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STRUCTURAL, FUNCTIONAL AND EVOLUTIONARY STUDIES OF ANTIMICROBIAL PEPTIDES

Pasupuleti, Mukesh

2009

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With due permission from the Medical Faculty at Lund University this doctoral thesis is to be defended for the public on September 30, 2009, at 9.15 A.M. in the GK-salen, Biomedicinsk Centrum, Sölvegatan 19.

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Organization	Document name
LUND UNIVERSITY Department of Clinical Sciences, Lund Faculty of Medicine Lund University	DOCTORAL DISSERTATION
	Date of issue September 30,2009
	Sponsoring organization
Author(s) Mukesh Pasupuleti	

Title and subtitle

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Key words: Antimicrobial peptides, Complement system, Kininogen, Tagging , activity enhancement

Classification system and/or index termes (if any):

Supplementary bibliographical information:	Language English
ISSN and key title: 1652-8220	ISBN 978-91-86253-73-8

Recipient's notes	Number of pages	Price
	Security classification	

Distribution by (name and address) Mukesh Pasupuleti, BMC B14, Tornavagen 10, Lund

Signature _____ Date 18, Aug'2009

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Front Image :
Scanning EM picture of *S.aurues* kindly provided by Matthias Morgelin (left top), Molecular models C3a molecule kindly provided by Bjorn Walse (left bottom), Helical wheel projection of CNY peptides.

Printed by Media-tryck, Lund University, Sweden

ISSN 1652-8220
ISSBN 978-91-86253-73-8

Lund University, Faculty of Medicine Doctoral Dissertation Series 2009:85

At
Floral feet of
Mother Saraswati

Mankind in the conquest to fly like a bird, swim like a fish, forgot to live like human

Mukesh Pasupuleti

Abstract

Antimicrobial peptides represent a heterogeneous group that displays multiple modes of action such as bacteriostatic, microbicidal and cytolytic properties that are sequence and concentration dependent. Life threatening infectious disease is now a worldwide crisis and treating them effectively is becoming difficult day by day, due to the emergence of antibiotic resistant strains at alarming rates. Hence, there is an urgent need for new class of antibiotics and, antimicrobial peptides (AMPs) are an ideal candidate for this job. AMPs are gene encoded short (<100 amino acids), amphipathic molecules with hydrophobic and cationic amino acids arranged spatially which exhibit broad-spectrum antimicrobial activity. AMPs form an ancient non-specific type of innate immunity found universally in all living organisms and used as the principal first line of defense against the invading pathogen. AMPs have been in the process of evolution, as have the microbes, for hundreds of years. Despite the long history of co-evolution, AMPs have not lost their ability to kill the microbes totally nor have the microbes learnt to avoid the lethal punch of AMPs. Based upon accumulating positive data, we are encouraged to believe that antimicrobial peptides have a great potential to be the next breakthrough and first novel, truly biological in nature, class of antibiotics.

The purpose of this study was twofold; primarily to elucidate the factors involved in governing the peptide activity and toxicity against membranes, and secondly to design a simple approach where we can boost and spread the spectrum of antimicrobial activity against pathogens such as *S. aureus* and *P. aeruginosa* for a peptide that is otherwise non-lethal to the bacteria. Results presented in this thesis show that antimicrobial domains of the anaphylatoxin C3a are structurally and evolutionary conserved. Moreover antimicrobial activity is not governed by a single factor, but instead by a combination of net charge, amphipathicity and helicity. By utilizing a low number of amino acid substitutions at strategic positions in an antimicrobial peptide derived from C3a, CNY20, we were able to develop peptides, which exert a significant activity on both *S. aureus* and *C. albicans* in contrast to the parent peptide. Although, antimicrobial activity is not governed by single parameter, the activity can still be boosted by end-tagging of a peptide with hydrophobic oligopeptide stretches. This modification promotes peptide binding to bacteria and subsequent cell wall rupture, but does not increase the toxicity or the protease susceptibility of the peptide. It is noteworthy that end tagging of ultra short peptides spanning 5-7 amino acids with hydrophobic amino acids

enhances bactericidal activity, while preserving low toxicity and protease resistance.

