	Final concentration
DNA product	1.5 µl	
2X Rapid ligation buffer	2.5 µl	1X
pGEM-T Easy Vector	0.5 µl	25 ng
T4 DNA ligase	0.5 µl	3 Weiss units/µl

All reagents were gently centrifuged to the bottom of the tube without air bubbles. The reaction was incubated at room temperature for 1 h and then incubated at 4℃ overnight.

2.1.2.7 Transformation

Preparation:

LB plates Preparation: (5 LB Plates with ampicillin/ IPTG/ X-Gal for a sample.)

A 500 ml glass bottle received 200 ml of double deionised water and 6.4 g LB agar. After a thorough mix, the glass bottle was autoclaved. Before use, the LB agar in the glass bottle was reheated to dissolve by using a microwave oven. When this cooled to 50-60 ℃, 550 µl ampicillin (100 µg/mL) was added into the bottle. Then 11 ml mixture was transferred to each Petri dish. After cooling, plates were tied together and put at 4 ℃ before use. 100 µl IPTG (Promega USA; 0.1 M) and 20 µl X-Gal (Promega USA; 50 mg/ml) were spread on LB agar plates and incubated at 37 ℃ for 30 min before use.

Transformation was used to transfer the recombinant DNA into the host bacterial cells by using JM109 *E. coli* cells to amplify targeted individual DNA fragments

(Figure 2.2). A labelled tube received 2.3 µl ligation product obtained from cells (JM109, Promega, USA; original concentration: $>10^8$ cfu/µg). These were taken out of -80 ℃ and put into an ice box immediately. After the cells had thawed, 50 µl competent cells were transferred into the tube. (Competent cells have the ability to take up extracellular DNA from the environment.) Then, the tube was gently flicked and incubated on ice for 20 min. After that, the tube was incubated at 42 ℃ for 47 s exactly on a heating block, while a thermometer was employed to ensure the accurate temperature. Then the tube was put back on ice for 2 min exactly and 950 µl SOC medium (Invirtrogen) was added into tube slowly down the wall. The reaction was set on a shaking incubator at 150 rpm 37 ℃ for 2.5 h.

Transformation culture was gently mixed, then 100 µl of culture was quickly transferred into Petri dishes and spread. LB Plates with ampicillin/ IPTG/ X-Gal and transformation culture were tied together and incubated (upside down) at 37 ℃ for 22 h in an incubator.

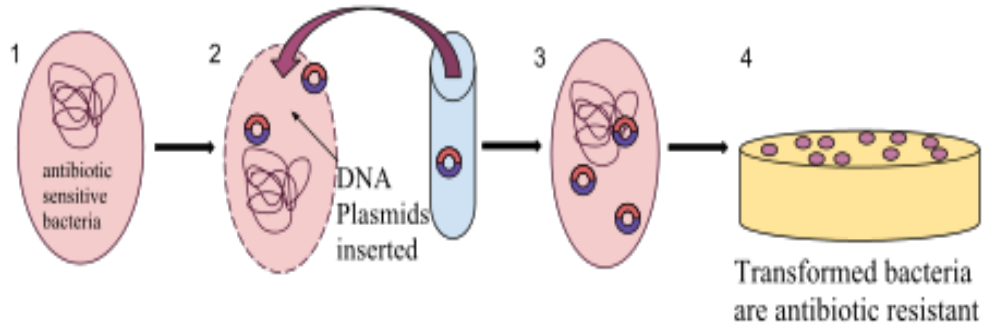

Figure 2.2: Transformation of recombinant DNA

2.1.2.8 Blue and white screening for recombinants

As the coding information holder of β-galactosidase, lacZ sequence has a multiple cloning site where the foreign DNA can be inserted. The completeness of lacZ sequence determines the composition of β-galactosidase. When the plasmid vector is

taken up by competent cells, α-complementation process will be triggered by the lacZΔM15 deletion mutation and the functional β-galactosidase enzyme will be produced.

If the foreign DNA was not inserted, X-gal in plates would be hydrolysed by β-galactosidase to 5, 5'-dibromo-4, 4'-dichloro-indigo which was an insoluble blue pigment. Therefore, the non-recombinant cells colonies would appear blue in colour while the recombinant ones appear white.

Preparation:

LB plates Preparation: (3 LB Plates with ampicillin/ IPTG/ X-Gal for a sample.)

Each LB agar plate was spread with 100 μl IPTG and 20 μl X-Gal. Then all the plates were incubated at 37 ℃ for 45 min before white colonies were transformed.

A Bunsen burner was opened and moved around to sterilise the air. Then ethanol was sprayed to clean the bench. New plates were taken out of the incubator and drawn with squares and labelled (Figure 2.3). Plates with colonies were taken from the incubator and pure white colonies were selected and labelled. Pure white colonies were sampled and streaked on the square. In addition, each colony was located on each square on the grid that was marked with a number. Finally, new plates were put into incubator upside down with an untied plastic bag at 37 ℃ overnight.

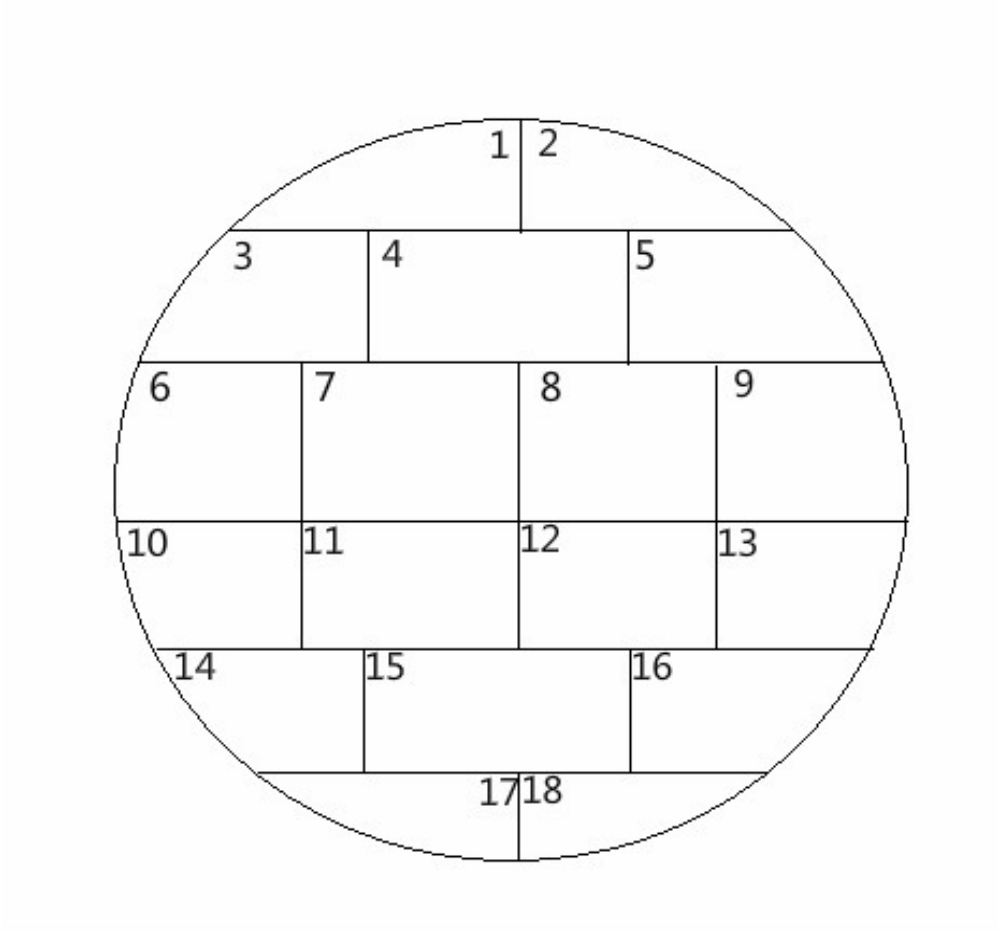

Figure 2.3: The grids on the blue and white colony selected Petri dish

*(The NO.18 grid was used to cool down the inoculation loop and marked with the name of frog, primer and time.)

2.1.2.9 Isolation of the recombinant DNA from JM109 cells

Pure white colonies were counted and labelled. The same number of autoclaved tubes were labelled and received 20 µl of double deionised water. Pure white colonies were harvested from each square by 200 µl autoclaved tips. After that, tips were put into tubes and shaken in the double deionised water. Then cells were shaken off and were heated up to 100 ℃ for 5 min by a heating block and put on ice for 5 min. After 5 min cells were vortexed for 30 s and centrifuged at 20000 x g for 5 min in an Eppendorf Centrifuge 5424 (Eppendorf, Germany).

2.1.2.10 Cloning PCR

The cloning PCR reaction was carried out using GoTaq® cloning Enzyme System (Clontech). The reagents were added to a 1.5 ml tube as shown in Table 2.5, vortexed for 2 to 3 s and centrifuged briefly. 12 PCR tubes were prepared. 47.5 µl master mix and 2.5 µl supernatant of broken cells were added to each PCR tube, pipetted, and then centrifuged briefly to remove the bubbles. PCR tubes were put in the PCR machine for 2.5 h. The cloning PCR products were stored in the fridge at 4℃.

Table 2.5 The volume and final concentration of the components in the cloning PCR reaction.

Reagents	**Volume**	**Final concentration**
10X PCR Buffer	10 µl	1 X
50X dNTP Mix	1 µl	0.2 mM
M-13 former primer	2.5 µl	5 µM
M-13 reverse primer	2.5 µl	5 µM
GoTaq® Polymerase	0.25 µl	
Isolation DNA product	2.5 µl	10-1000 ng
PCR-Grade Water	31 µl	

PCR tubes were putted into the PCR machine which then performed the following programme:

- 94ºC for 1 min.
- 31 cycles: denaturation 30s at 94℃, primer annealing 30s at 55℃, extension for 180s at 72℃.

- Final extenxion step: 72℃ for 3min and storage at 4℃ prior to use.

2.1.2.11 Gel analysis of cloned PCR products

Details of how to perform gel analysis have been described in section 2.1.2.4.

2.1.2.12 Purification of cloned PCR products

This step was performed as described in section 2.1.2.5. However, the volume of PCR water which was added into the tube before incubation was different. 20µl of PCR water was added into the tubes in this procedure.

2.1.2.13 Sequencing Reaction

A BigDye® Terminator v3.1 Cycle Sequencing Kit was used in this step. For each sample, 12.4 µl DD water, 3.57 µl Sequencing buffer, 2.86 µl ready reaction mix and M13 forward primer or M13 reverse primer were added into a 1.5 ml tube before allocating 18.4 µl into the PCR tube. 2.5 µl of DNA sample was added into each tube (Table 2.6). Then the tubes were placed in the PCR machine and the programme was set as follows: 26 cycles of Rapid thermal ramp at 96℃ for 20 s, Rapid thermal ramp at 55℃ for 10 s and Rapid thermal ramp at 60℃ for 4 min.

Table 2.6 The reagents and corresponding information of the mixture were added into each PCR sample. This kind of mixture was added into each tube. If the result was showed DT, the M13-F should be replaced by M13-R.

Reagents	Each volume
5X Sequencing buffer	3.57 µl
DD water	12.4 µl
M13-F primer/M13-R	1.14 µl
Ready reaction mix	2.86 µl
DNA sample	2.5 µl
total	20.9 µl

2.1.2.14 Purification of extension products

A certain number of tubes received 10 µl PCR-grade water. Meanwhile, each sequencing reaction PCR tube received 72 µl 95 % ethanol. Then solution in PCR tubes was transferred to tubes which contained 10 µl PCR grade-water one-to-one. After that, each tube was vortexed for 30 s and incubated at room temperature for 20 min. Then, solutions were centrifuged at 20000 x g in Eppendorf Centrifuge 5424 (Eppendorf, Germany) for 20 min. Supernatant was removed as quickly as possible.

Then each tube received 260 µl 70% ethanol and was vortexed for 30 s. After that, each tube was centrifuged at 20000 x g in an Eppendorf Centrifuge 5424 (Eppendorf, Germany) for 10 min. Supernatant was removed as quickly as possible. Tubes were put on ice for 1 min. Then tubes were put on a heating block for 1 min at 95 ℃ with lids opened. One minute later, tubes were cooled to room temperature. These three steps were repeated 2- 3 times. Samples were kept at room temperature until no ethanol remained.

2.1.2.15 Preparation of samples for sequencing

10.3µl of HIDI were added to each sample tube. The tubes were heated in a heating block at 95℃ for 4.5 min. At the end of the 4.5 min, the tubes were placed in ice for 3 min. The contents were then transferred to a 96-well plate. Then, the samples were sequenced using an automated ABI 3100 capillary DNA Sequencer. The principle of Sanger sequencing is shown in Figure 2.4. This method was based on the selective incorporation of chain-terminating dideoxynucleotides by DNA polymerase during DNA replication. It required a single-strand DNA template, a short primer (M13F/M13R), dNTP, ddNTP, a DNA polymerase. ddNTP lacks 3’ hydroxy group required for the formation of a phosphodiester bond between two nucleotides,

causing DNA Polymerase to cease extension of DNA when a modified ddNTP is incorporated. The ddNTP may be radioactively or fluorescently labelled for detection in automated sequencing machines.

