Identifying Novel Antimicrobial Peptides with Therapeutic Potential Against Multidrug-Resistant Bacteria by Using the SPOT Synthesis

Kai Hilpert*

KIT (Karlsruhe Institute of Technology), Institute of Functional Interfaces, P.O.Box 3640, 76021 Karlsruhe, Germany

Abstract: Developing of new lead structures against multidrug-resistant bacteria is an urgent need and cationic antimicrobial peptides (AMPs) have the potential to become such a lead structure. Here we provide an overview of a method to screen peptides for antimicrobial activity based on the SPOT synthesis. Three different strategies to study and optimize antimicrobial activity of peptides have been reported, substitution analysis of known AMPs, scrambling of known AMPs, and screening peptide libraries for novel AMP sequences. This screening method has great potential for discovery and optimization of novel antibiotics; many different peptides with low cytotoxicity and superior activity against different pathogenic bacteria have already been discovered and selected candidates were successfully tested in a mouse infection model using *Staphylococcus aureus*. The combination of this screening method together with quantitative structure-activity relationship (QSAR) approach led to superior active peptide against several clinical isolates of different multidrug-resistant bacteria. A variant of this screening method can also be used to discover peptides that are antimicrobial when attached to surfaces, a potentially important application for prevention of implant-associated infection.

Keywords: Antimicrobial peptide, host defence peptide, multidrug-resistant, *Pseudomonas aeruginosa*, high throughput screening, QSAR, spot synthesis, drug development.

MULTIDRUG-RESISTANT BACTERIA

Conventional antibiotics are a pillar of modern medicine. With their introduction, infections of individuals to great plagues caused by pathogenic bacteria lost their horror, and in the world antibiotics continue to save millions of lives every year. In Germany, 250-300 tons of antibiotics were used in human medicine and 750 tons in veterinary medicine alone in the year 2007 [1]. However, the introduction of conventional antibiotics to the market was closely followed by antibiotic resistance, to the extent that there is now a global threat of multidrug-resistant bacteria. Many bacteria that adversely affect human health are multidrug-resistant to varying degrees; these bacteria include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococci* (VRE), multidrug-resistant *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [2, 3].

Bacterial resistance to conventional antibiotics is quickly becoming a global public health emergency, resulting in a push to develop new treatment strategies and substances with novel modes of action. One class of substances that may offer a last resort for treating infections of multidrug-resistant bacteria is cationic antimicrobial peptides (AMPs) or also called host-defence peptides. The most clinically advanced host-defence peptide at present is omiganan, a derivative of indolicidin [4].

CATIONIC ANTIMICROBIAL PEPTIDES

Cationic AMPs, part of innate immunity, are associated with many different forms of life, from plants to vertebrates [5]. Many microbes are able to synthesize powerful antimicrobial peptides, such as polymyxin B and gramicidin S, using multi-enzyme complexes [6]. The cationic peptides of various bacteria and fungi aid in the defence and maintenance of their ecological niche. AMPs form a critical part of the invertebrate immune system; they are found in hemolymph, phagocytic cells and different epithelial cells [7]. Vertebrate AMPs are found on skin and mucosal surfaces, and within granules of immune cells [8]. Amphibian skin is a rich source of these peptides; for example, the skin of the frog *Odorrana grahami* contains 107 different peptides [9].

*Address correspondence to this author at the KIT (Karlsruhe Institute of Technology), Institute of Functional Interfaces, P.O.Box 3640, 76021 Karlsruhe, Germany; Tel: 07247 82-4039; Fax: 07247 82-4842; E-mail: kai.hilpert@kit.edu

1570-193X/11 $58.00+.00

In recent years, it has become clear that cationic AMPs or host defence peptides are not only able to kill Gram-positive and Gram-negative bacteria, fungi, parasites and enveloped viruses, but can also alter immune response in mammals [10-15]. They have been used successfully in animal models, towards prevention of septic shock, they have been shown to be chemotactic, promoting wound healing and angiogenesis, and they have been found to selectively modulate chemokine and cytokine production. Despite these findings and the potential importance of these substances to human health, the mode of action of these AMPs is not yet understood [16]. Until now, the peptide function most studied has been antibacterial activity. The primary interaction site of these peptides with bacteria is thought to be the bacterial membrane, but increasing evidence suggests that many peptides may have internal cell targets [17]. It has been shown that certain peptides inhibit RNA-, DNA- and/or protein-synthesis, or inhibit enzyme activities, such as the chaperone function of DnaK [17, 18].

In the event that peptides have an internal target, they must still interact with the bacterial membrane; various models have been developed to explain the interaction of host-defence peptides with bacterial membranes, including the Barrel-Stave, Aggregate, Carpet and Torodial Pore models [19]. Over 1000 different naturally occurring peptides have been discovered, and are described in different databases (http://aps.unmc.edu/AP/main.php, http://www.bbcm.units.it/~tossi/pag1.htm). Some examples of natural cationic host-defence peptides are provided in Table **1**. These naturally occurring peptides show enormous variety in their sequences and structures (Fig. **1**), sharing only some common features, such as short length (12-60 amino acids), excess positive charge and amphiphilicity. Based on shared properties, four broad groups of antimicrobial peptides have been classified [20, 21]: (A) β-stranded cysteine-containing peptides, which are stabilized by two or more disulphide bonds (*e.g.* α- and β- defensins), (B) linear molecules that fold upon membrane insertion into amphipathic helices (*e.g.* insect cecropins, frog magainins and human LL-37), (C) loop structures formed by one disulphide bridge, and often possessing tails (*e.g.* frog brevinin and bovine bactenecin), and (D) extended structures that are Trp-, Pro- and/or His-rich (*e.g.* human histatins and bovine indolicidin).

SCREENING APPROACHES

In order to screen large numbers of peptides, two different approaches can be used: biological or chemical synthesis. Both approaches can be used to synthesize and screen large numbers of

Table 1.