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Abbreviations

AMP **:** Antimicrobial peptides

CLR **:** C-type lectin

FTIR **:** Fourier Transform Infrared Spectroscopy

HNP **:** Human neutrophil peptide

PRR **:** Pattern recognition receptors

PAMP **:** Pathogen–Associated Molecular Pattern

TLRs **:** Toll-like receptors

Original papers

This thesis is based on the following papers

I. **Pasupuleti M**, Walse B, Nordahl EA, Morgelin M, Malmsten M, Schmidtchen A. (2007) Preservation of antimicrobial properties of complement peptide C3a, from invertebrates to humans. *J Biol Chem*,**282**:2520-2528.

II. **Pasupuleti M**, Walse B, Svensson B, Malmsten M, Schmidtchen A. (2008) Rational design of antimicrobial C3a analogues with enhanced effects against Staphylococci using an integrated structure and function-based approach. *Biochemistry*,**47**:9057-9070.

III. Schmidtchen A, **Pasupuleti M**, Mörgelin M, Davoudi M, Alenfall J, Chalupka A, Malmsten M. (2008) Boosting antimicrobial peptides by hydrophobic amino acid end-tags. *J Biol Chem* **284**:17584-17594.

IV. **Pasupuleti M**, Schmidtchen A, Chalupka A, Ringstad L, Malmsten M. (2008) End-tagging of ultra-short antimicrobial peptides by W/F stretches to facilitate bacterial killing. *PLoS One*,**4**:e5285

The Immune system

The immune system is as old as life on earth itself; all along the evolution paradise certainly did not last, as one organism was potential rich source of food for other. In order to defend and eliminate the intrusion of another organism there was a need for a system, which could help the host in defense. Even today, the ability to protect oneself is key factor for survival, and all living forms, from bacteria to humans, resist the invasion of their body by another organism through either a simple or complex defense mechanism called the immune system. The immune system is a remarkably versatile defense system evolved to protect the individual from pathogenic organism and to clear damaged host self-components. In higher animals, the immune system consists of specialized cells capable of killing pathogens, this is, in contrast to unicellular organisms, which utilize simple mechanisms such as restriction enzymes, phagocytosis, antimicrobial peptides, and RNA interference[1].

Nowadays for more didactical reasons, the immune system in higher animals is divided into two types, "innate and adaptive system" based upon whether receptors of the system are encoded in the germ line or generated by recombination/diversification of the gene segments[2]. Both the innate and adaptive systems differ from each other with regard to many aspects such as the recognition system, the mechanism and kinetics of action, the recruitment to the site, and the type of cells involved in controlling the response etc. This classification is mostly found in immunology text books only and doesn't exist in nature, as both systems orchestrate together by cross communication. For unknown reasons, the older immune system was not replaced in higher verterberates and was instead supplemented to, thus creating an extra layered structure of immune system with cross talk[3]. Thus, during evolution, immune system complexity increased with addition of new and diverse components that have acquired the ability to co-operate inorder to provide an efficient and promount response.

Adaptive immunity

Adaptive immunity is a highly complex form of immunity, which exist in higher vertebrates[2]. It can discriminate host components from pathogens and differentiate among pathogen types e.g. virus, bacteria in order to mount a required defense by using both non-specific and highly specific cell mediated responses. By using a wide variety of cells and control mechanisms, the adaptive immune system responds to the challenge of pathogenic organisms with a high degree of specificity and "memory". It can recognize two antigens

differing by just a single amino acid. Once the adaptive immune system has recognized and responded to an antigen, it exhibits immunological memory, thus a higher immune response in a shorter time is mounted when exposed to the same antigen again.