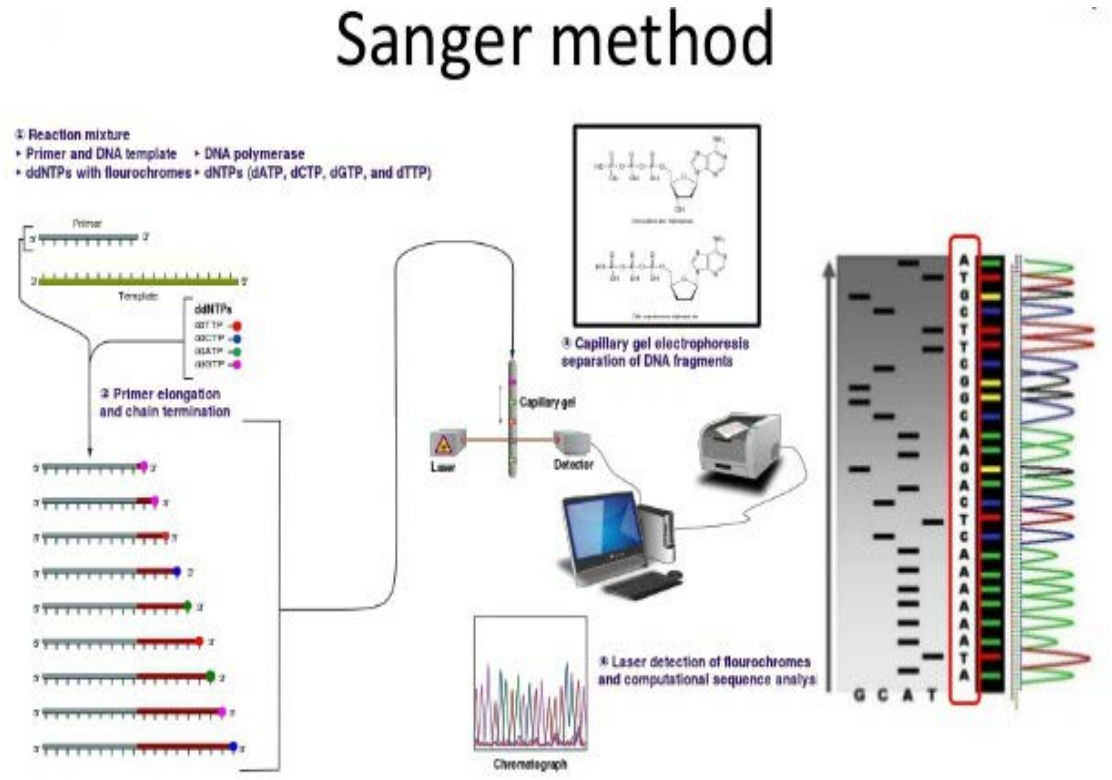

Figure 2.4 The Sanger (chain-termination) method for DNA sequencing.

2.2 Results

A cDNA encoding the biosynthetic precursor of QUB-3005 was successfully cloned from the skin secretion of *Rana amurensis*. The nucleotide and translated open-reading frame amino acid sequence of this cDNA are shown in Figure 2.5. The open-reading frame consisted of 72 amino acid residues, including a putative 22-residue signal peptide at the N-terminus, followed by a 21-residue acidic residue-rich spacer domain , and a predicted mature peptide of 29-residues at the C-terminus. A BLAST search indicated that the amino acid sequence of deduced mature peptide QUB-3005 shared the highest identity with ranatuerin-2Toc, ranatuerin-2Toa, ranatuerin-2TOb, preproranatuerin-2Ob and preproranatuerin-2Oe. Meanwhile, multi-sequence

alignment indicated that QUB-3005 shared moderate similarity with other AMPs. The sequence identities between QUB-3005 and ranatuerin-2Toc, ranatuerin-2Toa, ranatuerin-2TOb, preproranatuerin-2Ob and preproranatuerin-2Oe, were 71%, 74%, 72%, 79% and 79%, respectively (Figure 2.6). Thus, together, the mature peptide sequence was confirmed as: GLMDFLKGAGKKLLAAGLDKLNCKLTGKC-COOH, a peptide which was named QUB-3005, according to it molecular mass.

```
     M  F  T  A   K  K  S   M  L  L   L  F  F  L   G  T  I ·
   1 ATGTTCACCG CGAAGAAATC CATGTTACTC CTTTTCTTTC TTGGGACCAT
     TACAAGTGGC GCTTCTTTAG GTACAATGAG GAAAAGAAAG AACCCTGGTA
     · T  L  S   L  C  Q  E   D  E  R   G  A  D   E  D  D  E ·
  51 CACCTTATCT CTCTGTCAGG AAGATGAGAG AGGTGCCGAT GAAGACGATG
     GTGGAATAGA GAGACAGTCC TTCTACTCTC TCCACGGCTA CTTCTGCTAC
     ·  G  E  M   T  E  E   Q  K  R  G   L  M  D   F  L  K
 101 AAGGGGAAAT GACCGAGGAA CAAAAAAGAG GTCTCATGGA TTTTTTGAAG
     TTCCCCTTTA CTGGCTCCTT GTTTTTTCTC CAGAGTACCT AAAAAACTTC
      G  A  G  K   K  L  L   A  A  G   L  D  K  L   N  C  K ·
 151 GGCGCAGGCA AGAAATTGTT AGCAGCTGGT CTGGATAAGC TAAATTGTAA
     CCGCGTCCGT TCTTTAACAA TCGTCGACCA GACCTATTCG ATTTAACATT
     · L  T  G   K  C  *
 201 ACTTACTGGT AAATGTTAAA ACCTGAATGG AAAGTCTCCT GATGTGGGAT
     TGAATGACCA TTTACAATTT TGGACTTACC TTTCAGAGGA CTACACCCTA
 251 ATCATTAAGC TACAAAAATG CTAAATGTCT AATAAAAAAA AAAAAAAAAA
     TAGTAATTCG ATGTTTTTAC GATTTACAGA TTATTTTTTT TTTTTTTTTT
```

Figure 2.5 Nucleotide and translated open-reading frame amino acid sequence of the cDNA encoding the biosynthetic precursor of QUB-3005 cloned from a *Rana amurensis* skin secretion-derived library. The putative signal peptide is double-underlined, the mature peptide is single-underlined and the stop codon is identified by an asterisk.

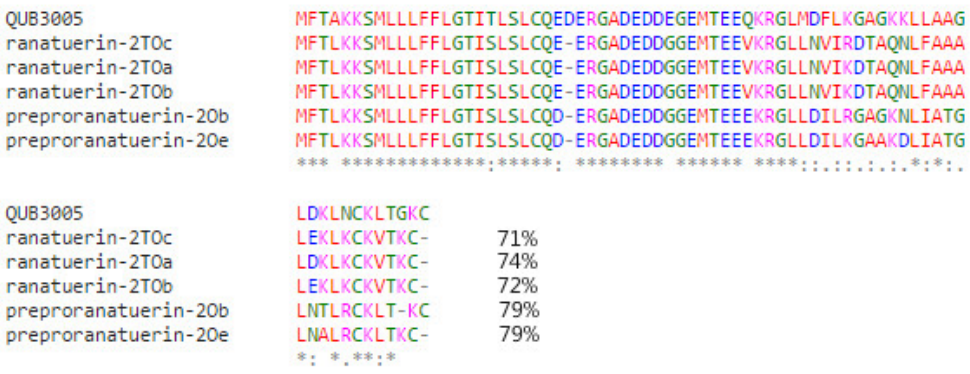

Figure 2.6 The sequence alignment of QUB3005 with other AMPs. The identical amino acid residues were indicated by a star (*). The sequence identities of ranatuerin-2Toc, ranatuerin-2Toa, ranatuerin-2TOb, preproranatuerin-2Ob and preproranatuerin-2Oe to QUB3005, are labelled on the right.

Chapter 3

Synthesis and purification of peptide, QUB-3005

3.1 Materials and Methods

3.1.1 Solid Phase Peptide Synthesis (SPPS) of QUB-3005

Solid phase peptide synthesis (SPPS) was employed using a Tribute Peptide Synthesiser (Protein Technologies, Inc., AZ, USA).

The first amino acid of the chain was attached to a solid polymer by a covalent bond. In addition, Wang resin and amino resin were often used as the solid polymer. Wang resin was located at the most carboxyl (C') terminal end of the first amino acid. A base-labile 9-fluorenylmethoxycarbonyl (Fmoc) group was located at the amino (N') terminal end and protected each amino acid. Another acid-labile group, usually located in the amino acid's side chains to protect each amino acid, were varied and included such as tertiary-butyl (tBu). Peptides were extended from the C' terminal end to the N' terminal end by forming "peptide bonds" between amino acids and finally, the completed peptide was removed from the resin (Figure 3.1).

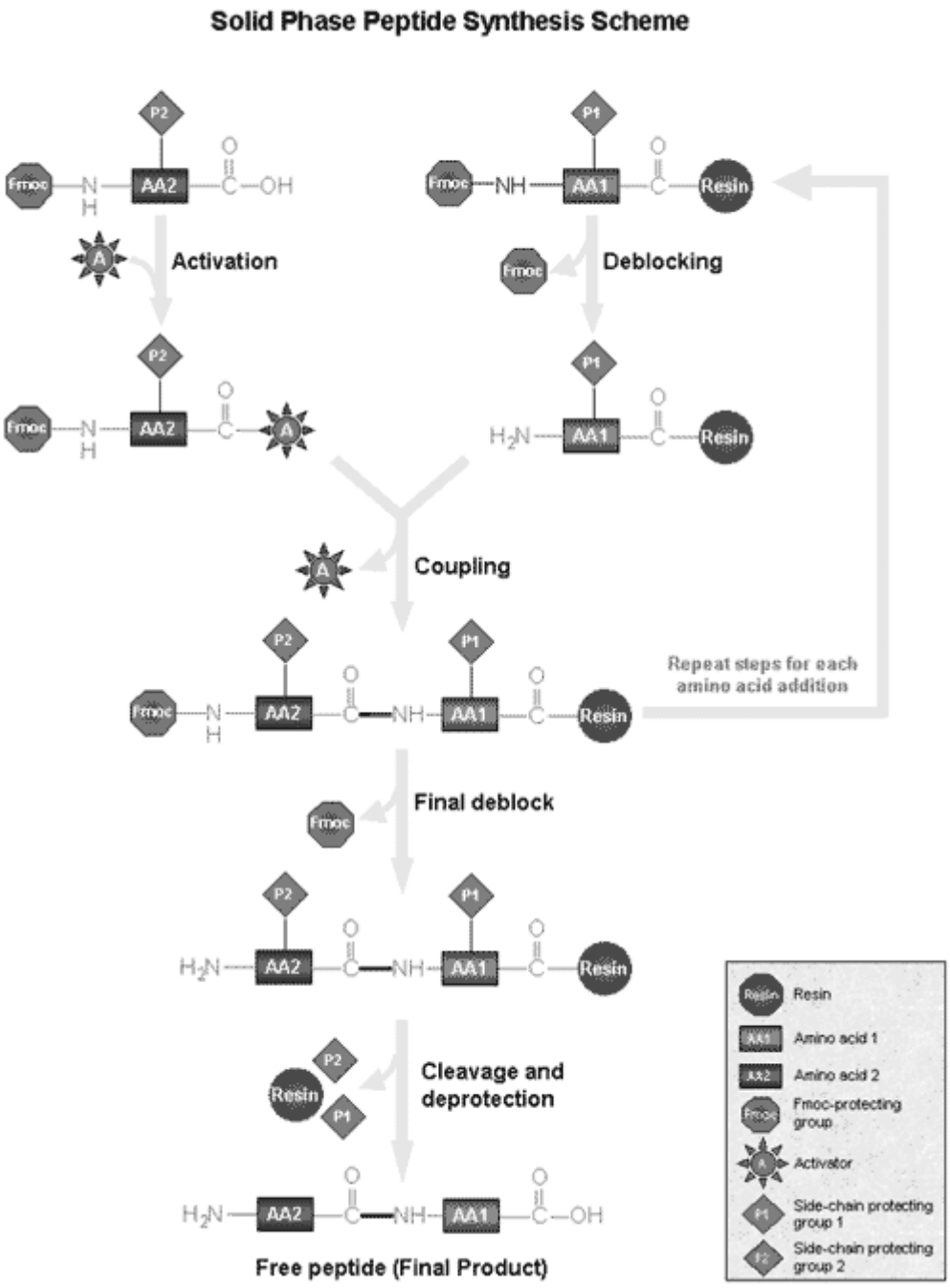

Figure 3.1 Solid phase peptdie synthesis

3.1.1.1 Calculations and weighings

To synthesise 0.3 mmol peptide, each amino acid should be used in a 4 fold molar excess, thus 1.2 mmol of each amino acid in the sequence was weighed. Also, 1.2 mmol of HBTU (activator) should also be weighed out for the purpose of catalysing each coupling reaction of amino acid. The molar quantity of resin should be

sufficient to carry out a 0.3 mmol level synthesis and the weight of resin was calculated by the formula:

$$\text{resin (g)} = \text{peptide (mmol)} / \text{loading capacity (mmol/g)}$$

After the resin was weighed, it was transferred into a clean 40 ml reaction vessel. Finally, after each amino acid and HBTU were weighed and they were added to the same clean amino acid vials with caps and septums.

3.1.1.2 Reagents and programmes used in the Tribute peptide synthesiser

The peptide was synthesised by the Tribute peptide synthesiser [Figure 3.2] and the programmes used are shown in Table 3.2. Preparations were carried out follows before starting programmes:

- Check the inline solvent filters and the source of Nitrogen.
- Vent the reagent bubbles and prepare enough reagents for synthesis.
- Pressurise and prime the reagent bottles.
- Insert the reaction vessels with resin and amino acid vials on the carousel.
- Select a reaction vessel, start and stop position on the carousel, and the coupling programme for each amino acid.
- Press RUN to start the synthesis.

Five bottles were installed in the machine and different reagents were in each bottle as shown in Table 3.1.

Table 3.1 The composition of each bottle in the machine.