Name	Ref.	Sequence*	Source	N. Ch.	H (%)	L.
α-Defensin-1 (HNP-1)	[56]	ACYCRIPACIAGERRYGTCI-YQGRLWAFCC	human, mammals, animals, *Homo sapiens*	3	53	30
Androctonin	[57]	RSVCRQIKICRRRGGCYYKCTNRPY	scorpions, arachnids, invertebrates, animals, *Androctonus Australis*	8	28	25
Apidaecin IA	[58]	GNNRPVYIPQPRPPHPRI	insect, honeybee, invertebrates, animals, *Apis mellifera L.*	4	23	18
Aurein 2.6	[59]	GLFDIAKKVIGVIGSL	frog, amphibians, animals, *Litoria aurea* and *Litoria raniformis*	1	56	16
Bactenecin	[45]	RLCRIVVIRVCR	cow, mammals, animals; *Bos taurus*	4	66	12
Bombinin	[60]	GIGALSAKGALKGLAKGLAEHFAN	toad, amphibians, animals, yellow-bellied toad	3	50	24
Cecropin P1	[61, 62]	SWLSKTAKKLENSAKKRISEGIAI-AIQGGPR	pig, mammals, animals, *Sus*	5	35	31
Clavanin B	[63]	VFQFLGRIIHHVGNFVHGFSHVF	Invertebrate, Sea squirt, tunicate, animals, *Styela clava*	5	52	23
Ginkbilobin	[64]	ANTAFVSSAHNTQKIPA-GAPFNRNLRAMLADLRQNAAFAG	Ginkgophyta, Ginkgoales, plant, *Ginkgo biloba*	4	47	40
Gramicidin S	[65, 66]	cyclo-(fPVOL)$_2$	*Bacilli, Bacillales,* bacteria *Bacillus brevis*	2	60	10
Indolicidin	[67, 68]	ILPWKWPWWPWRR-CONH$_2$	cow, mammals, animals; *Bos taurus*	*3*	*53*	*13*
LfcinB6	[69]	RRWQWR	designed, corresponding to residues 4-9 of Lactoferrin B (PDB 1LFC)	3	33	6
LL37	[70]	LLGDFFRKSKEKIGKE-FKRIVQRIKDFLRNLVPRTES	human, mammals, animals, *Homo sapiens*	*6*	*35*	*37*
Magainin2	[71]	GIGKFLHSAKKFGKAFVGEIMNS	frog, amphibians, animals, *Xenopus laevis*skin	4	43	23
Melittin	[72]	GIGAVLKVLTTGLPALISWIKRKRQQ	insect, invertebrates, animals, insect, Honeybee venom, *Apis mellifera*	5	46	26
Pleurocidin	[73]	GWGSFFKKAAHVGKHVGKAALTHYL	fish, animals, Winter flounder, *Pleuronectes americanus*	7	44	25
PMAP-23	[74]	RIIDLLWRVRRPQKPKFVTVWVR	pig, mammals, animals, *Sus*	6	47	23
Polymyxin B	[75]	RD*TD*D*D*fLD*D*T	*Bacilli, Bacillales,* bacteria *Bacillus polymyxa*			
Tachyplesin I	[76]	KWCFRVCYRGICYRRCR	crabs, *crustacea*, invertebrates, animals, *Tachypleus tridentatus*	6	47	17
Temporin G	[77]	FFPVIGRILNGIL	frog, amphibians, animals, *Rana temporaria*	1	61	13
VESP-VB1	[78]	FMPIIGRLMSGSL	insects, invertebrates, animal, *Vespa bicolor*	1	53	13
VrD2 (Vigna radiata defensin 2)	[79]	KTCENLAN-TYRGPCFTTGSCDDHCKNKEHLRSGRC RDDFRCWCTRNC	plant defensin, mung bean, Vigna radiata	5	29	47

O = Ornithine, D* = diaminobutyric acid, *Small letter indicates D-Amino acid, Ref. = references, N. Ch. = netto charge, H (%) = percentage hydrophobic amino acids in a molecule, L. = peptide chain length, most data were obtained from http://aps.unmc.edu/AP/main.php

peptides; however, one major advantage of using a biological synthesis is the low price of each peptide, since it is ribosomally-synthesized. Also, in a well-designed biological assay, highly active peptides can be enriched by performing several rounds of screening. Disadvantages of the biological approach include the following: only gene-encoded amino acids may be used, limited numbers of selected sequences permit only partial activity information, biological peptide libraries are tricky to handle, and often these libraries utilise peptides fused to proteins, rather than single, independent peptide molecules. Biological techniques such as phage display [22] and ribosome display [23] have been successfully used to screen for AMPs [24, 25].

Chemically synthesizing large numbers of peptides is less straightforward than using a biological approach, since standard fully automated peptide synthesis on resin following Boc- or Fmoc-chemistry is not designed for large numbers of peptides. In an attempt to solve this restriction, several different modified procedures have been developed, including pin synthesis, digital photolithography, tea bag synthesis and SPOT^{TM} synthesis on cellulose [26-29]. These methods can incorporate more than 600 commercially-available peptide building blocks, and it is possible to systematically investigate the interaction of interest; in this way, peptides can easily be transformed to peptoids, a possible direction toward developing lead structures for new drugs.

SCREENING PEPTIDES FOR ANTIMICROBIAL ACTIVITY USING SPOT SYNTHESIS

Peptide Synthesis

SPOT synthesis follows standard Fmoc-chemistry. It is a method that permits synthesis of up to 8000 addressable peptides on a cellulose membrane, in a highly parallel fashion [30, 31]. Small

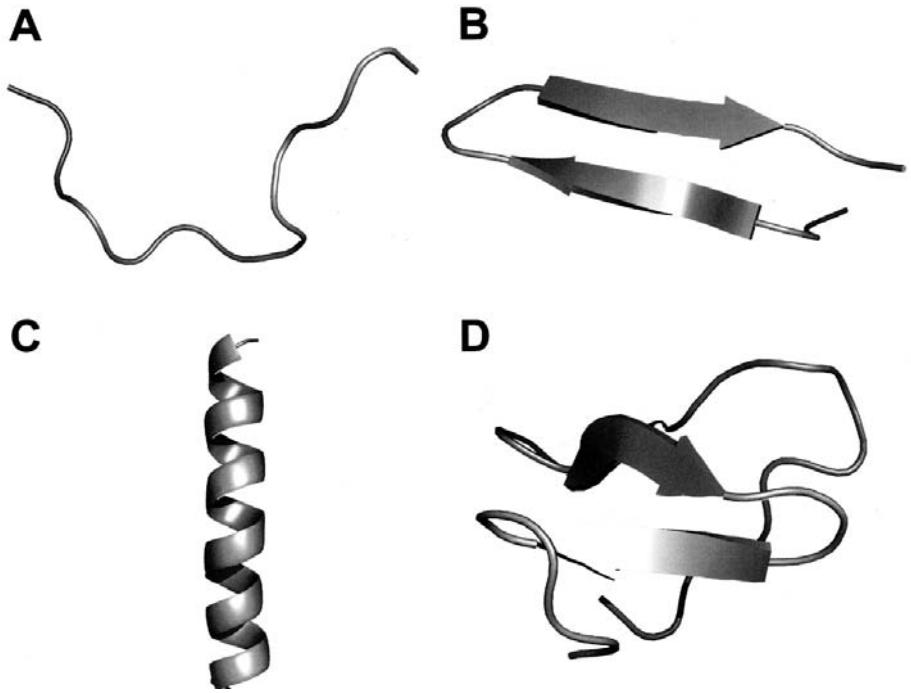

Fig. (1). Cartoon structures of antimicrobial peptides **A**) indolicidin in dodecylphosphocholine micelles (pdb code: 1G89) [53] **B**) Tachyplesin I in dodecylphosphocholine micelles (pdb code: 1WO1) **C**) Piscidin 1 in sodium dodecyl sulfate micelles (pdb code: 2OJO) [54] **D**) Kalata B1 bound to dodecylphosphocholine micelles (pdb code: 1ZNU) [55].