Table 1: Overview of immune system found in living organisms

			Adaptive immunity	Innate immunity	Antimicrobial peptides
Prokaryotes		Bacteria	-	-	+
		Fungi	-	-	+
Eukaryotes		Plants	-	*	+
	Invertebrate animals	Protozoa	-	*	?
		Porifera	-	*	?
		Annelida	-	*	+
		Arthropods	-	+	+
		Mollusca	-	+	+
		Echinodermata	-	+	+
	Vertebrate animals	Jawless fishes	-	+	?
		Jawed fishes	+	+	+
		Amphibians	+	+	+
		Reptiles	+	+	+
		Birds	+	+	+
		Mammals	+	+	+

- = Failure to demonstrate its presence + = Definitive demonstration

* = Partial components are present ? = Presence or absence to be demonstrated

Typically, adaptive immunity comes into action with a delay of 4-5 days after the host is antigenically challenged by the pathogen, and this delay is compensated by antigen specificity and memory that makes it unique and special. Even though it is highly advanced and equipped, adaptive immunity is not independent of innate immunity. Both systems operate in a highly interactive and cooperative way, producing a combined response, which is more effective than each system could produce by itself. Therefore, adaptive immunity is just one half of the arsenal of the immune system that an

individual has.

About 500 million years ago, the adaptive immune system first appeared in jawed vertebrates[2], when a transposon carrying the early form of recombinase activating gene was introduced into the germline[4, 5]. The ability to generate unlimited variability of immune receptors in each individual by random recombination and diversification of gene segments and, clonal expansion of cells bearing a specific receptor in response to an antigen, might have given jaw fishes temporary advantages that lead to their large spread in the animal kingdom. Probably during that time, the adaptive immune system might have provided better protection than innate immunity at the individual level, due to multicellular complexity in architecture, diversity of pathogen/microbes encountered and high age of individual survival in higher vertebrates[2]. As a result of this, it become an virtual universal characteristic of all vertebrates[4]. Primitive forms of lymphoid tissues are present in invertebrates, however to date no antibody or cell mediated long lasting immunity has been discovered in lower invertebrates[6].

Table 2: Comparison between adaptive and innate immunity

	Adaptive	Innate
Presence	In higher vertebrates only*	All living forms
Response time needed after infection	Days	Hours
Specificity	Highly diverse	Limited and Fixed
Response to repeated infection	Faster than primary response	Identical to primary response
Memory of pathogen / infectious agent	Yes	No
Germline encoded	No	Yes
Components	Antibodies, Antigens, leukocytes	AMPs, cytokines, complement components, phagocytes

*: As on date

Innate immunity

In comparison to adaptive immunity, innate immunity is a universal and ancient form of host defense against pathogens. Unlike adaptive immunity,

innate immunity is less specific without any memory or clonal expansion, but it is a fast and effective means of defense against the pathogen[7]. Previously it was thought that the major role of innate immunity is to prevent and hold the microbial growth shortly after the infection, until a sufficient amount of adaptive immunity is mobilized to the site of infection[8]. Very recently the significance of the innate immune system is being understood, particularly when it becomes clear that it is the first defense mechanism that is activated against the pathogen and has a remarkably broad spectrum of effectiveness[9, 10]. Moreover successful evolution and extraordinary survival of plants and invertebrates in harsh conditions for more than 400 million years without a counterpart and/or adaptive immunity, emphasizes the extreme effectiveness and significance of innate immunity[7, 11, 12]. In addition, the similarity in mode of action and molecules used for defense in plants and animals indicates that the innate immune system evolved long before the split of the evolutionary tree into plant and animal kingdoms [13, 14]. Although rare, defects in innate immunity are always lethal to an individual, suggesting the presence of redundancy. Interestingly, the side effects of the adaptive immune system, such as autoimmune disease, allergy and allograft rejection has never been seen in the lower invertebrates and plants despite numerous efforts to show this by various researchers[13].

Components of innate immunity

In order to control both endogenous and exogenous bacteria, innate immunity has developed numerous ways. In a broad sense, the innate immune system is composed of physical barriers (e.g. skin, mucosal lining), effectors molecules (e.g. AMPs, cytokines), and cells (neutrophils, macrophages). Most components of innate immunity are present before the onset of an attack by pathogens and are not directed against a particular pathogen, but against various Pathogen–Associated Molecular Patterns (PAMPs) such as LPS of Gram-negative bacteria, bacterial flagellin, glycolipids of mycobacteria, lipoteichoic acids of Gram-positive bacteria, mannans of yeast, double stranded RNA of viruses[13] etc. Interestingly, pathogen and normal microbial flora have fairly similar structural features and how innate immunity discriminates between them is not clear.