Bottle Number	Reagent
1	DMF
2	DMF
3	20% piperidine and 80% DMF

4		11%NMM and 89% DMF
5		DCM

Table 3.2 Single coupling

Step	Action	Bottle	Time	Repeat	Process
1	RV-Top	1	10min	3	Wash machine
2	RV-Rop	3	150s	3	Deprotect Fmoc
3	Wash	1	30s	1	Remove piperidine
4	RV-Top	1	30s	3	Remove Fmoc and piperidine thoroughly
5	AA-Delivery and Mix	4	25min		Activation and Coupling
6	Wash	1	30s	1	Wash NMM
7	RV-Top	1	30s	3	Remove NMM thoroughly
8	RV-Top	3	150s	3	Final deprotection
9	Wash	1	30s	1	Wash

					piperidine
10	RV-Top	1	30s	3	Remove piperidine and Fmoc thoroughly
11	RV-Top	5	30s	3	Bubbling and wash DMF

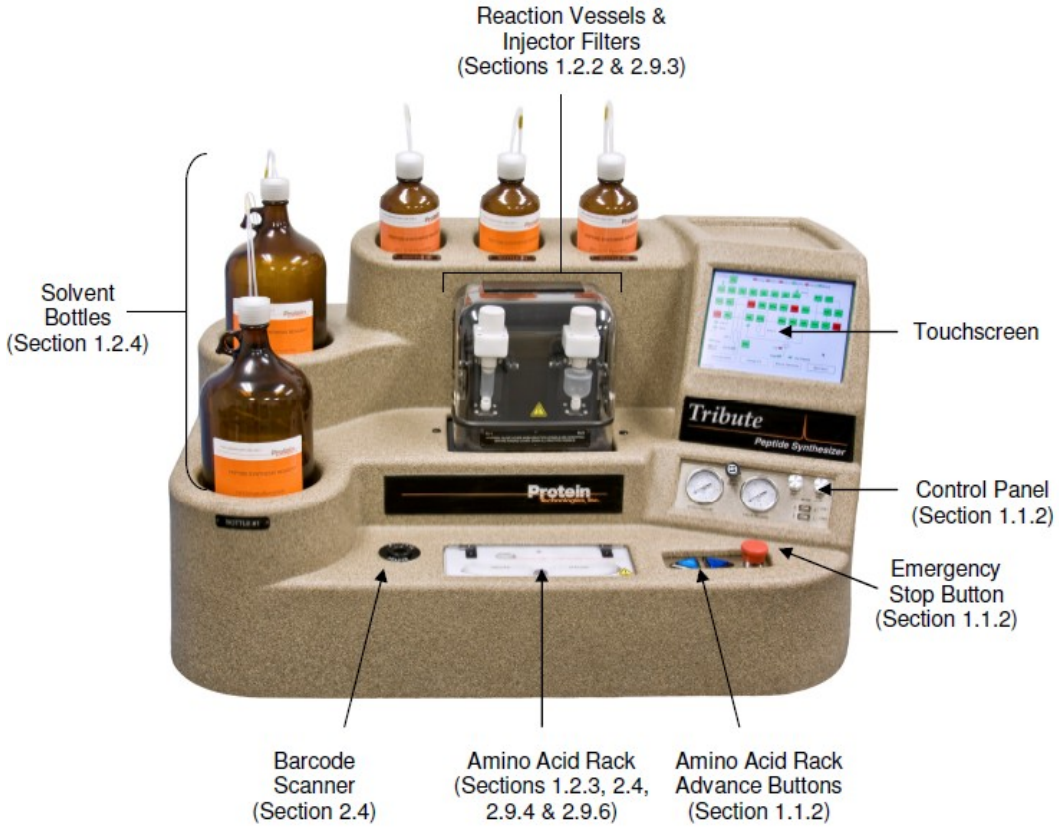

Figure 3.2 The Tribute peptide synthesiser

3.1.1.3 Cleavage and deprotection of the peptide

The peptide was collected after deprotecting the last Fmoc. To separate the peptide from resin, the side-chain protecting groups and peptide were removed from the resin by incubating in trifluoroacetic acid.

(1) The crude peptide/resin was incubated with the following cocktail recipe to cleave the side-chain protecting groups and resin, 95% TFA, 2.5% TIPS (Triisopropylsilane), 2.5% H_2O v/v. The cocktail volume was based on 1 g per 25 ml.

(2) The round bottomed flask was placed on a magnetic stirrer and the cleavage/ deprotection reaction was performed at room temperature for 6-8 h with stirring to remove the resin as well as the side-chain protecting groups from the peptide. A Buchner funnel was then used to filter the peptide solution and filtrate was collected in a 50 mL round bottomed flask. DCM was used to rinse the interior of the round bottom flask for the duration.

(3) The filtrate was concentrated by rotary evaporation to near dryness and then placed in a 50mL centrifuge tube. 45 mL of Et_2O (stored in a refrigerator at -20℃) was added and kept standing overnight to complete the peptide precipitation. If the sequence contained two or more cysteines, the 50 mL tube should be placed at room temperature with a porous cover to complete the oxidation reaction, which should be allowed to take place for at least three days after concentration. The mixture was shaken at least twice each day.

3.1.1.4 Precipitate washing

To collect the product, the Et_2O makes the peptide into an insoluble precipitate.

(1) The tube was filled with approximately 45 ml of Et2O then centrifuged at 5000 x g for 5 min.

(2) The supernatant was discarded carefully to collect the peptide, and then 45 ml of Et_2O was added to the tube a second time.

(3) The complete process was repeated 3 times, and the last time the Et_2O should be as clean as possible.

3.1.1.5 Lyophilisation

To transport and store the peptide more conveniently, it needs to be lyophilised using a laboratory freeze dryer.

(1) 7.5 ml of Buffer B and then of 7.5 ml Buffer A were added to the tube, then it was vortexed to dissolve the sample.

(2) The tube contents were snap frozen in liquid nitrogen, covered with pierced foil then placed into the laboratory freeze dryer for 60 h.

3.1.2 Reversed-Phased High Performance Liquid Chromatography (RP-HPLC) of QUB-3005

High-performance liquid chromatography (HPLC) is essential for identification and purification of peptides. It is becoming increasingly important in analytical chemistry. A component in a mixture can be isolated, identified and quantified by HPLC. Liquid is used as mobile phase, by using the high-pressure infusion system. The sample, dependent on the pump pressure, can pass through the packed pressure column. There are six main parts in an HPLC system: pump (s), mixer, injector, column, detector and collector (Figure 3.3).

This technology is widely used in chemistry, medicine, industry, agriculture and other disciplines.

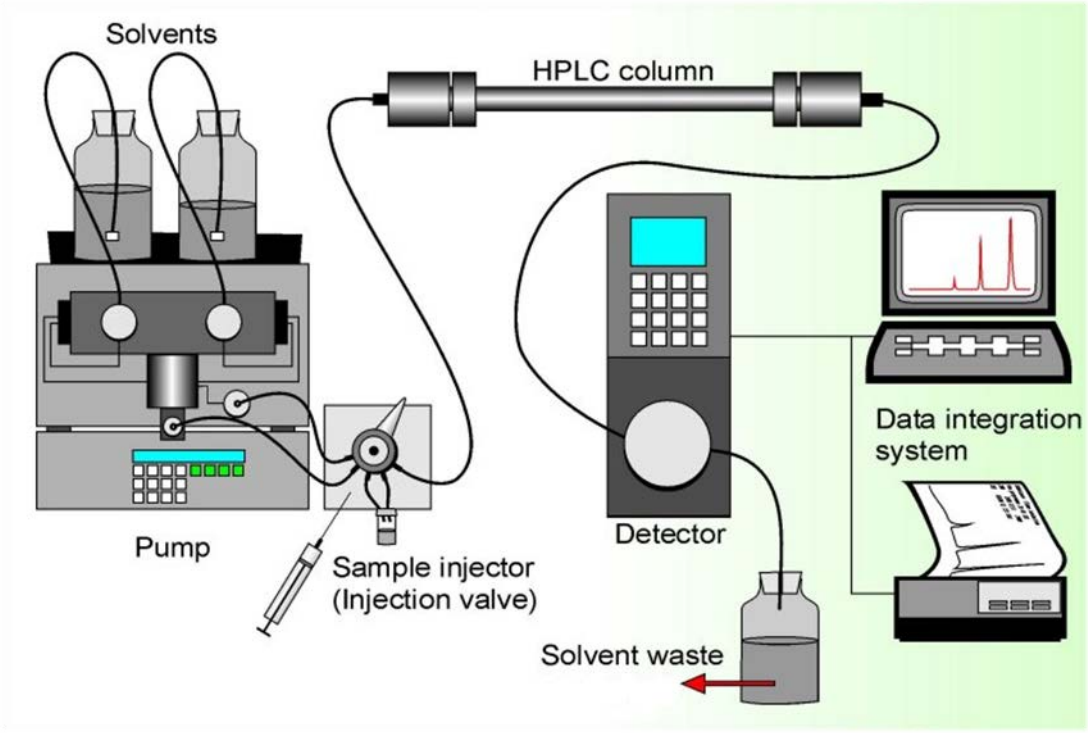

Figure 3.3 HPLC apparatus

Reagents were prepared as below:

Solution A: trifluoroacetic acid (TFA)/water (0.05:99.95, v/v)

Solution B: trifluoroacetic acid (TFA)/ water/Acetonitrile (0.05/19.95/80.00, v/v/v)

One ml Buffer A was added into a 1.5 ml tube which contained 1 mg of synthesised peptide. The tube was vortexed and centrifuged at 6000 x g for 10 min. The supernatant was retained for injection.

Before injecting the sample, the HPLC column was washed by Solution B for 30 min, and then washed by Solution A for 30 min. The purpose of this step was to clean the column. Next, the sample was injected into the HPLC system from the injector. The flow rate was 1 ml/min and the fractions were collected at 1 min intervals for 80 min.

3.1.3 Matrix Assisted Laser Desorption Ionisation-Time of flight (MALDI-TOF) Mass Spectrometer Analysis of QUB-3005

MALDI-TOF MS is a matrix-assisted laser desorption/ionisation-time-of-flight mass spectrometry technique. On the basis of matrix, it can analyse the masses of molecules by the time-of-flight results of ions. The linear time-of-flight Voyager DE mass spectrometer (Perseptive Biosystems, Framingham, MA, USA) use an electrical field to accelerate the ions. MALDI-TOF MS can be employed not only to analyse the masses of peptides, but also to examine the purity of the samples.

Desorption and ionisation are the main processes of MALDI-TOF MS. Frist of all, under the irradiation of the laser, the matrix absorbs its energy. Then the upper layer molecules ablate from the mixture. After that, sample molecules are protonated by the hot plume which is caused by desorption.

In this study, α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix and it was prepared as 10 mg/ml solution of CHCA in acetonitrile/TFA/Water (50/0.05/49.95, v/v/v). The linear time-of-flight Voyager DE mass spectrometer was in positive detection mode. Internal mass calibration of the instrument was achieved using standard peptides of established molecular mass providing a determined accuracy of + 0.1 %.

The masses of the peptides contained in HPLC fractions can be determined rapidly by using matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry, on a linear time-of-flight Voyager DE mass spectrometer equipped with a nitrogen laser.

3.2 Results

Solid phase peptide synthesis of QUB-3005 was successfully accomplished by using the Tribute peptide synthesiser. The product was subjected to MALDI-TOF (Figure 3.4) mass spectrometry and RP-HPLC (Figure 3.5) analysis, which indicated a remarkable high degree of purity of the peptide, QUB-3005. This demonstrated that the crude synthesised peptide had achieved high purity sufficient for subsequent assays.

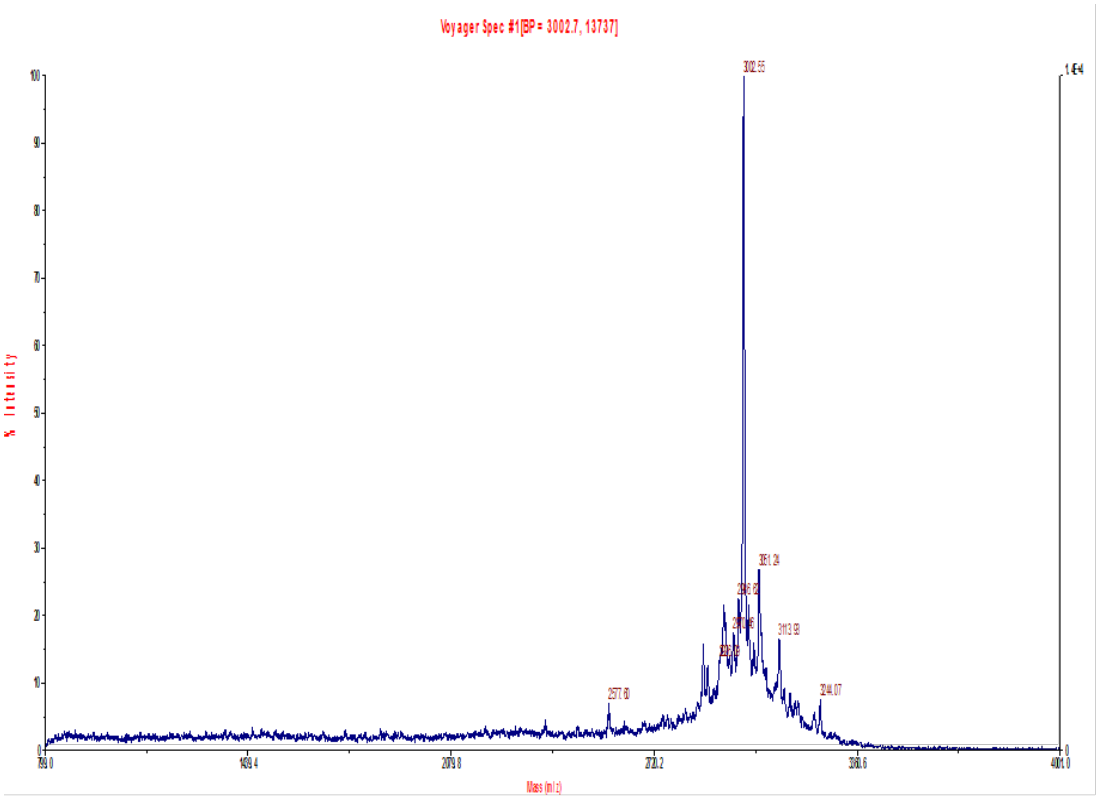

Figure 3.4 MALDI-TOF mass spectrum of synthetic peptide QUB-3005.