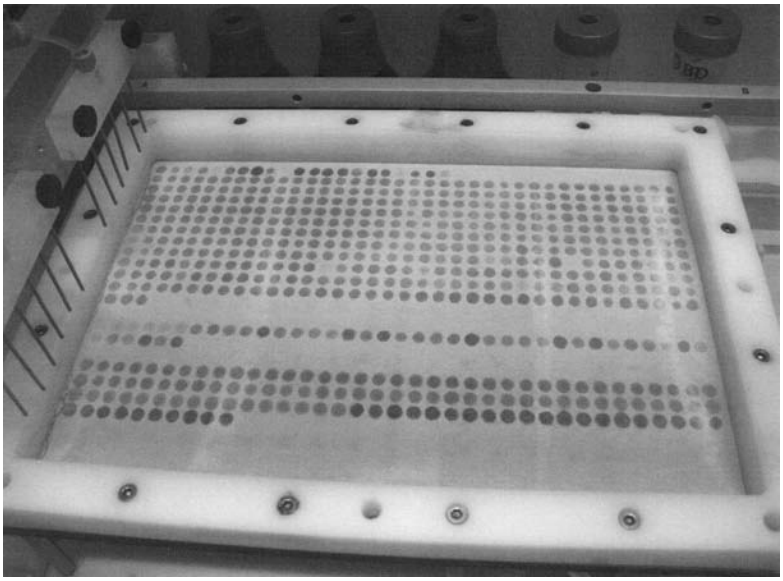

Fig. (2). Large tray of a fully automated peptide synthesizer. The picture shows the large spots used for the antimicrobial assay.

spots (ca. 0.1 μl amino acid per spot) are ideal for binding assays, such as with studying antibody epitopes [32, 33]. Larger spots (ca. 1.2 μl per spot) are ideal for biological assays in 96-well microtiter plates [34]. On a sheet of cellulose (18x29 cm), 800-1000 large peptide spots can be synthesized. In antimicrobial peptide screening, large spots (diameter ca. 0.6cm) and a glycine linker have been used [35]; using the glycine as a linker between cellulose (hydroxyl-groups) and the first amino acid (carboxyl-group) yields a high peptide density, up to 1.9 μmol/cm^2 [36].

Manual SPOT synthesis is easily integrated into a laboratory, as it requires no special equipment; however, it is a labour-intesive procedure. Manual SPOT synthesis is only used for synthesizing and screening small numbers of peptides. Fortunately, SPOT synthesis is now automated and can be used for screening much larger numbers of peptides or peptide mixtures. Both semi- and fully automated synthesizers are available for purchase (*e.g.* Intavis, Köln, Germany).

Peptide synthesis described for antimicrobial screening has been performed on a fully automated system that uses a large tray (Fig. **2**) and glycine linkers. After the synthesis and final side chain de-protection step, the ester bond between the glycine linker and the cellulose is cleaved using ammonia gas, resulting in a peptide with an amidated C-terminus [31]. The peptides are then punched out and transferred into a 96-well microtiter plate, after which distilled water is added to each well. Out of this stock solution, peptides can then be used for different assays.

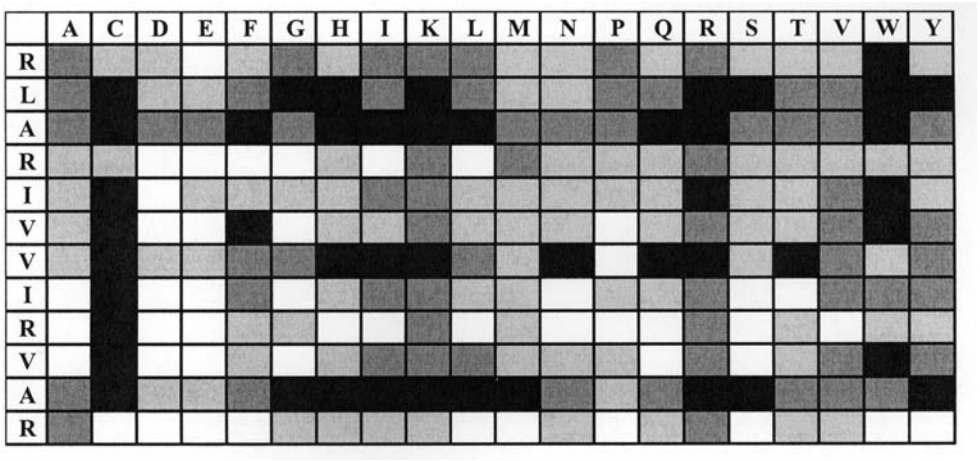

Fig. (3). Substitution analysis for peptide Bac2A, a variant of the naturally occurring peptide bactenecin, modified from [37]. The first column shows the single-letter code sequence of the original peptide Bac2A, the second and third rows show column number and amino acids, respectively, substituted at each amino acid position. Results are colour-coded as follows: dark grey = equivalent activity compared to the parent peptide Bac2A, black = superior activity compared to the parent peptide Bac2A, light grey = weaker activity compared to the parent peptide Bac2A, and white, indicating very little activity.

For each cellulose sheet, 1000 peptides can be synthesized over four days in a fully automated manner, and around 90,000 peptides per synthesizer per year. Fifteen different bacterial or fungal strains can be used at different dilutions for each peptide, and up to seven dilution steps have been reported, [37, 38] resulting in 1,350,000 possible dilution curves, and a total of 10,800,000 potential data points showing the antimicrobial activity of each peptide against different microbes. In order to manage this amount of data, a special statistical toolbox has been developed [39].

Screening Peptides for Antimicrobial Activity

Peptides that are synthesized using SPOT technology can be used in two slightly different assays. One assay is very sensitive and fast (luminescence based), with a read-out time of four hours; the downside is that it requires genetically modified bacteria [35]. The other assay is less sensitive (based on chemical stain) and involves additional pipetting steps and a longer read out time, but it can be used with all microbes (Mania, D., Hilpert, K., Ruden, S., Fischer, R. and Takeshita, N.; manuscript accepted at Appl Environ Microbiol.). In the following, I will focus on the sensitive assay.