In humans, innate immunity consists of proteins such as antimicrobial peptides, complement, cytokines, toll-like receptors (TLRs) etc.

Toll-like receptors

TLRs are the "eyes" of the innate immune system because they sense the infection by a pathogen and initiate the host defense mechanism[15]. TLRs were first identified in *Drosophila* as transmembrane receptor molecules involved in dorso-ventral polarity establishment during embryo development and later their role in innate immunity was described [15]. Evolutionary and functional analysis revealed rudimentary type of Toll/TLR systems in cnidarians, thereby pushing the evolution of this system back to 550 million years or perhaps beyond [5, 16]. Strangely to date, no developmental role has been ascribed to mammalian TLRs, apart from their direct role in immunity [13].

Innate immunity limits the infectious challenge rapidly using a wide armamentarium of pattern recognition receptors (PRR) such as Toll-like receptors (TLRs), C-type lectin (CLRs) etc. TLRs are a unique and essential type of PRR in animals and humans, which recognise PAMP molecules shared by most pathogenic microbes. They are usually expressed by cells of the immune system, but in case of injury to host, cells at the site of damage can respond by expressing TLRs[17, 18]. TLRs are transmembrane receptors with an extracellular leucine rich repeat domain and an intracellular Toll/IL-1 receptor domain[15]. TLRs (animals) bind to bacterial elicitors through leucine-rich repeats and signal through adaptor/effectors proteins containing TIR domains. This, initiates transcriptional programs including inflammatory cytokines, chemokines, AMPs, co-stimulatory molecules etc[13, 16, 19].

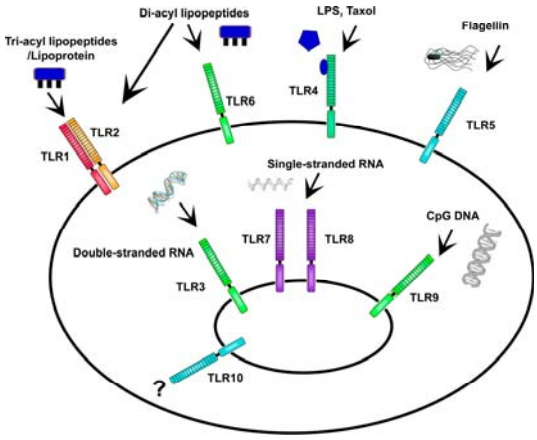

Figure 1: TLRs location in the cell and their ligands

To date 12 TLRs have been described in humans and mice. They are divided into 5 groups based upon amino acid sequence similarity. Each TLR has a different PAMP or ligand specificity and activates a different target gene. Interestingly, not all TLRs are expressed at a single location in the cell. They are expressed at various locations including the surface of the cell, endosome and intracellular regions in order to cover various routes of pathogen entry into the cell. The advantage of TLRs is that, a limited number of receptors are able to recognise a large variety of molecular structures. It is widely accepted that innate immunity recognise self and non-self by using TLRs since they are equipped to recognize PAMPs shared by different microorganisms[16].

Complement system

The complement system is an evolutionary old, powerful, integral and vital part of the innate immune system, as it senses danger and disturbed homeostasis of the body. The complement system is one of the major effectors of the humoral branch of the immune system. Thus, it plays an important role not only in defense but also as an essential instrument of self-surveillance. Research on the complement system in mammals has lead to the discovery of more than 35 different soluble and cell bound proteins which function in an orchestrated pattern to eliminate the pathogen or maintain homeostasis. Complement has three physiological functions (a) defense against invading pathogen (b) clearance of debris and immune complexes (c) integration and enhancement of the adaptive response [20, 21]. Complement consists of a complex machinery with an arsenal of both positive and negative regulatory factors, activating proteins and receptors. The complement system is initiated by three different mechanisms known as, the classical pathway, the alternate pathway or the lectin pathway. All pathways merge near the enzymatic activation of C3. the acknowledged molecular pillar of the complement system, followed by the common termination pathway which leads to the formation of a membrane attack complex[22]. The alternate pathway is initiated by binding of the complement protein C3 to the surface of the pathogen[23]. On the other hand, the lectin pathway is activated by the binding of lectin proteins to the mannose residues in the glycoprotein or carbohydrate present on the surface of the bacteria or fungi[24]. The classical pathway is initiated by an antigen-antibody complex, and is dependent on adaptive immunity whereas the alternate or lectin pathways are independent of this[23].