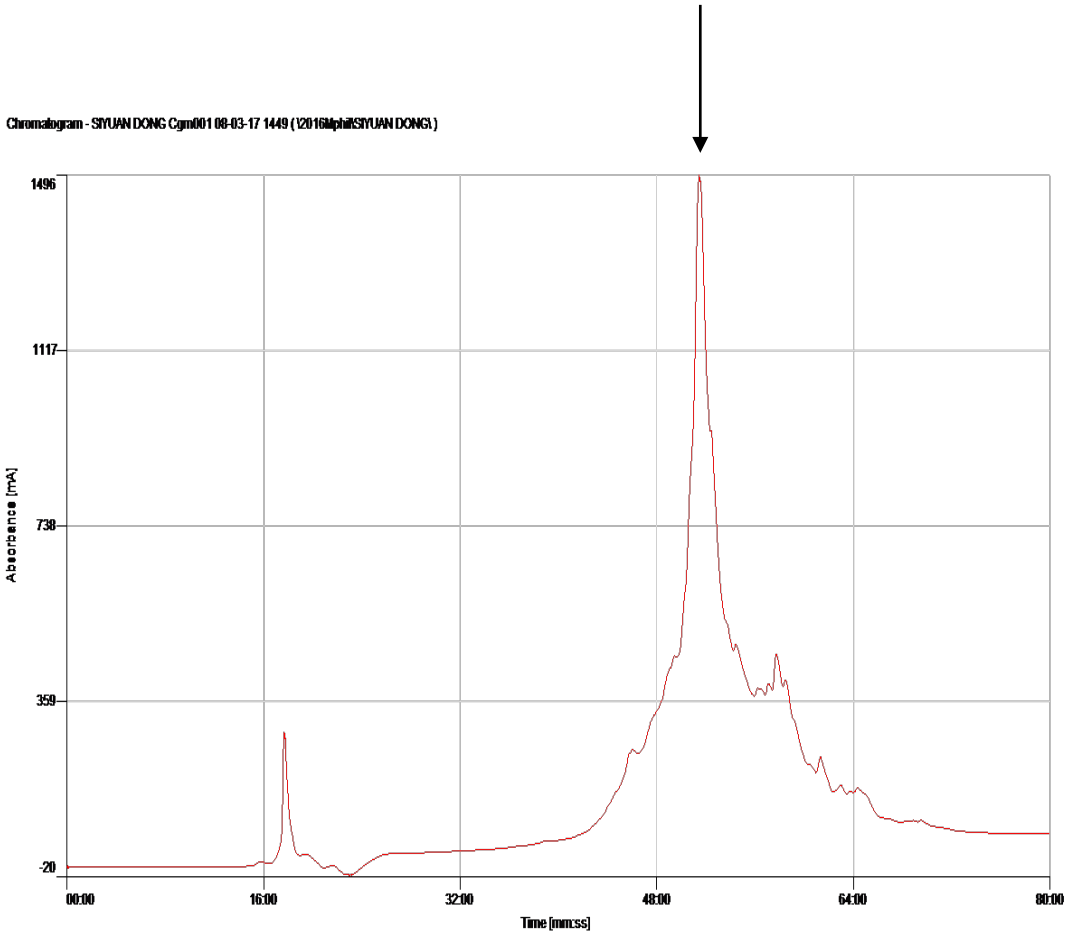

Figure 3.5 Reverse phase HPLC chromatography of synthetic peptide. The arrow indicates the retention time of the peak that contains the bioactive peptide, QUB-3005.

Chapter 4

Assessing the bioactivities of QUB-3005

4.1 Materials and methods

4.1.1 Antimicrobial assays

4.1.1.1 Minimal inhibitory concentration (MIC) assay

(1) Inoculation

Four types of model microorganisms were used in this thesis: *Staphylococcus aureus* (a Gram-positive bacterium), *Escherichia coli*, *P. aeruginosa* (Gram-negative bacteria) and *Candida albicans* (a yeast). A bead of each microorganism stock culture was taken from frozen storage and placed into separate marked flasks containing 100ml MHB (Mueller Hinton Broth). The cultures were incubated overnight (16~20h) in the 37°C orbital incubator.

(2) Subculture

500μl of initial growth cultures were separately transferred into separate new pre-warmed 200ml MHB McCartney bottles which were then placed in the orbital shaking incubator (37°C) for 1~1.5h. Once log phase of growth had been reached, the optical density (OD) was obtained using a spectrometer (λ=550nm). The time for this and the OD values are summarised in Table 4.1. (cfu represents colony-forming units)

Table 4.1 The timescales of logarithmic phases of growth and their optical densities

Organism	Subculture time	Optical density (OD)
S. aureus	1.5h	1×10^8 cfu/ml
E.coli	45min	1×10^8 cfu/ml
P.aeruginosa	45min	1×10^8 cfu/ml
C.albicans	1h	5×10^6 cfu/ml

(3) MIC assay

Eleven mg of peptide (QUB-3005) were dissolved in an appropriate volume of the reagent PBS and diluted to produce a series of concentrations - 512, 256, 128, 64, 32, 16, 8, 4, 2, 1(μM), which were diluted 100-fold when added to the required plate. As the microorganisms reaching their log phase, 100μl subculture product was transferred to each labelled Petri dish and enough fresh MHB was measured for diluting to 5×10^5 cfu/ml. Then 100μl of above diluted cultures were added into one microtube as the replicate for the next viable count assay.

A 96-well plate was prepared and marked number (Figure 4.1). 5 replicates would be included in this step. 1μl of different concentrations of peptide solution were added into each well respectively, and 99μl of diluted bacterial subculture which had reached the standard optical density was subsequently added into each well to mix with the peptide solution. Thereafter, 100μl of MHB was added into the blank group and the same volume of diluted bacterial subculture was added into the growth group as the growth control. The plate was incubated at 37°C overnight (16~20h), after mixing in the shaking incubator for 5~7min. After that, the plate was observed directly, slots containing clear liquid represented no bacterial growth. On the other hand, the absorbance of each well was monitored by an EL× 808^{TM} Absorbance Microplate Reader (Bio Tec, USA) at 550 nm. Both results were considered in determining the minimum inhibitory concentration.

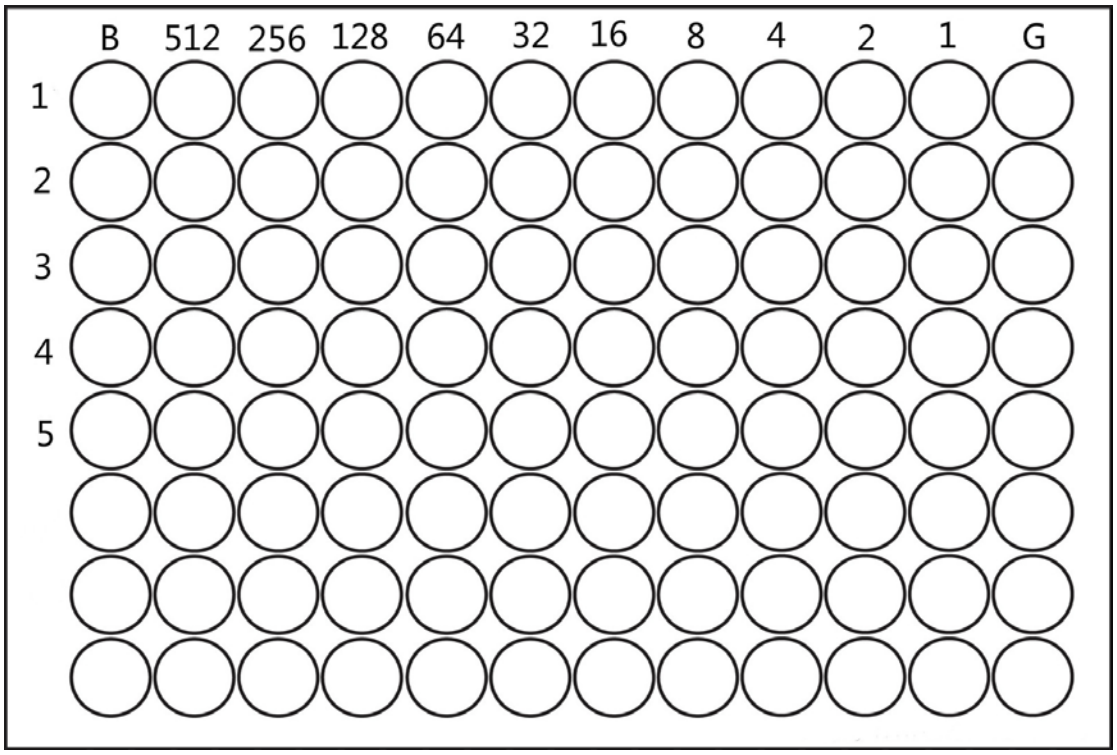

Figure 4.1 96-well plate (G: growth control)

4.1.1.2 Viable cell counting

6 consecutive microtubes received 900µl PBS (Phosphate Buffer Saline), and one of these contained the replicate of subculture products prepared in the former operation. The initial replicate was diluted in ten-fold dilutions from 10^{-1} to10^{-6}.

Sufficient preheated MHA (Mueller Hinton Agar) was poured into a Petri dish and let solidify. There were three round drops of diluted contents dropped separately onto each labelled region by pipetting 20µl of each cell dilution [Figure 4.2]. All liquid drops were air-dried naturally near the gas burner and then transferred into an incubator overnight at 37℃ .

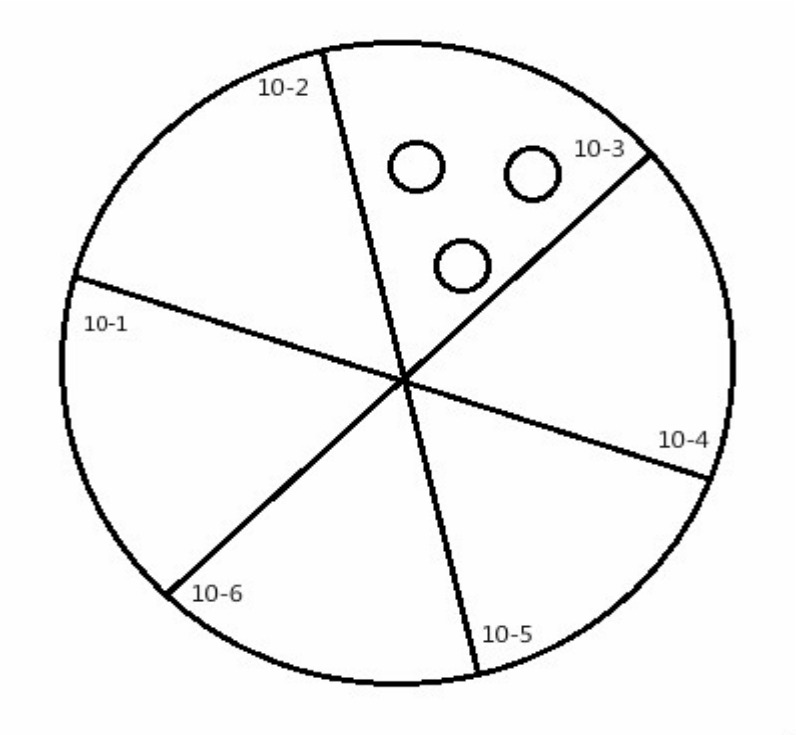

Figure 4.2 Viable cell count on the Petri dish.

4.1.1.3 Minimum bacterial concentration (MBC) assay

After the plate was read, the MIC value was obtained. Subsequently, the mixture in each clear well was mixed thoroughly and 20 µl of mixture was transferred to an MHB plate (Figure 4.3). Then plates were incubated at 37 ℃ for 17-22 h. The minimum bactericidal concentration could be obtained by observing which sample did not have bacterial colonies. The peptide concentration of the sample was the MBC value.

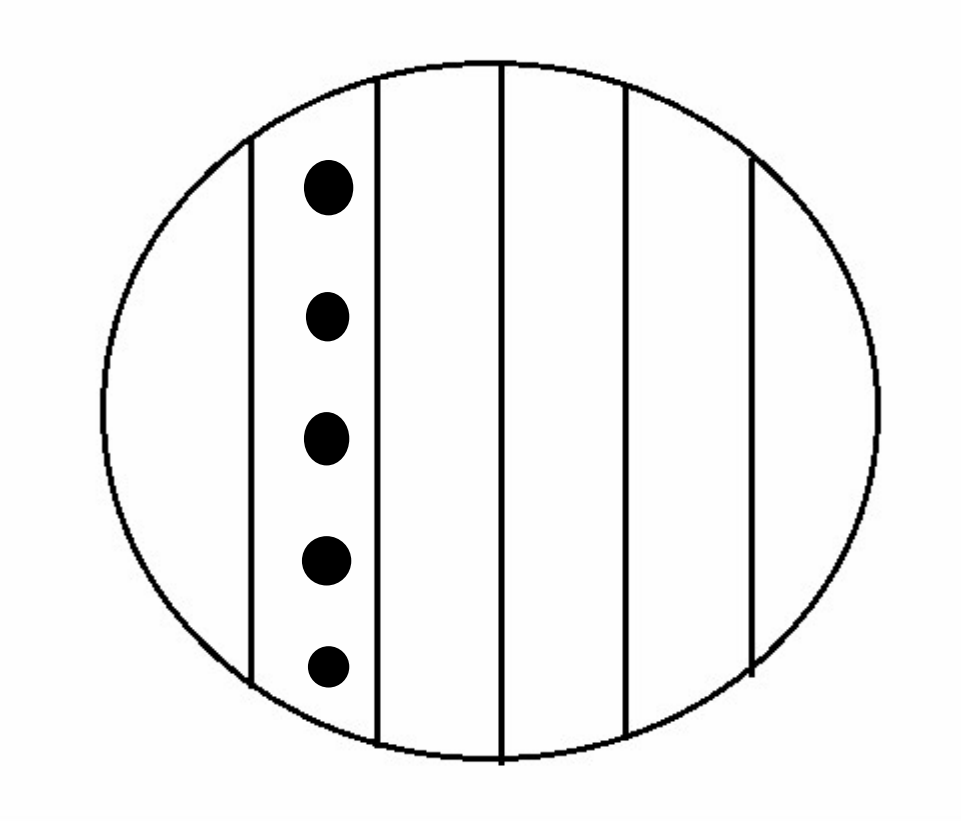

Figure 4.3 MBC om the Petri dish.

4.1.2 Haemolysis assay

4.1.2.1 Preparation of horse red blood cell suspension

Two ml of horse blood were transferred into a 50-ml centrifuge tube after the fresh horse blood was gently shaken on a rotating mixer. Then the tube was centrifuged at 1000 x g for 5 min and the supernatant containing plasma was discarded gently by pipette. Next, 30 ml of autoclaved phosphate buffered saline (PBS) was transferred gently to the tube. Subsequently, the rotating mixer was employed to resuspend erythrocytes and then the tube was centrifuged at 1000 x g for 5 min.