Previously, luminescence-based assays have been used with great success to rapidly screen for antimicrobial substances [40-42]. To screen for antimicrobial peptides a bioluminescence assay was used where the amount of available energy was monitored. The bioluminescence reaction introduced by the *luxCDABE* gene cassette is dependent on oxidation of reduced riboflavin mononucleotide ($FMNH_2$) and a long-chain fatty aldehyde. This gene cassette was incorporated into the *Pseudomonas aeruginosa* genome [43]. *Pseudomonas aeruginosa* strain H1001, with a *Tn5-luxCDABE* gene inserted into the *fliC*, grows normally and expresses luminescence in abundance and, most importantly, in a constitutive fashion. As long as the bacterium remains under good growth conditions, light is produced. Any peptide that is able to interfere with the metabolism and/or energy state of the bacterium is observed as decreased light production, since the level of $FMNH_2$ are consequently reduced; this reaction has been shown to correspond very well with bacterial mortality [37]. Bioluminescence can be measured in a microtiter plate reader set up for high throughput reading, and the entire assay can be performed within four hours.

Screening Results

Three different strategies to study and optimize antimicrobial activity of peptides have been reported up to now A) substitution analysis of known AMPs, B) sequence scrambling of known AMPs, and C) screening peptide libraries for novel AMP sequences. Substitution analysis provides insight into the positional importance of each amino acid in a peptide sequence and its associated antimicrobial activity. Each position in the parent peptide sequence is exchanged against all other 19 gene-encoded amino acids. When substitution analysis indicates that a particular amino acid can only be substituted with itself or very similar amino acids, without leading to a loss of biological activity, this amino acid it is described as a key amino acid. When substitutions lead to improved antimicrobial activity, the amino acid in this position can be subsequently optimized: two such substitution analyses have been performed [37, 44].

A) Substitution Analysis of Known AMPs

The peptide Bac2A (RLARIVVIRVAR) is a modified variant of the naturally occurring peptide bactenecin (RLCRIVVIRVCR) [45]. Bac2A and a scrambled variant of Bac2A, Bac034 (VRLRIRVAVIRA), were analyzed using luminescence-based substitution analysis (Figs. **3** & **4**). While both peptides were comprised of identical amino acids, had the same length, net charge and number of hydrophilic and hydrophobic amino acids, their substitution analysis revealed divergent activity patterns: Bac2A could be optimized at most of the positions, whereas Bac034 showed optimization possibilities mainly restricted to the C-terminus of the peptide [37, 44]. In contrast to Bac2A, the important region of antimicrobial activity in Bac034 was in the core of the sequence, -IRV-, where R represents a key amino acid. The C-terminal arginine was important to the antimicrobial action of Bac2A, but the C-terminal alanine in Bac034 was not and could be optimized. In the peptide Bac2A, two valines, at positions 6 and 7 respectively, showed differing importance to antimicrobial activity: the valine at position 6 could only be substituted by 3 other amino acids, whereas valine 7 could be substituted by 8 other amino acids. This example highlights the possibilities for both broad and highly detailed identification of amino acid positional and compositional importance for achieving antimicrobial activity using substitution analysis. The information from these two analyses has been used to develop novel peptides with superior activity against different human pathogenic microorganisms, including different bacteria and one fungus [37, 44]. The resultant patented peptides have the potential to become lead structures for novel antimicrobial drugs [46].

B) Sequence Scrambling of Known AMPs

The effect of a substitution at one position along a peptide can be dependent on the positions of other amino acids, even when located at a distance within the molecule (Fig. **5**). This presents

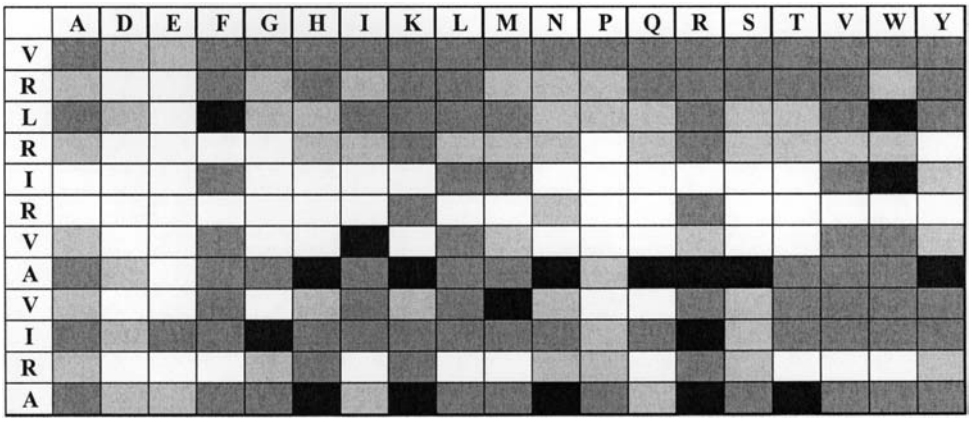

Fig. (4). Substitution analysis for Bac034, a scrambled variant of Bac2A, modified from [44]. The first column shows the single-letter code sequence of the original peptide Bac034. The second and third rows provide column number and amino acids respectively in the single-letter code substituted at each amino acid position. Results are colour-coded as follows: dark grey = equivalent activity compared to the parent peptide Bac034, black = superior activity compared to the parent peptide Bac034, light grey = weaker activity compared to the parent peptide Bac034, and white, representing very little antimicrobial activity.

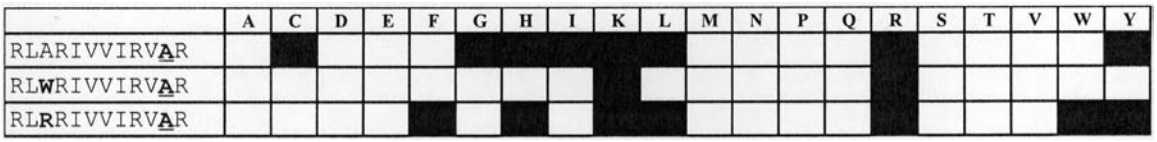

Fig. (5). Comparison of three substitution analyses of a single position (underlined and bold) of three different peptides modified from [44]. The underlined positions in the peptides represent substitutions. The first peptide is Bac2A and the other two peptides are different variants of Bac2A, where one position (bold) was substituted by a trypthophan (W) or phenylalanine (F) respectively. Black boxes represent peptides with antimicrobial activity at least 30% stronger than the parent peptide.

challenges to linear optimization of antimicrobial activity [44]. Based on the assumption that peptide sequence is important for achieving antimicrobial activity, scrambled versions of a peptide were expected to show either weak or no activity; otherwise, when peptide sequence was unimportant for achieving antimicrobial activity, scrambled peptides should have resulted in at least some antimicrobial activity. Within a set of 49 scrambled Bac2A variants activities ranging from superior to inactive were found, indicating that antimicrobial activity was not solely dependent on the composition of amino acids, but rather required particular linear sequence patterns that accounted for overall charge and ratio of hydrophobic/hydrophilic amino acids [44]. While suitable amino acid composition, including charge and ratio of hydrophobic/hydrophilic amino acids was necessary, it was not necessarily sufficient to produce antimicrobial activity.