Structure based evolutionary comparison studies of the proteins involved in the complement pathways have shown that this system originated in primitive

organisms containing the most rudimentary innate immune system[10, 25]. It is perceived that complement proteins and non-specific protease inhibitor α-2-microglobulins, found in invertebrates such as arthropods and mollusks, have originated from a common progenitor gene which was lost during evolution[2, 26] or exists in other forms in lower animals and has yet to be discovered [5]. Complement genes then expanded by gene duplication and various pathways including activation/inactivation components have been incorporated in the system. Human complement factor C3 is a large protein with characteristic canonical thioester domain structure, which is similar to those found in lower cnidarians[10]. Notably, in comparison to humans, not all domains are present in lower vertebrates, thus confusing whether the evolution of C3 molecules occurred earlier or later[5]. Regardless, a true functional homologue of C3 was found in the horse shoe crab, (living fossil), thereby revealing the presence of the opsonic complement defense system in higher invertebrates[27, 28]. Agnathas, the most ancient living fish verterberates appers to have only alternate and lectin pathways. However cartilgious fishes, the most primitive species to posses immunoglobilins, have all the three-complement components. Thus, complement system rose to a higher level with the incorporation of antibodies during the evolution of Jaw vertebrates[2]. Of course, this is mere speculation at this time, and more studies on the immunological response and discrimination power of self and non-self in primitive lower invertebrates will undoubtedly reveal a greater understanding of this system.

During complement activation, proteolytic cleavage of the precursor molecules generates 3 types of anaphylatoxins e.g. C3a, C4a, and C5a. Anaphylatoxins are able to trigger degranulation of endothelial cells, mast cells, or phagocytes producing a local inflammatory response, which could lead to lethality depending upon the concentration. An important finding presented in this thesis, is that the antimicrobial activity of the C-terminal region of C3a and C4a but not C5a from various animals is conserved all along evolution from invertebrates to vertebrates and that this activity is more connected to structure rather than sequence[28]. Notably, even though anaphylatoxins share a partial structural identity, these factors are immunologically distinct molecules having no antigenic determinants in common[29].

Cytokines

Cytokines are low molecular weight regulatory or glycoproteins secreted by blood cells and various other cell types in response to a number of stimuli. Cytokines bind to specific receptors on the target membrane and alter the gene

expression by triggering signal transduction. Due to the often-high affinity exhibited by various cytokine receptors, cytokines can mediate their biological effects at picomolar concentrations. To date, more than 200 different cytokines have been discovered, which are grouped into four classes; hematopoietins, interferon's, chemokines, and tumor necrosis factor[30]. Interferons (IFNs) and chemokines are highly investigated due to their wide availability and immediate role in infections. Interferons are produced by the cells of the immune system of most vertebrates in response to challenge by foreign agents such as parasites, tumor cells and double-stranded RNA, a key indicator of viral infection. Interferons assist the immune response by inhibiting viral replication within host cells, activating natural killer cells and macrophages, increasing antigen presentation to lymphocytes, and inducing the resistance of host cells to viral infection. They are part of the non-specific immune system and are induced at an early stage in viral infection – before the specific immune system has time to respond.

Therapies based on cytokines and their receptors have entered into clinical practice. Interestingly, many chemokines have moderate antimicrobial activity and many AMPs have chemotactic activity. Albeit controversial, some researchers argue that chemokines and antimicrobial peptide defensins have originated from a single gene or by a gene duplication whereby one lead to the development of other [31, 32]. Although there is a quite similarity in activity and overall tertiary structure, an evolutionary relationship between defensins and chemokines remains to be determined[31].