When the supernatant became clear after repeating the washing step several times, 50 ml PBS was added to make a 4% erythrocyte suspension.

4.1.2.2 Calculations and peptide dilution

Peptide was dissolved in sterilised PBS and used to make peptide solutions at different concentrations: 1024 µM, 512 µM, 256 µM, 128 µM, 64 µM, 32 µM, 16 µM, 8 µM, 4 µM, and 2 µM. For each concentration, 200 µl of peptide solution were added into a 1.5 ml tube and there were 5 replicates for each concentration. Finally, 200 µl of red cell suspension were added into each tube.

The blank group was prepared with 4 ml of red cell suspension. In addition, 4 µl of Triton X-100 was added into 1996 µl of PBS and then 2000 µl of red cell suspension was also mixed in to serve as a positive control. All the tubes were incubated at 37℃ for 2 h and then centrifuged at 900 x g for 5 min.

4.1.2.3 96-well-plate haemolysis assay

Supernatants were added into wells of a 96-well-plate (ThermoFisher Scientific) and the absorbance was detected at λ=570 nm with an ELx808^{TM} Absorbance Microplate Reader (BioTek, USA). The data were analysed by Prism-6 software and the HC_{50} (the half maximal haemolytic concentration) of the peptide was acquired.

4.1.3 Anticancer activity assay

4.1.3.1 Resuscitation of frozen cell lines

1) The human cancer cells were taken from the −80 ºC freezer and immediately put into a water bath at 37 ºC and gently and continuously agitated to ensure the cells thawed as soon as possible.

2) All thawed cells were immediately transferred to a 15-ml centrifuge tube containing 10 ml of pre-warmed complete medium. The tube was centrifuged at 200 × g for 5 min at room temperature to get rid of the DMSO.

3) The supernatant containing the DMSO was carefully discarded whilst ensuring no disturbance to cells at the bottom of the tube.

4) A volume of 1 ml of medium was added to the centrifuge tube and mixed completely by gently pipetting. Then, all cells were transferred and seeded into 75 cm^2 culture flasks.

4.1.3.2 Subculture of Adherent Cell Lines

The cells were sub-cultured when they had grown to confluency. The appropriate time of cell subculture was determined by cell behaviour and by observation.

1) An inverted microscope was used to evaluate the degree of confluence and check the absence of fungal and microbial contaminants.

2) The spent medium was removed from the culture and 10 ml of sterile PBS were added into the flask to wash the cell monolayer and then the PBS was discarded.

3) 1 ml of trypsin/EDTA (Invitrogen, UK) solution was added to cover the cell monolayer and the flask was stored in the incubator for 2–10 min at 37 ºC.

4) After the incubation step, the microscope was used to confirm that all the cells were detached and floating, followed by the addition of 10 ml of complete medium to the flask to inactivate and dilute the trypsin and to release any attached cells.

5) A sterile 15 ml centrifuge tube was prepared to hold the transferred cell suspension and subsequently centrifuged at 250 × g for 5 min. The supernatant was discarded immediately. Then, the cells were re-suspended in 5 ml of fresh medium and dispersed by vortexing.

6) A specific volume of the resultant cell culture was passed to another clear flask and diluted to a final volume depending on the cell growth behaviour.

4.1.3.3 Cell quantification

Cell quantification is important to confirm the number of the cells in the culture to allow comparisons between parallel experiments and also repeatability.

1) An AS1000 Improved Neubauer haemocytometer (Hawksley, UK) and coverslip were cleaned with water and dried using tissue [Figure 4.4].

2) A volume of 50 µl of the cell suspension was added to a well in the plate and mixed with an equal volume of Trypan Blue (Invitrogen, UK) and then gently pipetted.

3) By use of the sterile tip of a pipette to transfer the cell suspension to both sides of the notches in the counting chamber, excess liquid overflowed to the overflow rails.

4) A microscope was employed for counting. There were nine squares on each side of the counting area, from which the bright and clear viable cells could be counted, while the dead cells were seen as dyed blue. Three squares were randomly selected and counted by using a counter [Figure 4.5].

5) The cells in each millilitre were computed using the following formula:

C (cells/ml) = average number of the cells per large square $\times 10^4 \times 2$ (dilution factor)

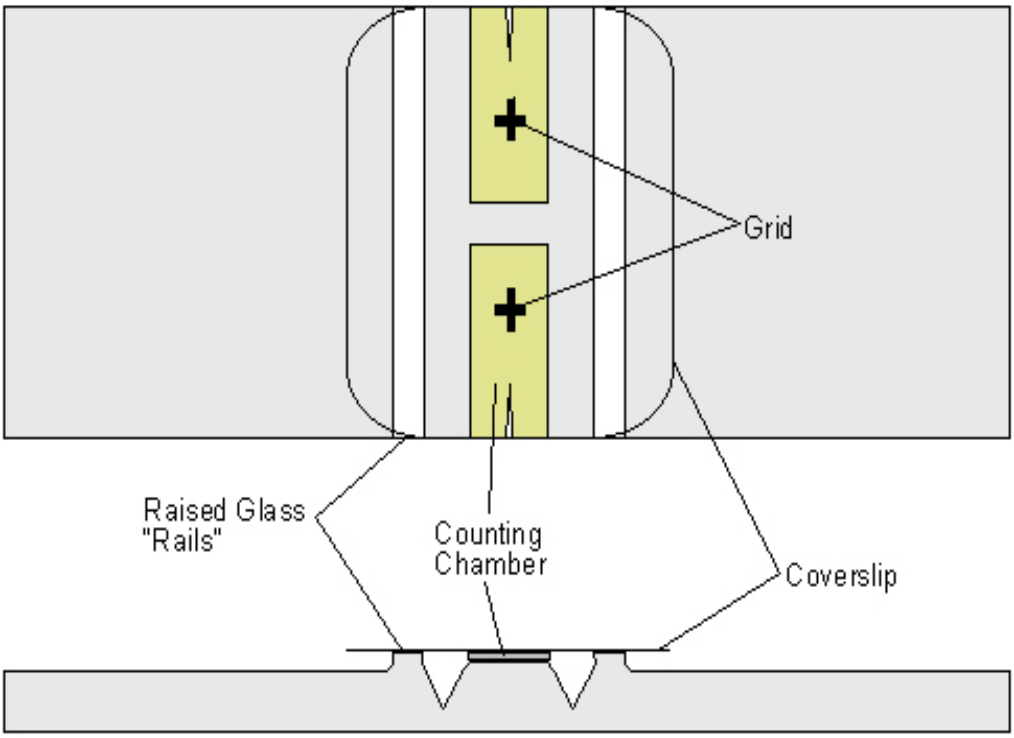

Figure 4.4 Haemocytometer

(From: http://www.di.uq.edu.au/sparqhaemocytometer)

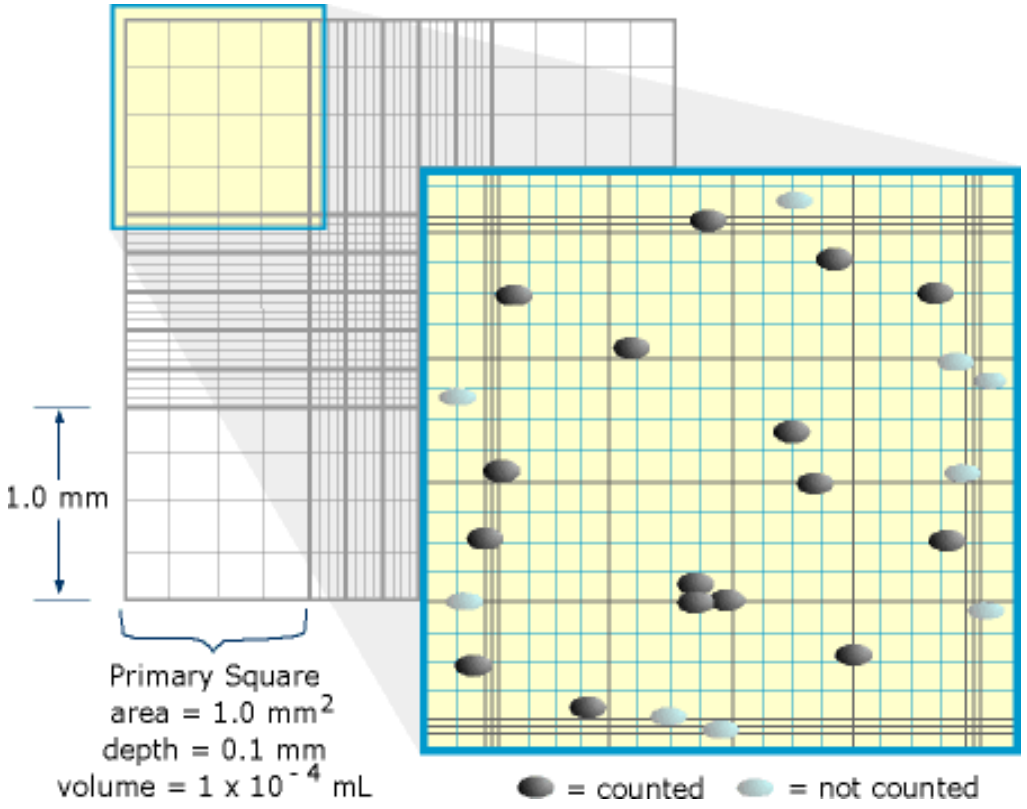

Figure 4.5 The cells under the microscope for counting

4.1.3.4 MTT vialibity assay

This assay can give a good indication of the number of viable cells when compared with drug-untreated cells in cytotoxicity assays. Yellow MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) is reduced to purple formazan by mitochondrial reductase enzymes inside the cell. Hence only living cells can catalyse this reaction and produce the formazan crystals. These crystals are dissolved in DMSO and can be measured spectrophotometrically at certain wavelengths (λ500-600nm).

(1) Cell seeding

1) Cells were counted as in section 4.1.3.3 and diluted to 5000 cells /100 ul (for each well) with the medium.

2) The diluted cell suspension was transferred to a tank and the suspension was added with a multichannel pipette to the 96-well plate.

3) The 96-well plate was placed in an incubator at 37 °C under 5% CO_2 overnight.

(2) Cell starving

1) The 96-well plate was taken out of the incubator.

2) The medium was discarded every 2 lines and FBS-free medium was added to 96-well plate as soon as possible to protect the cells.

3) The 96-well plate was placed in an incubator for 12 h at 37°C under 5% CO_2.

(3) Peptide addition

1) The lyophilised peptide was weighed and dissolved in PBS to obtain a stock peptide solution with a concentration of 10^{-2} mol/L.

2) The peptide solution was ten-fold diluted to obtain solutions in a range of concentrations from 10^{-4} mol/L to 10^{-9} mol/L with FBS-free medium.

3) After cell starving, peptide solutions from 10^{-4} mol/L to 10^{-9} mol/L were added to the 96-well plate (each well 100 ul), each concentration occupying a single line.

4) FBS-free medium was added to two columns of the plate as control and PBS was added to the last column of the plate as control.

5) The plate was placed in an incubator for 24 h at 37°C under 5% CO_2.

(4) MTT addition

1) After 24 h of treatment, a volume of 10 ul MTT (5nM) reagent was added to each well free from the light.

2) The plate was placed in an incubator for 4 h – 6 h at 37°C under 5% CO_2.

3) After incubation, the mixture of MTT and medium was discarded and then 100 ul of DMSO were transferred to each well.

4) The plate was removed to a shaking incubator for 10 min.

5) The plate was then placed on the ELx808™ Absorbance Microplate Reader (BioTek, USA) and the absorbance of wells measured at λ550nm.

6) Each peptide concentration was repeated in triplicate.

7) The cancer cell inhibition rate formula used was as follows:

Inhibition (%) = (OD control- OD sample)/OD control *100%

4.2 Results

4.2.1 Antimicrobial activity of QUB-3005

The activities of QUB-3005 against the Gram-negative bacteria (*E.coli* and *P. aeruginosa*), the Gram-positive bacterium (*S.aureus*) and the yeast (*C.albicans*) are shown below in Figures 4.6 - 4.9. Each test was performed in triplicate and the results demonstrated that QUB-3005 had a high potency to inhibit *E.coli*, *S.aureus* and *C.albicans* and a relatively low ability to inhibit *P. aeruginosa*. The MIC values

were 16μM, 16μM, 16μM and 128μM against *E.coli*, *S.aureus*, *C.albicans* and *P. aeruginosa*, respectively. The MBC of the first three organisms were 64μM, 64μM and 16μM respectively, while no bactericidal effect of QUB-3005 was detected against *P. aeruginosa*.