C) Screening Peptide Libraries for Novel AMP Sequences

Novel sequences for antimicrobial activity can be discovered using peptide libraries. A completely random peptide library with 200 members was synthesized and antimicrobial activity was determined: 53% of the peptides showed weak antimicrobial activity, and 47% were inactive [47, 48]. Two semi-random peptides libraries, "A" and "B", with 943 and 500 different peptides respectively, were synthesized based on results described in [37, 44]; here, the success rate was higher: in A and B, 3.2% of the peptides showed superior activity, while 18.2% (A) and 26.7% (B) were active, 68.4% (A) and 61.1% (B) weakly active, and 10.2% (A) and 8.9% (B) inactive. Since sequences of peptides in libraries can vary greatly, these results demonstrate that peptide libraries can be used to find novel antimicrobial peptides with therapeutic potential [47].

Further Analysis of Peptide Library Screening Results

A quantitative structure-activity relationship (QSAR) approach can be applied to the results from peptide library screening in order to discover additional novel AMPs [47]. QSAR studies are strongly dependent on the available data set and descriptors used to train the program towards model development. Descriptors are used to translate the molecule or atom into meaningful numbers; for example, amino acid or peptide charge can be easily transformed in this way. The data sets produced by the luminescence based screening assay reviewed here deliver ideal training sets for QSAR studies, since the antimicrobial activity level of each peptide is distinct and known. Another particular advantage of combining the screening assay described here with QSAR is that predicted peptides can be easily synthesized and antimicrobial activity determined, in order to test the model's predictive powers.

The data sets presented here included superior activity, active, weakly active and inactive peptides, and QSAR was performed using 'inductive' descriptors [47]. This inductive set of QSAR descriptors was recently developed to introduce full 3-D-sensitive properties of peptides. This may dramatically improve peptide activity prediction, by allowing model construction that incorporates a wide range of peptide diversity, rather than a narrow range of nearly identical peptides. Inductive QSAR descriptors have been used previously: from an extensive set of 2686 chemical structures, up to 97% separation accuracy was observed for the three types of molecular antimicrobial activities [49]. These novel descriptors have been used in different types of models for classification, and, in some cases, such as with artificial neural networks (ANNs), predictions are generally more accurate, followed closely by the application of *k*-nearest neighbors methods [50].

Data sets A and B were used to train ANN using inductive descriptors. After reaching a plateau in the learning curve, 100,000 virtual peptides were first analyzed and ranked according to their predicted activity, then divided into four parts. The top 50 peptides from each quartile were synthesized and antimicrobial activity was determined: accuracy in identifying high activity peptides was 94% [51]. Several of these novel peptides were highly active against 19 different multidrug-resistant strains of eight species of "superbugs" that have been very problematic in clinical settings [47]. The effect of antimicrobial peptides identified by QSAR has also been demonstrated in a mouse infection model, using *S. aureus* (ATCC 25923):

all mice survived infection when treated with an antimicrobially active peptide [47]. Hemoltytic assays performed with these peptides showed a very weak effect, and very low toxicity was found in human peripheral blood mononuclear (PBMC) cells [47].

Another potential application of QSAR is screening for peptides that are antimicrobial while tethered to a surface. This particular application can be very useful for designing surfaces coated with novel antimicrobials, such as with surgical implants [52].

SUMMARY

Multidrug-resistant bacteria pose a substantial risk to human health. Cationic antimicrobial peptides are gaining attention for their potential to treat such bacterial infections. Understanding the sequence requirements of short cationic AMPs will bring research much closer towards the drug development stage; different screening approaches can be applied to achieve this. The SPOT technology, a rapid, fully automated procedure, can be used to chemically synthesize large numbers of peptides or peptide mixtures. The resultant peptide libraries can then be used to screen for antimicrobial activity, using luminescence gene-encoded bacteria (fast and sensitive method) or wild type bacteria which then will be counted or stained (slow and relative insensitive). The data arising out of this assay can be used to establish sequence requirements of short AMPs, as well as build predictive computer models (*e.g.* using QSAR) to discover additional novel AMPs.

REFERENCES

[1] GERMAP, *Antibiotika-Resistenz und-Verbrauch*. Antiinfectives Intelligence, Gesellschaft für klinisch-mikrobiologische Forschung und Kommunikation mbH: Rheinbach, Germany, **2008**.

[2] Rossolini, G. M.; Mantengoli, E. Antimicrobial resistance in Europe and its potential impact on empirical therapy. *Clin. Microbiol. Infect.* **2008**, *14* (Suppl 6), 2-8.

[3] Moet, G. J.; Jones, R. N.; Biedenbach, D. J.; Stilwell, M. G.; Fritsche, T. R. Contemporary causes of skin and soft tissue infections in North America, Latin America, and Europe: report from the SENTRY Antimicrobial Surveillance Program (1998-2004). *Diagn. Microbiol. Infect. Dis.* **2007**, *57* (1), 7-13.

[4] Rubinchik, E.; Dugourd, D.; Algara, T.; Pasetka, C.; Friedland, H. D. Antimicrobial and antifungal activities of a novel cationic antimicrobial peptide, omiganan, in experimental skin colonisation models. *Int. J. Antimicrob. Agents* **2009**, *34* (5), 457-461.

[5] Hancock, R. E.; Lehrer, R. Cationic peptides: a new source of antibiotics. *Trends Biotechnol.* **1998**, *16* (2), 82-88.

[6] Finking, R.; Marahiel, M. A. Biosynthesis of nonribosomal peptides1. *Annu. Rev. Microbiol.* **2004**, *58*, 453-488.

[7] Kawabata, S.; Beisel, H. G.; Huber, R.; Bode, W.; Gokudan, S.; Muta, T.; Tsuda, R.; Koori, K.; Kawahara, T.; Seki, N.; Mizunoe, Y.; Wai, S. N.; Iwanaga, S. Role of tachylectins in host defense of the Japanese horseshoe crab Tachypleus tridentatus. *Adv. Exp. Med. Biol.* **2001**, *484*, 195-202.

[8] Nochi, T.; Kiyono, H. Innate immunity in the mucosal immune system. *Curr. Pharm. Des.* **2006**, *12* (32), 4203-4213.

[9] Li, J.; Xu, X.; Xu, C.; Zhou, W.; Zhang, K.; Yu, H.; Zhang, Y.; Zheng, Y.; Rees, H. H.; Lai, R.; Yang, D.; Wu, J. Anti-infection peptidomics of amphibian skin. *Mol. Cell. Proteomics* **2007**, *6* (5), 882-894.

[10] Easton, D. M.; Nijnik, A.; Mayer, M. L.; Hancock, R. E. Potential of immunomodulatory host defense peptides as novel anti-infectives. *Trends Biotechnol.* **2009**, *27* (10), 582-590.

[11] Steinstraesser, L.; Kraneburg, U. M.; Hirsch, T.; Kesting, M.; Steinau, H. U.; Jacobsen, F.; Al-Benna, S. Host defense peptides as effector molecules of the innate immune response: a sledgehammer for drug resistance? *Int. J. Mol. Sci.* **2009**, *10* (9), 3951-3970.

[12] Nijnik, A.; Pistolic, J.; Wyatt, A.; Tam, S.; Hancock, R. E. Human cathelicidin peptide LL-37 modulates the effects of IFN-gamma on APCs. *J. Immunol.* **2009**, *183* (9), 5788-5798.

[13] Schiemann, F.; Brandt, E.; Gross, R.; Lindner, B.; Mittelstadt, J.; Sommerhoff, C. P.; Schulmistrat, J.; Petersen, F. The cathelicidin LL-37 activates human mast cells and is degraded by mast cell tryptase: counter-regulation by CXCL4. *J. Immunol.* **2009**, *183* (4), 2223-2231.

[14] Mookherjee, N.; Hamill, P.; Gardy, J.; Blimkie, D.; Falsafi, R.; Chikatamarla, A.; Arenillas, D. J.; Doria, S.; Kollmann, T. R.; Hancock, R. E. Systems biology evaluation of immune responses induced by human host defence peptide LL-37 in mononuclear cells. *Mol. Biosyst.* **2009**, *5* (5), 483-496.

[15] Mookherjee, N.; Rehaume, L. M.; Hancock, R. E. Cathelicidins and functional analogues as antisepsis molecules. *Expert Opin. Ther. Targets* **2007**, *11* (8), 993-1004.

[16] Mookherjee, N.; Hancock, R. E. Cationic host defence peptides: innate immune regulatory peptides as a novel approach for treating infections. *Cell. Mol. Life Sci.* **2007**, *64* (7-8), 922-933.

[17] Brogden, K. A. Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? *Nat. Rev. Microbiol.* **2005**, *3* (3), 238-250.

[18] Subbalakshmi, C.; Sitaram, N. Mechanism of antimicrobial action of indolicidin. *FEMS Microbiol. Lett.* **1998**, *160* (1), 91-96.

[19] Hale, J. D.; Hancock, R. E. Alternative mechanisms of action of cationic antimicrobial peptides on bacteria. *Expert Rev. Anti. Infect. Ther.* **2007**, *5* (6), 951-959.

[20] Hancock, R. E. Cationic peptides: effectors in innate immunity and novel antimicrobials. *Lancet Infect. Dis.* **2001**, *1* (3), 156-164.

[21] Boman, H. G. Innate immunity and the normal microflora. *Immunol. Rev.* **2000**, *173*, 5-16.

[22] Paschke, M. Phage display systems and their applications. *Appl. Microbiol. Biotechnol.*, **2006**, *70* (1), 2-11.

[23] Yan, X. H.; Xu, Z. R. Ribosome-display technology: applications for directed evolution of functional proteins. *Drug Discov. Today* **2006**, *11* (19-20), 911-916.

[24] Pini, A.; Giuliani, A.; Falciani, C.; Runci, Y.; Ricci, C.; Lelli, B.; Malossi, M.; Neri, P.; Rossolini, G. M.; Bracci, L. Antimicrobial activity of novel dendrimeric peptides obtained by phage display selection and rational modification. *Antimicrob. Agents Chemother.* **2005**, *49* (7), 2665-2672.

[25] Xie, Q.; Matsunaga, S.; Wen, Z.; Niimi, S.; Kumano, M.; Sakakibara, Y.; Machida, S. *In vitro* system for high-throughput screening of random peptide libraries for antimicrobial peptides that recognize bacterial membranes. *J. Pept. Sci.* **2006**, *12* (10), 643-652.

[26] Houghten, R. A. General-Method for the rapid solid-phase synthesis of large numbers of peptides - specificity of antigen-antibody interaction at the level of individual amino-acids. *Proc. Natl. Acad. Sci. USA*, **1985**, *82* (15), 5131-5135.

[27] Pellois, J. P.; Zhou, X. C.; Srivannavit, O.; Zhou, T. C.; Gulari, E.; Gao, X. L. Individually addressable parallel peptide synthesis on microchips. *Nature Biotechnol.*, **2002**, *20* (9), 922-926.

[28] Geysen, H. M.; Meloen, R. H.; Barteling, S. J. Use of peptide-synthesis to probe viral-antigens for epitopes to a resolution of a single amino-acid. *Proc. Natl. Acad. Sci. USA*, **1984**, *81* (13), 3998-4002.

[29] Frank, R. Spot-Synthesis - an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. *Tetrahedron* **1992**, *48* (42), 9217-9232.

[30] Hilpert, K.; Winkler, D. F.; Hancock, R. E. Cellulose-bound peptide arrays: preparation and applications. *Biotechnol. Genet. Eng. Rev.* **2007**, *24*, 31-106.

[31] Hilpert, K.; Winkler, D. F.; Hancock, R. E. Peptide arrays on cellulose support: SPOT synthesis, a time and cost efficient method for synthesis of large numbers of peptides in a parallel and addressable fashion. *Nat. Protoc.* **2007**, *2* (6), 1333-1349.

[32] Hoffmuller, U.; Knaute, T.; Hahn, M.; Hohne, W.; Schneider-Mergener, J.; Kramer, A. Evolutionary transition pathways for changing peptide ligand specificity and structure. *EMBO J.* **2000**, *19* (18), 4866-4874.