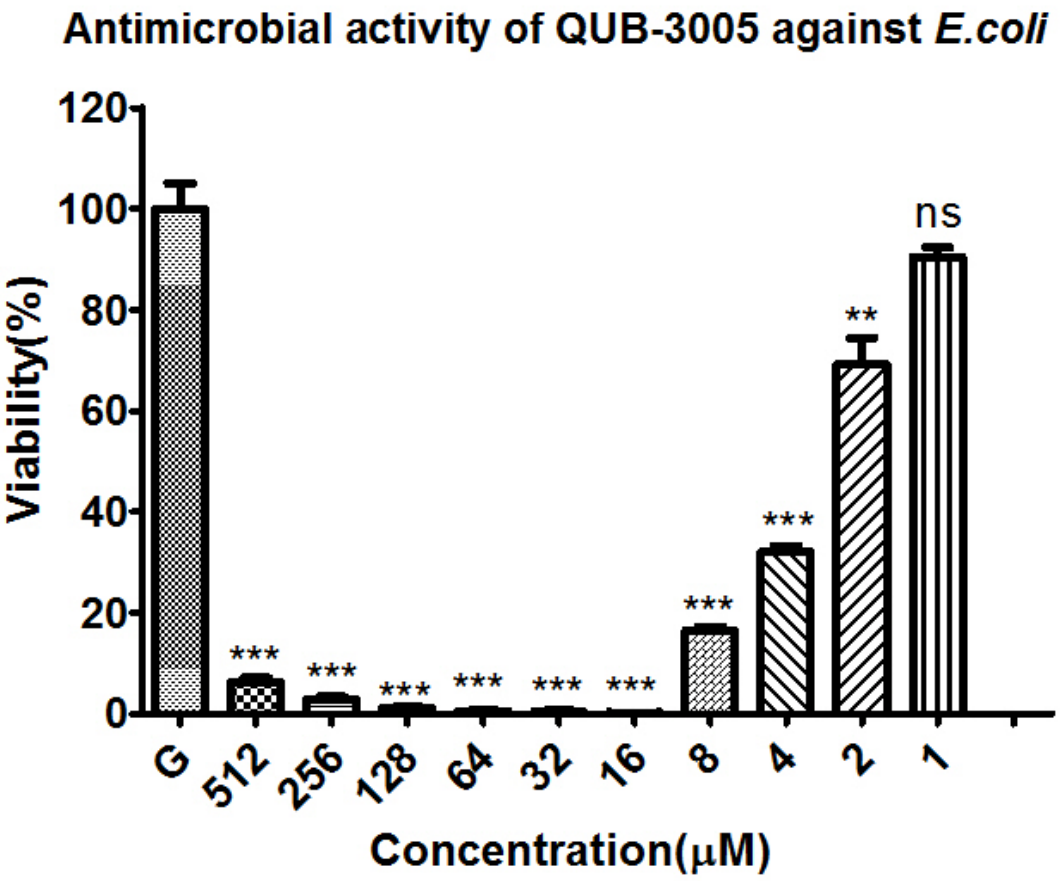

Figure 4.6 Minimal inhibitory concentration of QUB-3005 against *E.coli.* Each column represents the mean ±SEM of 3 replicates. Student T-test of peptide groups compared to positive controls. (The p value is indicated as *p<0.05,**p<0.01,***p<0.001; ns represents there was no significant difference between two groups; one-way ANOVA analysis showed that p < 0.0001. G: growth control)

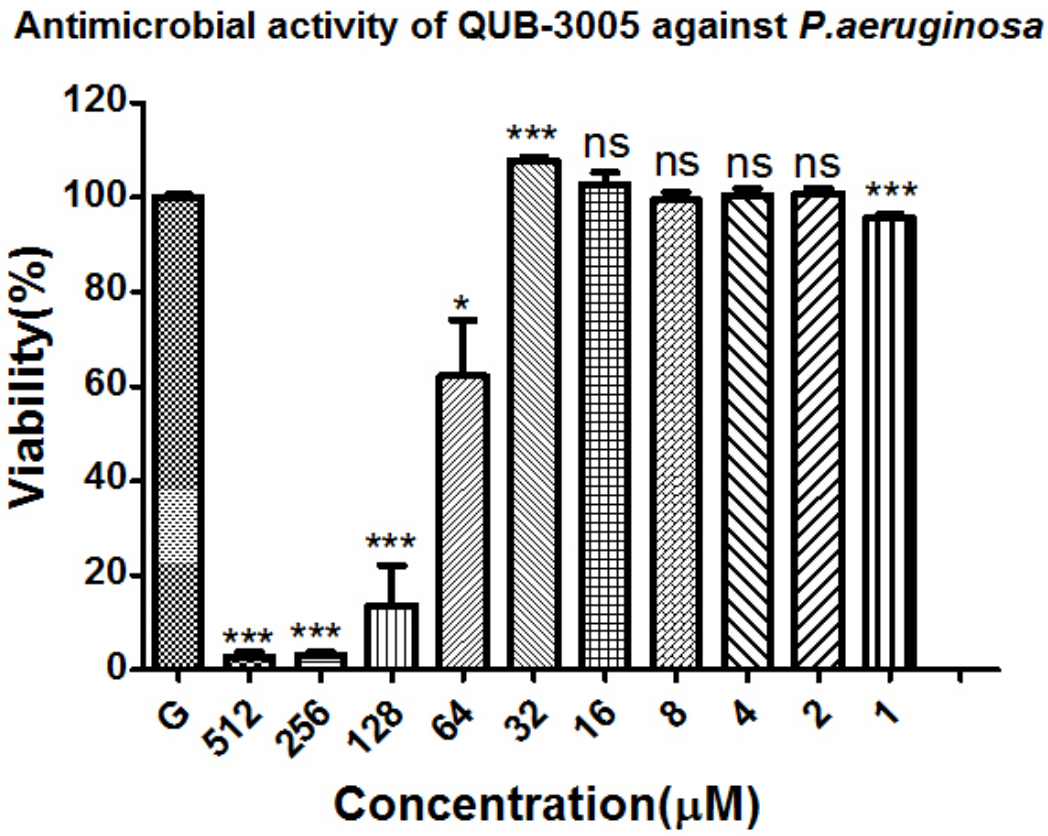

Figure 4.7 Minimal inhibitory concentration of QUB-3005 against *P. aeruginosa.* Each column represents the mean ±SEM of 3 replicates. Student T-test of peptide groups compared to positive controls. (The p value is indicated as *p<0.05,**p<0.01,***p<0.001; ns represents there was no significant difference between two groups; one-way ANOVA analysis showed that p < 0.0001. G: growth control)

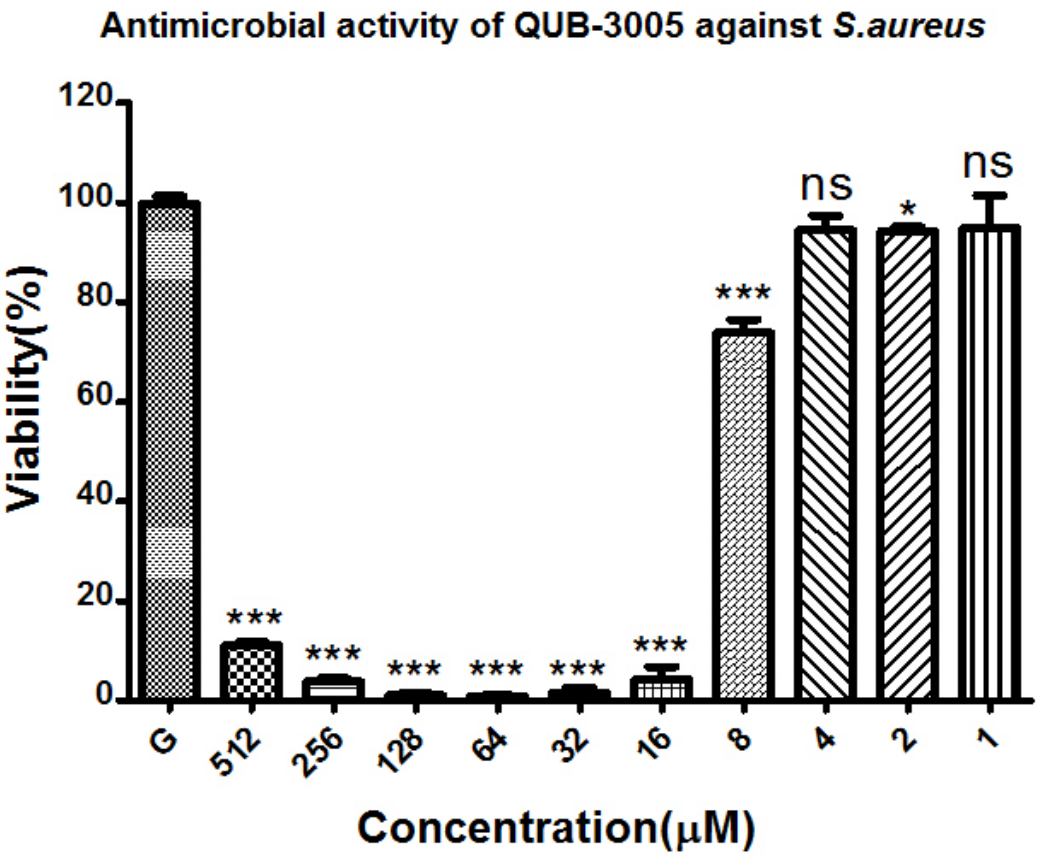

Figure 4.8 Minimal inhibitory concentration of QUB-3005 against *S.aureus.* Each column represents the mean ±SEM of 3 replicates. Student T-test of peptide groups compared to positive controls. (The p value is indicated as *p<0.05,**p<0.01,***p<0.001; ns represents there was no significant difference between two groups; one-way ANOVA analysis showed that p < 0.0001. G: growth control)

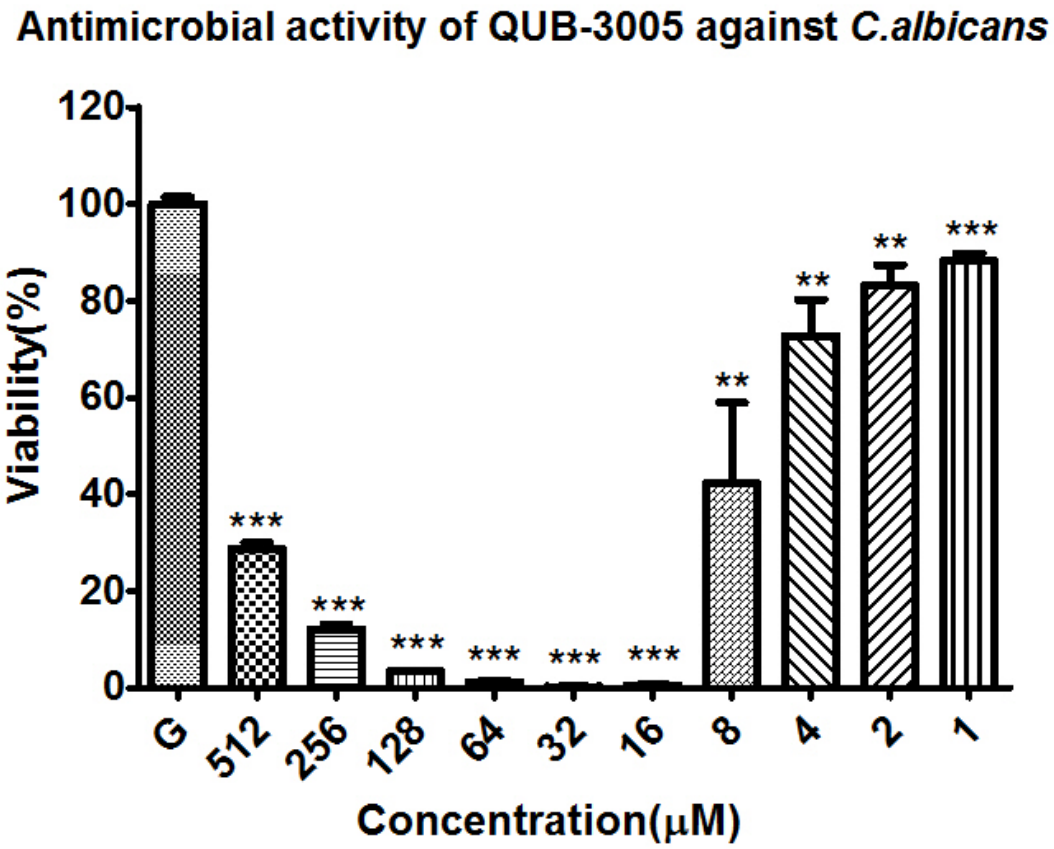

Figure 4.9 Minimal inhibitory concentration of QUB-3005 against *C.albicans.* Each column represents the mean ±SEM of 3 replicates. Student T-test of peptide groups compared to positive controls. (The p value is indicated as *p<0.05,**p<0.01,***p<0.001; one-way ANOVA analysis showed that p < 0.0001. G: growth control)

4.2.2 Haemolytic activity of QUB-3005

Haemolytic assay using horse red blood cells is an important way to evaluate the potential risks of peptide drugs *in vivo*. The haemolytic activity of QUB-3005 is shown in Figure 4.10. According to the MIC values for microorganisms tested, the haemolysis was 17% at 16μM.

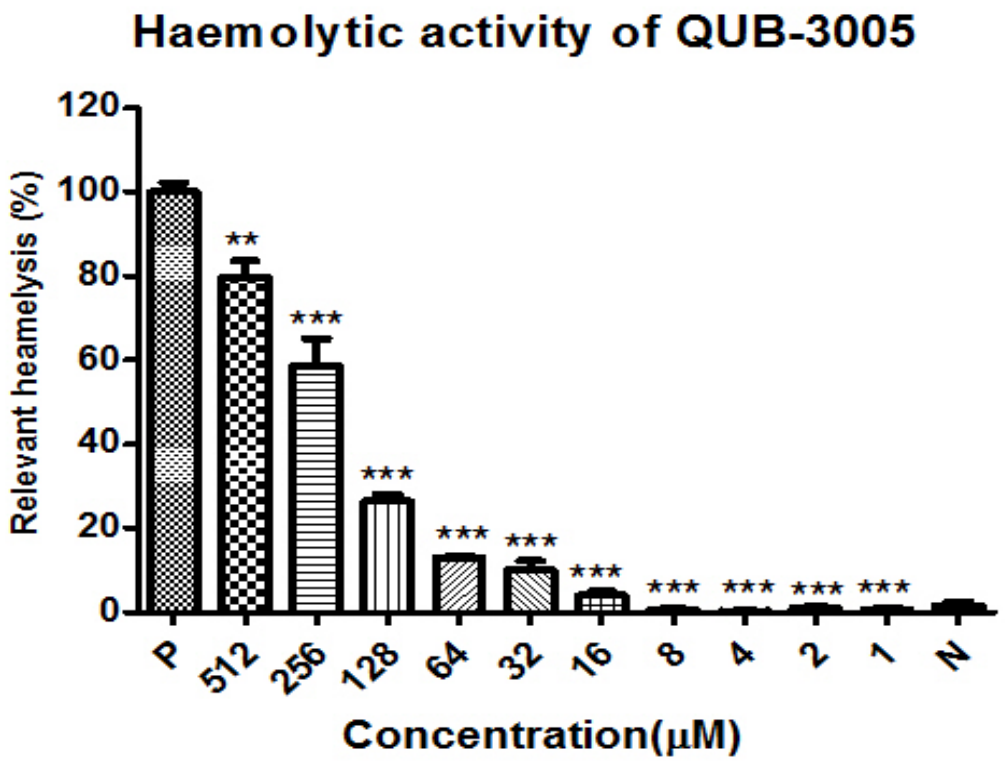

Figure 4.10 Haemolytic activity of QUB-3005. Each column represents the mean ±SEM of 3 replicates. Student T-test of peptide groups compared to positive controls. (The p value is indicated as *p< 0.05,** $p < 0.01$,*** $p < 0.001$; one-way ANOVA analysis showed that p < 0.0001. P: positive control: 1% Triton X-100 N: negative control, PBS)

4.2.3 MTT cell viability assay of QUB-3005

MTT assay was employed to determine whether the peptide QUB-3005 could inhibit the proliferation of human cancer cells. The anticancer activity of QUB-3005 against the H157 cell line, is shown in Figure 4.11. From this figure, it was revealed that at concentrations of 10^{-5} and 10^{-4} M, a significant growth inhibitory effect was observed on H157 cancer cells with cell viability rates of 40.67% and 22.1%, respectively.