[33] Hilpert, K.; Hansen, G.; Wessner, H.; Kuttner, G.; Welfle, K.; Seifert, M.; Hohne, W. Anti-c-myc antibody 9E10: epitope key positions and variability characterized using peptide spot synthesis on cellulose. *Protein Eng.* **2001**, *14* (10), 803-806.

[34] Hilpert, K.; Wessner, H.; Schneider-Mergener, J.; Welfle, K.; Misselwitz, R.; Welfle, H.; Hocke, A. C.; Hippenstiel, S.; Hohne, W. Design and characterization of a hybrid miniprotein that specifically inhibits porcine pancreatic elastase. *J. Biol. Chem.* **2003**, *278* (27), 24986-24993.

[35] Hilpert, K.; Hancock, R. E. Use of luminescent bacteria for rapid screening and characterization of short cationic antimicrobial peptides synthesized on cellulose using peptide array technology. *Nat. Protoc.* **2007**, *2* (7), 1652-1660.

[36] Kamradt, T.; Volkmer-Engert, R. Cross-reactivity of T lymphocytes in infection and autoimmunity. *Mol. Divers.* **2004**, *8* (3), 271-280.

[37] Hilpert, K.; Volkmer-Engert, R.; Walter, T.; Hancock, R. E. High-throughput generation of small antibacterial peptides with improved activity. *Nat. Biotechnol.* **2005**, *23* (8), 1008-1012.

[38] Mikut, R.; Hilpert, K. Interpretable features for the activity prediction of short antimicrobial peptides using fuzzy logic. *Int. J. Peptide Res. Ther.* **2009**, *15* (2), 129-137.

[39] Mikut, R. Computer-based analysis, visualization, and interpretation of antimicrobial peptide activities. *Methods Mol. Biol.* **2010**, *618*, 287-299.

[40] Naveh, A.; Potasman, I.; Bassan, H.; Ulitzur, S. A new rapid and sensitive bioluminescence assay for antibiotics that inhibit protein synthesis. *J. Appl. Bacteriol.* **1984**, *56* (3), 457-463.

[41] Wheat, P. F.; Spencer, R. C.; Hastings, J. G. A novel luminometer for rapid antimicrobial susceptibility tests on gram-positive cocci by ATP bioluminescence. *J. Med. Microbiol.* **1989**, *29* (4), 277-282.

[42] Shawar, R. M.; Humble, D. J.; Van Dalfsen, J. M.; Stover, C. K.; Hickey, M. J.; Steele, S.; Mitscher, L. A.; Baker, W. Rapid screening of natural products for antimycobacterial activity by using luciferase-expressing strains of *Mycobacterium bovis* BCG and *Mycobacterium intracellulare*. *Antimicrob. Agents Chemother.* **1997**, *41* (3), 570-574.

[43] Lewenza, S.; Falsafi, R. K.; Winsor, G.; Gooderham, W. J.; McPhee, J. B.; Brinkman, F. S.; Hancock, R. E. Construction of a mini-Tn5-luxCDABE mutant library in *Pseudomonas aeruginosa* PAO1: a tool for identifying differentially regulated genes. *Genome Res.* **2005**, *15* (4), 583-589.

[44] Hilpert, K.; Elliott, M. R.; Volkmer-Engert, R.; Henklein, P.; Donini, O.; Zhou, Q.; Winkler, D. F.; Hancock, R. E. Sequence requirements and an optimization strategy for short antimicrobial peptides. *Chem. Biol.* **2006**, *13* (10), 1101-1107.

[45] Wu, M.; Hancock, R. E. Improved derivatives of bactenecin, a cyclic dodecameric antimicrobial cationic peptide. *Antimicrob. Agents Chemother.* **1999**, *43* (5), 1274-1276.

[46] Hancock, R. E. W.; Hilpert, K. Antimicrobial Peptides. Patent Application PCT/CA2005/001731, WO/2006/050611, 2006.

[47] Cherkasov, A.; Hilpert, K.; Jenssen, H.; Fjell, C. D.; Waldbrook, M.; Mullaly, S. C.; Volkmer, R.; Hancock, R. E. Use of artificial intelligence in the design of small peptide antibiotics effective against a broad spectrum of highly antibiotic-resistant superbugs. *ACS Chem. Biol.* **2009**, *4* (1), 65-74.

[48] Mikut, R.; Reischl, M.; Ulrich, A. S.; Hilpert, K. In *Data-based activity analysis and interpretation of small antibacterial peptides*, 18th workshop computational intelligence, Universitaetsverlag Karlsruhe: **2008**; pp. 189-203.

[49] Karakoc, E.; Sahinalp, S. C.; Cherkasov, A. Comparative QSAR- and fragments distribution analysis of drugs, druglikes, metabolic substances, and antimicrobial compounds. *J. Chem. Inf. Model.* **2006**, *46* (5), 2167-2182.

[50] Karakoc, E.; Cherkasov, A.; Sahinalp, S. C. Distance based algorithms for small biomolecule classification and structural similarity search. *Bioinformatics* **2006**, *22* (14), e243-51.

[51] Fjell, C. D.; Jenssen, H.; Hilpert, K.; Cheung, W. A.; Pante, N.; Hancock, R. E.; Cherkasov, A. Identification of novel antibacterial peptides by chemoinformatics and machine learning. *J. Med. Chem.* **2009**, *52* (7), 2006-2015.

[52] Hilpert, K.; Elliott, M.; Jenssen, H.; Kindrachuk, J.; Fjell, C. D.; Korner, J.; Winkler, D. F.; Weaver, L. L.; Henklein, P.; Ulrich, A. S.; Chiang, S. H.; Farmer, S. W.; Pante, N.; Volkmer, R.; Hancock, R. E. Screening and characterization of surface-tethered cationic peptides for antimicrobial activity. *Chem. Biol.* **2009**, *16* (1), 58-69.

[53] Rozek, A.; Friedrich, C. L.; Hancock, R. E. Structure of the bovine antimicrobial peptide indolicidin bound to dodecylphosphocholine and sodium dodecyl sulfate micelles. *Biochemistry* **2000**, *39* (51), 15765-15774.

[54] Lee, S. A.; Kim, Y. K.; Lim, S. S.; Zhu, W. L.; Ko, H.; Shin, S. Y.; Hahm, K. S.; Kim, Y. Solution structure and cell selectivity of piscidin 1 and its analogues. *Biochemistry* **2007**, *46* (12), 3653-3663.

[55] Shenkarev, Z. O.; Nadezhdin, K. D.; Sobol, V. A.; Sobol, A. G.; Skjeldal, L.; Arseniev, A. S. Conformation and mode of membrane interaction in cyclotides. Spatial structure of kalata B1 bound to a dodecylphosphocholine micelle. *FEBS J.* **2006**, *273* (12), 2658-2672.