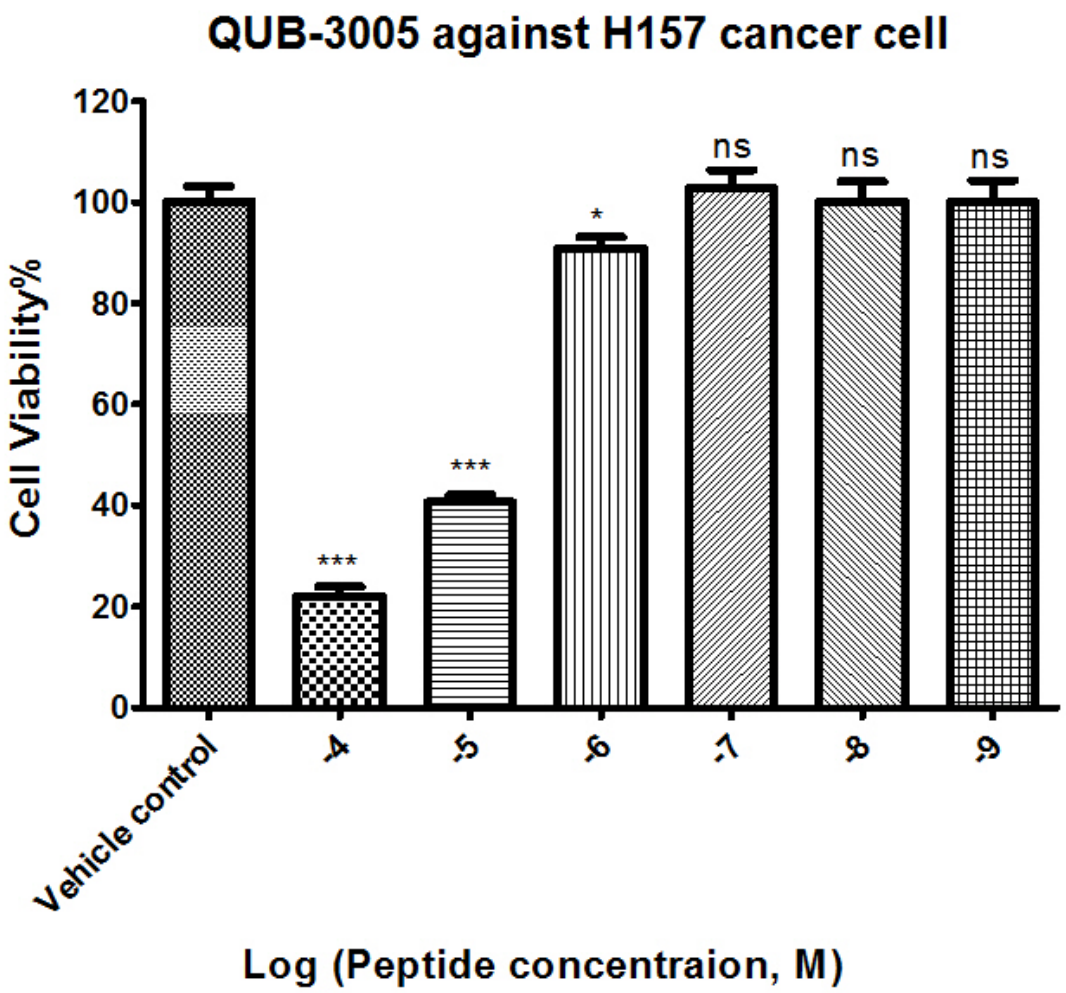

Figure 4.11 MTT cell viability assay: H157 cells were treated with different concentrations of QUB-3005 from 10^{-9} M to 10^{-4} M for 24 h. Each column represents the mean ±SEM of 3 replicates. Student T-test of peptide groups compared to positive controls. (The p value is indicated as *p<0.05,**p<0.01,***p<0.001; ns represents there was no significant difference between two groups; one-way ANOVA analysis showed that $p < 0.0001$.)

Chapter 5

Discussion

5.1 Discussion

To survive in hostile surroundings, amphibians equip themselves with protective skins, which can secrete various gene-encoded bioactive peptides and other pharmacological compounds, to serve as perfect natural shields. Thousands of such peptides have been identified in the last 20 years, such as antioxidant peptides, wound-healing peptides, AMPs, immunomodulatory peptides and bradykinin-related peptides (54). Among these peptides, AMPs are the preferred alternatives for the development of novel therapeutic reagents and traditional antibiotic agents. AMPs have a variety of activities, such as bacterial killing and their growth inhibition (55).

There are many methods used by AMPs to kill cells, such as damaging the structure of membranes, inhibiting DNA, RNA and protein synthesis, as well as interacting with certain intracellular targets.

The cell membranes of bacteria are rich in acidic phospholipids, such as phosphatidylglycerol and cardiolipin (56, 57). There are many negative charges present in these phospholipid head groups. Therefore, when AMPs with the positive charges contact with the bacterial membrane, they tend to work from the outside by firstly tackling the bi-layer's exterior leaflets to surround the membrane. Two interactions occur during the process. One is the electrostatic interaction between positive and negative charges carried by the AMPs and the bacterial membrane respectively, initiating the cellular association. The other one is the hydrophobic interaction between the hydrophobic regions of the peptides and the zwitterionic phospholipids surface of the membrane. However, most lipids with negatively-charged head groups outside the membrane can rarely contact the inner leaflet of the plasma membrane (58). As a result, an overwhelming proportion of these are free of net charges. The exterior of the membrane is mainly composed of sphingomyelin and

zwitterionic phosphatidylcholine, which can be found in other mammalian cells. Hence, the hydrophobic interaction plays a major role in the formation of peptide-cell binding (59). Nevertheless, due to the fact that electrostatic interaction is stronger than the hydrophobic interaction, AMPs are much more likely to engage membranes of the bacterial cell instead of those host cells, a desirable outcome which directly leads to the termination of microorganisms while maintaining the safety of mammalian cells (56). Therefore, it is the trait of selectivity that makes AMPs a favourable choice of antibiotics in the process of host defence against bacteria.

To help the AMPs differentiate between the membranes of mammalian cells and those of bacteria and finally target the bacteria precisely, the cationic property plays a crucial role, thanks to the fact that negative charges outside bacterial membranes outnumber those outside mammalian cells (58).

There are other factors contributing to the AMP's selective capability. Cholesterol, for instance, functions as a unique stabilising agent and exists in mammalian cells to help shield the latter from the attacks of AMPs by reducing their activities, stabilising the lipid bi-layer and interacting with the peptide (60).

Another helpful factor is the transmembrane potential, an established element in peptide-lipid interaction (61). The existence of an inside-negative transmembrane potential guarantees the successful infiltration of peptides with positive charges outside the membrane into the inner leaflet to make reactions occur. Thus, promoting the membrane permeabilisation. The fact that bacterial cells carry more negative charges than mammalian cells does help the AMPs with positive charges to target bacterial membranes under this circumstance.

Moreover, the activities of most AMPs can be reduced by increasing ionic strength, which leads to the selectivity of the AMPs by weakening the electrostatic interactions required for the initial interaction (60).

On the other hand, even if intracellular targets are considered as a way to kill cells, an initial cell membrane interaction with peptides is essential for the antimicrobial activities of AMPs; and this interaction determines the spectrum of target cells (62).

Here, a bioactive peptide, named QUB-3005 has been identified using "shotgun" cloning of its biosynthetic precursor-encoding cDNA from a skin secretion-derived cDNA library of *Rana amurensis.* The primary structure of this peptide was subsequently confirmed based on experiments and BLAST analysis. Then the peptide was chemically synthesised using solid-phase synthesis methodology and the product was subjected to MALDI-TOF mass spectrometry to confirm its molecular mass. Meanwhile, RP-HPLC was employed to purify it, and the bioactivities of this peptide were examined by means of antimicrobial assays, haemolytic assays and anticancer assays.

In the antimicrobial assays, QUB-3005 was active against *E.coli, S.aureus, C.albicans* and *P. aeruginosa* with MIC values with 16μM, 16μM, 16μM and 128μM, respectively. However, in the *C. albicans* assays, QUB-3005 presented a precipitation phenomenon at the concentration of 512μM. Meanwhile, at such effective concentrations, the haemolytic effects were 17% at 16μM and 36.6% at 128μM which suggested that the peptide may have potential to be applied *in vivo*.

In the anticancer assays, QUB-3005 presented a potent anti-proliferative effect *in vitro* against an H157 cancer cell line at the concentration of 10^{-5} M. The ablility of QUB-3005 to inhibit PC-3 cells, and MB435s cells was relatively low. However, no

significant effect on U251MG cell lines (Figure 5.1) by the treatment of QUB-3005 was observed. Based on these results, QUB-3005 should be subjected to further studies and may be developed as an effective drug for use on cancer cells. It is probably that this peptide might have a wound healing effect according to the results.

In recent years, the incidence of cancer is consistently increasing, among which lung cancer, prostate cancer and breast cancer take up to almost half of total cases. Many cancers are still incurable, and most of the available therapies on clinic are not efficiently. It's urgent to develop new anti-tumor drugs which can specically target on tumor cells with less toxicity on normal cells for the treatment of cancers. It is convenient for us to utilise the biological diversity of peptides to explore new drugs for cancer treatment. By studying the characteristics and modification of the biological structures, it is possible to design new anti-neoplastic drugs which have more specicity and less toxicity on the basis of their functions.

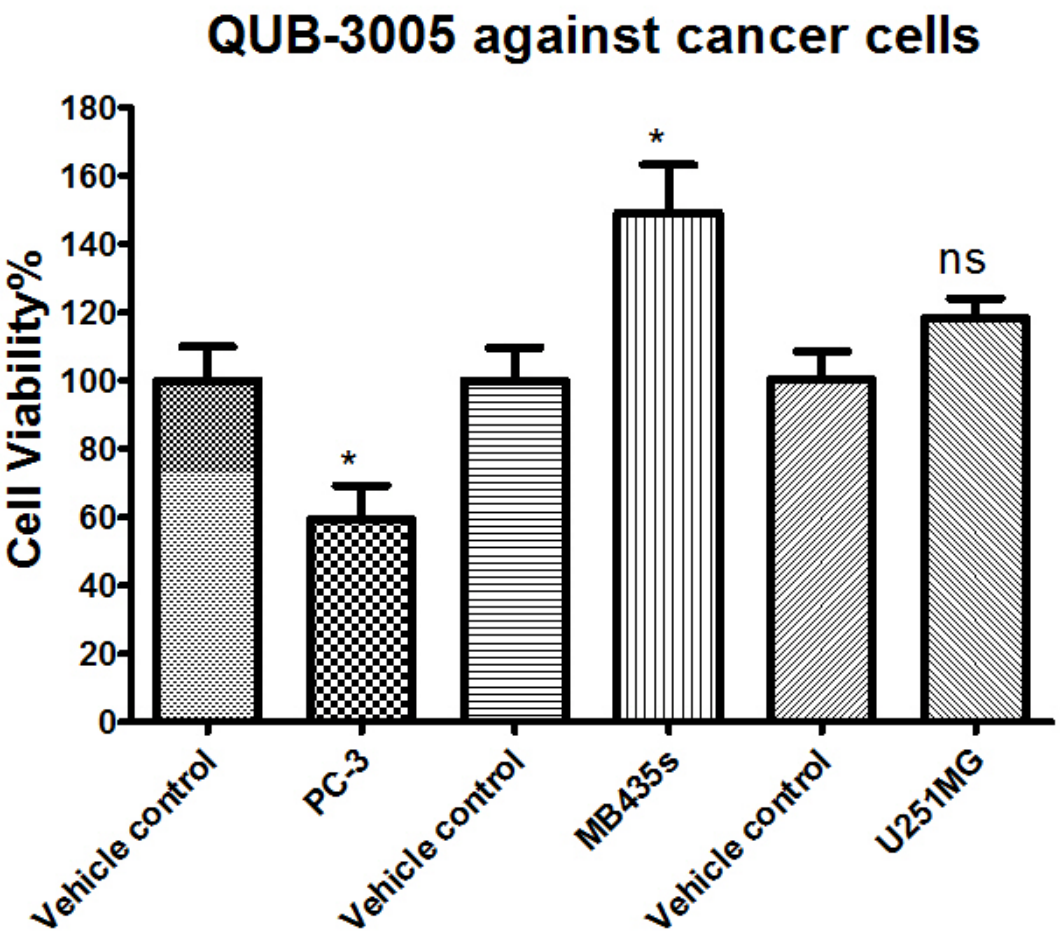

Figure 5.1 MTT cell viability assay: PC-3 cells, MB435s cells and U251MG cells were treated with QUB-3005 at a concentration of 10^{-5} M for 24 h. Each column represents the mean ±SEM of 3 replicates. Student T-test of peptide groups compared to positive controls. (The p value is indicated as *p<0.05,**p<0.01,***p<0.001; ns represents there was no significant difference between two groups; one-way ANOVA analysis showed that p = 0.0013.)

Much work has been done on the study of peptides, for example, discovering some novel molecules may uncover important properties, including pharmacological, pathological or antimicrobial activity, for drug development. The properties of AMPs, such as the broad-spectrum antibacterial activity and low level of induction of pathogen resistance, make AMPs promising substitutes for traditional antibiotics. In addition, some of these peptides can be used as biological food additives in farms. It has been proven that AMPs can enhance the immunity of pigs and poultry. As the

feed containing these AMPs properly regulates the metabolism of these livestock, for example, promoting digestion and absorption in the intestinal tract, altering the intestinal microbiota positively and improving the growth performance in a proper way.

The antimicrobial and immunomodulating activity of AMPs are the beneficial factors to affect growth performance, thereby promoting nutrient digestibility and health.

Also, there are some potential medical-pharmaceutical products produced by amphibian skin secretions. For example, the damaged skin or respiratory infections can be treated by some novel molecules which are found in amphibian skin secretions. However, in some tropical regions, severe infections could be caused by some local fungi and some bacteria and fungi have a resistance to antibiotics. Hence, molecules isolated from amphibian skin secretions might be developed as useful antimicrobial agents or some other therapeutic agents. Besides, there are many peptides with various undiscovered properties that might improve the prospects of the peptides. For instance, using the peptides as vaccines against viral diseases (63).

Despite the fact that there are many favourable factors for us to study AMPs, there still are some problems. First of all, there is not enough data about the toxicity of the peptides (64). The second is that the stability of peptide has not been studied thoroughly *in vivo*. Last but not the least, the cost of mass production of peptide preparations is a major obstacle and this problem has lasted for quite a long time. However, some of these matters have been gradually resolved. The problem of stability has been worked out by using the peptide preparations in combination with a protease inhibitor or by designing analogues, making them more difficult to be identified by the proteases for degradation. In addition, there are now cheaper and more effective technologies which have solved the problems of mass production (55).

Currently, however, although a large number of AMPs have been tested experimentally, few of them have entered into clinical trials. We should have a better understanding of the functions of skin secretions from amphibians, particularly their anticancer and antimicrobial roles. Most importantly, it is necessary to study on amphibians in a non-lethal way.

Chapter 6

References

1. Elkan E, Cooper JE. Skin Biology of Reptiles and Amphibians. P Roy Soc Edinb B. 1980;79:115-&.
2. Heatwole H. Amphibian biology. Chipping Norton, NSW: Surrey Beatty & Sons; 1994. v. <1-7, 9, pt. 3, 11, pt. 4> p.
3. Yeaman MR. Mechanisms of Antimicrobial Peptide Action and Resistance. Pharmacol Rev. 2003;55(1):27-55.
4. Papagianni M. Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnology Advances. 2003;21(6):465-99.
5. Sitaram N, Nagaraj R. Host-defense Antimicrobial Peptides: Importance of Structure for Activity. Current Pharmaceutical Design. 2002;8(9):727-42.
6. Dürr UHN, Sudheendra US, Ramamoorthy A. LL-37, the only human member of the cathelicidin family of antimicrobial peptides. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2006;1758(9):1408-25.
7. Dhople V, Krukemeyer A, Ramamoorthy A. The human beta-defensin-3, an antibacterial peptide with multiple biological functions. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2006;1758(9):1499-512.
8. Hancock REW, Rozek A. Role of membranes in the activities of antimicrobial cationic peptides. FEMS Microbiology Letters. 2002;206(2):143-9.
9. Varkey J, Singh S, Nagaraj R. Antibacterial activity of linear peptides spanning the carboxy-terminal β-sheet domain of arthropod defensins. Peptides. 2006;27(11):2614-23.
10. Morikawa N, Hagiwara K, Nakajima T. Brevinin-1 and -2, unique antimicrobial peptides from the skin of the frog, Rana brevipoda porsa. Biochem Biophys Res Commun. 1992;189(1):184-90.
11. Conlon JM, Sonnevend A, Patel M, Davidson C, Nielsen PF, Pal T, et al. Isolation of peptides of the brevinin-1 family with potent candidacidal activity from the skin secretions of the frog Rana boylii. J Pept Res. 2003;62(5):207-13.
12. Simmaco M, Mignogna G, Barra D, Bossa F. Antimicrobial peptides from skin secretions of Rana esculenta. Molecular cloning of cDNAs encoding esculentin and brevinins and isolation of new active peptides. J Biol Chem. 1994;269(16):11956-61.
13. Goraya J, Wang Y, Li Z, O'Flaherty M, Knoop FC, Platz JE, et al. 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. 2000;267(3):894-900.
14. Yasin B, Pang M, Turner JS, Cho Y, Dinh NN, Waring AJ, et al. Evaluation of the inactivation of infectious Herpes simplex virus by host-defense peptides. Eur J Clin Microbiol Infect Dis. 2000;19(3):187-94.
15. Kumari VK, Nagaraj R. Structure-function studies on the amphibian peptide brevinin 1E: translocating the cationic segment from the C-terminal end to a central position favors selective antibacterial activity. J Pept Res. 2001;58(5):433-41.
16. Clark DP, Durell S, Maloy WL, Zasloff M. Ranalexin. A novel antimicrobial peptide from bullfrog (Rana catesbeiana) skin, structurally related to the bacterial antibiotic, polymyxin. J Biol Chem. 1994;269(14):10849-55.
17. Giacometti A, Cirioni O, Greganti G, Quarta M, Scalise G. In vitro activities of membrane-active peptides against gram-positive and gram-negative aerobic bacteria. Antimicrob Agents Chemother. 1998;42(12):3320-4.

18. Giacometti A, Cirioni O, Barchiesi F, Scalise G. Anticryptosporidial activity of ranalexin, lasalocid and azithromycin alone and in combination in cell lines. J Antimicrob Chemother. 2000;45(3):375-7.
19. Conlon JM, Kolodziejek J, Nowotny N. Antimicrobial peptides from ranid frogs: taxonomic and phylogenetic markers and a potential source of new therapeutic agents. Biochim Biophys Acta. 2004;1696(1):1-14.
20. Conlon JM, Abraham B, Sonnevend A, Jouenne T, Cosette P, Leprince J, et al. Purification and characterization of antimicrobial peptides from the skin secretions of the carpenter frog Rana virgatipes (Ranidae, Aquarana). Regul Pept. 2005;131(1-3):38-45.
21. Goraya J, Knoop FC, Conlon JM. Ranatuerins: antimicrobial peptides isolated from the skin of the American bullfrog, Rana catesbeiana. Biochem Biophys Res Commun. 1998;250(3):589-92.
22. Nguyen LT, Haney EF, Vogel HJ. The expanding scope of antimicrobial peptide structures and their modes of action. Trends in Biotechnology. 2011;29(9):464-72.
23. O'Driscoll NH, Labovitiadi O, Cushnie TPT, Matthews KH, Mercer DK, Lamb AJ. Production and Evaluation of an Antimicrobial Peptide-Containing Wafer Formulation for Topical Application. Current Microbiology. 2012;66(3):271-8.
24. Hein KZ, Takahashi H, Tsumori T, Yasui Y, Nanjoh Y, Toga T, et al. Disulphide-reduced psoriasin is a human apoptosis-inducing broad-spectrum fungicide. Proceedings of the National Academy of Sciences. 2015;112(42):13039-44.
25. Nicolas P. Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. FEBS Journal. 2009;276(22):6483-96.
26. Brogden KA. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nature Reviews Microbiology. 2005;3(3):238-50.
27. Papo N, Shai Y. Can we predict biological activity of antimicrobial peptides from their interactions with model phospholipid membranes? Peptides. 2003;24(11):1693-703.
28. Yang L, Harroun TA, Weiss TM, Ding L, Huang HW. Barrel-stave model or toroidal model? A case study on melittin pores. Biophys J. 2001;81(3):1475-85.
29. Lee M-T, Chen F-Y, Huang HW. Energetics of Pore Formation Induced by Membrane Active Peptides†. Biochemistry-Us. 2004;43(12):3590-9.
30. Yang L, Harroun TA, Weiss TM, Ding L, Huang HW. Barrel-Stave Model or Toroidal Model? A Case Study on Melittin Pores. Biophys J. 2001;81(3):1475-85.
31. Ehrenstein G, Lecar H. Electrically gated ionic channels in lipid bilayers. Q Rev Biophys. 1977;10(1):1-34.
32. Wu Y, Huang HW, Olah GA. Method of oriented circular dichroism. Biophys J. 1990;57(4):797-806.
33. Pouny Y, Rapaport D, Mor A, Nicolas P, Shai Y. Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes. Biochemistry-Us. 1992;31(49):12416-23.
34. Shai Y. Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by alpha-helical antimicrobial and cell non-selective membrane-lytic peptides. Biochim Biophys Acta. 1999;1462(1-2):55-70.
35. Ladokhin AS, White SH. 'Detergent-like' permeabilization of anionic lipid vesicles by melittin. Biochim Biophys Acta. 2001;1514(2):253-60.

36. Bechinger B. The structure, dynamics and orientation of antimicrobial peptides in membranes by multidimensional solid-state NMR spectroscopy. Biochim Biophys Acta. 1999;1462(1-2):157-83.
37. Oren Z, Shai Y. Mode of action of linear amphipathic alpha-helical antimicrobial peptides. Biopolymers. 1998;47(6):451-63.
38. Yamaguchi S, Hong T, Waring A, Lehrer RI, Hong M. Solid-State NMR Investigations of Peptide−Lipid Interaction and Orientation of a β-Sheet Antimicrobial Peptide, Protegrin†. Biochemistry-Us. 2002;41(31):9852-62.
39. Kalfa VC, Jia HP, Kunkle RA, McCray PB, Jr., Tack BF, Brogden KA. Congeners of SMAP29 kill ovine pathogens and induce ultrastructural damage in bacterial cells. Antimicrob Agents Chemother. 2001;45(11):3256-61.
40. Ladokhin AS, Selsted ME, White SH. Sizing membrane pores in lipid vesicles by leakage of co-encapsulated markers: pore formation by melittin. Biophys J. 1997;72(4):1762-6.
41. Matsuzaki K, Yoneyama S, Miyajima K. Pore formation and translocation of melittin. Biophys J. 1997;73(2):831-8.
42. Kang JH, Shin SY, Jang SY, Lee MK, Hahm KS. Release of aqueous contents from phospholipid vesicles induced by cecropin A (1-8)-magainin 2 (1-12) hybrid and its analogues. J Pept Res. 1998;52(1):45-50.
43. Hristova K, Selsted ME, White SH. Critical Role of Lipid Composition in Membrane Permeabilization by Rabbit Neutrophil Defensins. J Biol Chem. 1997;272(39):24224-33.
44. Zhao HX, Mattila JP, Holopainen JM, Kinnunen PKJ. Comparison of the membrane association of two antimicrobial peptides, magainin 2 and indolicidin. Biophys J. 2001;81(5):2979-91.
45. Matsuzaki K, Murase O, Miyajima K. Kinetics of Pore Formation by an Antimicrobial Peptide, Magainin 2, in Phospholipid Bilayers. Biochemistry-Us. 1995;34(39):12553-9.
46. Matsuzaki K, Murase O, Fujii N, Miyajima K. An Antimicrobial Peptide, Magainin 2, Induced Rapid Flip-Flop of Phospholipids Coupled with Pore Formation and Peptide Translocation†. Biochemistry-Us. 1996;35(35):11361-8.
47. Kagan BL, Selsted ME, Ganz T, Lehrer RI. Antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. Proc Natl Acad Sci U S A. 1990;87(1):210-4.
48. Wu Y, Huang HW, Olah GA. Method of oriented circular dichroism. Biophys J. 1990;57(4):797-806.
49. He K, Ludtke SJ, Huang HW, Worcester DL. Antimicrobial peptide pores in membranes detected by neutron in-plane scattering. Biochemistry-Us. 1995;34(48):15614-8.
50. Ludtke SJ, He K, Heller WT, Harroun TA, Yang L, Huang HW. Membrane pores induced by magainin. Biochemistry-Us. 1996;35(43):13723-8.
51. Yang L, Harroun TA, Heller WT, Weiss TM, Huang HW. Neutron Off-Plane Scattering of Aligned Membranes. I. Method of Measurement. Biophys J. 1998;75(2):641-5.
52. Yang L, Weiss TM, Harroun TA, Heller WT, Huang HW. Supramolecular structures of peptide assemblies in membranes by neutron off-plane scattering: method of analysis. Biophys J. 1999;77(5):2648-56.
53. Chen FY, Lee MT, Huang HW. Evidence for membrane thinning effect as the mechanism for peptide-induced pore formation. Biophys J. 2003;84(6):3751-8.

54. Xu X, Lai R. The Chemistry and Biological Activities of Peptides from Amphibian Skin Secretions. Chemical Reviews. 2015;115(4):1760-846.
55. Reddy KV, Yedery RD, Aranha C. Antimicrobial peptides: premises and promises. Int J Antimicrob Agents. 2004;24(6):536-47.
56. Matsuzaki K. Control of cell selectivity of antimicrobial peptides. Biochim Biophys Acta. 2009;1788(8):1687-92.
57. Chou HT, Kuo TY, Chiang JC, Pei MJ, Yang WT, Yu HC, et al. Design and synthesis of cationic antimicrobial peptides with improved activity and selectivity against Vibrio spp. Int J Antimicrob Agents. 2008;32(2):130-8.
58. Hancock RE, Sahl HG. Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat Biotechnol. 2006;24(12):1551-7.
59. Tennessen JA. Molecular evolution of animal antimicrobial peptides: widespread moderate positive selection. J Evol Biol. 2005;18(6):1387-94.
60. Zasloff M. Antimicrobial peptides of multicellular organisms. Nature. 2002;415(6870):389-95.
61. Matsuzaki K, Sugishita K, Fujii N, Miyajima K. Molecular basis for membrane selectivity of an antimicrobial peptide, magainin 2. Biochemistry-Us. 1995;34(10):3423-9.
62. He K, Ludtke SJ, Worcester DL, Huang HW. Neutron scattering in the plane of membranes: structure of alamethicin pores. Biophys J. 1996;70(6):2659-66.
63. Francis MJ. Peptide vaccines for viral diseases. Sci Prog. 1990;74(293 Pt 1):115-30.
64. Hancock RE. Host defence (cationic) peptides: what is their future clinical potential? Drugs. 1999;57(4):469-73.