[56] Turner, J.; Cho, Y.; Dinh, N. N.; Waring, A. J.; Lehrer, R. I. Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrob. Agents Chemother.* **1998**, *42* (9), 2206-2214.

[57] Mandard, N.; Sy, D.; Maufrais, C.; Bonmatin, J. M.; Bulet, P.; Hetru, C.; Vovelle, F. Androctonin, a novel antimicrobial peptide from scorpion Androctonus australis: solution structure and molecular dynamics simulations in the presence of a lipid monolayer. *J. Biomol. Struct. Dyn.* **1999**, *17* (2), 367-380.

[58] Casteels-Josson, K.; Capaci, T.; Casteels, P.; Tempst, P. Apidaecin multipeptide precursor structure: a putative mechanism for amplification of the insect antibacterial response. *EMBO J.* **1993**, *12* (4), 1569-1578.

[59] Rozek, T.; Wegener, K. L.; Bowie, J. H.; Olver, I. N.; Carver, J. A.; Wallace, J. C.; Tyler, M. J. The antibiotic and anticancer active aurein peptides from the Australian Bell Frogs Litoria aurea and Litoria raniformis the solution structure of aurein 1.2. *Eur. J. Biochem.* **2000**, *267* (17), 5330-5341.

[60] Csordas, A.; Michl, H. Isolation and structural resolution of a haemolytically active polypeptide from the immune secretion of a European toad. *Monatsh. Chem.* **1970**, *101*, 182-189.

[61] Moore, A. J.; Beazley, W. D.; Bibby, M. C.; Devine, D. A. Antimicrobial activity of cecropins. *J. Antimicrob. Chemother.* **1996**, *37* (6), 1077-1089.

[62] Sipos, D.; Andersson, M.; Ehrenberg, A. The structure of the mammalian antibacterial peptide cecropin P1 in solution, determined by proton-NMR. *Eur. J. Biochem.* **1992**, *209* (1), 163-169.

[63] Lee, I. H.; Zhao, C.; Cho, Y.; Harwig, S. S.; Cooper, E. L.; Lehrer, R. I. Clavanins, alpha-helical antimicrobial peptides from tunicate hemocytes. *FEBS Lett.* **1997**, *400* (2), 158-162.

[64] Wang, H.; Ng, T. B. Ginkbilobin, a novel antifungal protein from Ginkgo biloba seeds with sequence similarity to embryo-abundant protein. *Biochem. Biophys. Res. Commun.* **2000**, *279* (2), 407-411.

[65] Lee, D. L.; Hodges, R. S. Structure-activity relationships of de novo designed cyclic antimicrobial peptides based on gramicidin S. *Biopolymers* **2003**, *71* (1), 28-48.

[66] Wadhwani, P.; Afonin, S.; Ieronimo, M.; Buerck, J.; Ulrich, A. S. Optimized protocol for synthesis of cyclic gramicidin S: starting amino acid is key to high yield. *J. Org. Chem.* **2006**, *71* (1), 55-61.

[67] Ryge, T. S.; Doisy, X.; Ifrah, D.; Olsen, J. E.; Hansen, P. R. New indolicidin analogues with potent antibacterial activity. *J. Pept. Res.* **2004**, *64* (5), 171-185.

[68] Selsted, M. E.; Novotny, M. J.; Morris, W. L.; Tang, Y. Q.; Smith, W.; Cullor, J. S. Indolicidin, a novel bactericidal tridecapeptide amide from neutrophils. *J. Biol. Chem.* **1992**, *267* (7), 4292-4295.

[69] Vogel, H. J.; Schibli, D. J.; Jing, W.; Lohmeier-Vogel, E. M.; Epand, R. F.; Epand, R. M. Towards a structure-function analysis of bovine lactoferricin and related tryptophan- and arginine-containing peptides. *Biochem. Cell Biol.* **2002**, *80* (1), 49-63.

[70] Travis, S. M.; Anderson, N. N.; Forsyth, W. R.; Espiritu, C.; Conway, B. D.; Greenberg, E. P.; McCray, P. B., Jr.; Lehrer, R. I.; Welsh, M. J.; Tack, B. F. Bactericidal activity of mammalian cathelicidin-derived peptides. *Infect. Immun.* **2000**, *68* (5), 2748-2755.

[71] Baker, M. A.; Maloy, W. L.; Zasloff, M.; Jacob, L. S. Anticancer efficacy of Magainin2 and analogue peptides. *Cancer Res.* **1993**, *53* (13), 3052-3057.

[72] Kreil, G. Structure of melittin isolated from two species of honey bees. *FEBS Lett.* **1973**, *33*, 241-244.

[73] Cole, A. M.; Weis, P.; Diamond, G. Isolation and characterization of pleurocidin, an antimicrobial peptide in the skin secretions of winter flounder. *J. Biol. Chem.* **1997**, *272* (18), 12008-12013.

[74] Zanetti, M.; Storici, P.; Tossi, A.; Scocchi, M.; Gennaro, R. Molecular cloning and chemical synthesis of a novel antibacterial peptide derived from pig myeloid cells. *J. Biol. Chem.* **1994**, *269* (11), 7855-7858.

[75] Regna, P. P.; Solomons, I. A.; Forscher, B.K.; Timreck, A.E. Chemical studies on polymyxin B. *J. Clin. Invest.* **1949**, *28* (5 Pt. 1), 1022-1027.

[76] Laederach, A.; Andreotti, A. H.; Fulton, D. B. Solution and micelle-bound structures of tachyplesin I and its active aromatic linear derivatives. *Biochemistry* **2002**, *41* (41), 12359-12368.

[77] Simmaco, M.; Mignogna, G.; Canofeni, S.; Miele, R.; Mangoni, M. L.; Barra, D. Temporins, antimicrobial peptides from the European red frog Rana temporaria. *Eur. J. Biochem.* **1996**, *242* (3), 788-792.

[78] Chen, W.; Yang, X.; Zhai, L.; Lu, Z.; Liu, J.; Yu, H. Antimicrobial peptides from the venoms of Vespa bicolor Fabricius. *Peptides* **2008**, *29* (11), 1887-1892.

[79] Lin, K. F.; Lee, T. R.; Tsai, P. H.; Hsu, M. P.; Chen, C. S.; Lyu, P. C. Structure-based protein engineering for alpha-amylase inhibitory activity of plant defensin. *Proteins* **2007**, *68* (2), 530-540.

Received: April 06, 2010 Revised: June 02, 2010 Accepted: July 14, 2010