Traditionally it is thought that innate immunity consists of phagocytic cells e.g. neutrophils and serum proteins e.g. complement, cytokines and interferon's. Nevertheless, during the last 4 decades it was found that, all sorts of living organism produce a large repertoire of antimicrobial peptides that are actively involved in clearance or inactivation of the microbes and/or play other significant roles in innate immunity.

Antimicrobial peptides

Antimicrobial peptides (AMPs) are gene encoded short (<100 amino acids), amphipathic molecules with broad-spectrum antimicrobial activity. Antimicrobial peptides represent a heterogeneous group that displays multiple modes of action including bacteriostatic, microbicidal and cytolytic properties that are sequence and concentration dependent. This ancient, non specific type

of innate immunity is the principal first line of defense used by many organisms against the invading pathogen[33]. Even though antimicrobial peptides are the first line of defense against the invading microbes, ironically they are a highly neglected aspect of immunology and are never addressed in typical immunology textbooks. The first AMPs to be isolated and characterized were those produced by bacteria. Logically, they don't protect the individual from infection since they kill other microbes, which might compete for space, food and other nutrients[34]. The wide recognition of AMPs started in the 1960s, when Spitznagel and Zeya discovered that basic proteins and peptides in polymorphnuclear leukocytes have antimicrobial properties[35, 36], which were later named as defensins[37, 38]. Seminal studies by Boman and colleagues in the 1980s demonstrated AMPs in invertebrates[39]. Since then more than more than 1400 AMPs have been isolated from bacteria, insects, and other invertebrates, amphibians, birds, fishes, and mammals including plants[33, 40].

Table 3: A list of AMPs produced by various organisms

Tree	Phylla/class	*Species*	AMP produced
Bacteria	Gram negative bacteria		Bacteriocins[33] Lantibiotics[34]
Fungi	Ascomycota saprophytic ascomycete	*Penicillium sp* *Pseudoplectania nigrella*	AF[41] Plectasin[42]
Plants		*Castanopsis chinensis*	TLPs[43, 44]
Animal kingdom	Porifera	*Stylissa caribica*	Stylisin[45]
		Discodermia kiiensis	Discodermin A[46]
	Cnidaria	*Hydra sp* *Aurelia aurita* *Sarcophyton glaucum*	Hydramacin-1[47] Aurelin[48] Sarcophytolide[49]
	Mollusk	*Mytilus galloprovincialis* *Conus mustelinus*	Myticin C[50] Conolysin-Mt[51]

	Annelida	*Nereis diversicolor* *Eisenia foetida* *Perinereis aibuhitensis* *Lumbricus rubellus*	Hedistin[52] OEP3121[53] Perinerin[54] Lumbricin[55]
	Arthopoda	*Carcinoscorpius rotundicauda* *Drosophila melanogaster*, *Pachycondyla goeldii* *Acalolepta luxuriosa* *Cupiennius salei* *Apis mellifera* *Androctonus australis* *Litopenaeus vannamei*, *Mytilus galloprovincialis* Insects belonging to lepidoptera and diptera, Marine protochordate and porcine intestine	Tachyplesins, Polyphemusin, and big defensin [56] Drosomycin, Cecropins, Diptericin, Drosocin, Attacin and Metchnikowin [4] Ponericidins[57] Acaloleptin A1, A2 and A3 [58], Lycotoxins and Cupiennin-1[59], Melittin[60], Androctonin[61], Penaeidins[62] Mytilin, Mytimycin[63] Cecropins[64]
	Echinodermata	*Strongylocentrotus droebachiensis*	Strongylocins[65]
	Fishes	Gadus morhua L *Ictalurus punctatus Rafinesque.* *Morone chrysops*	Hepcidin[66] HbbetaP-1[67] Piscidins[68]

		Oncorhynchus mykiss *Pleuronectes americanus*	Histone H2A[69] Pleurocidin[70]
	Reptiles	*Bungarus fasciatus* *Oxyuranus microlepidotus*	Cathelicidin-BF[71] Omwaprin[72]
	Amphibian	*Xenopus Sp*	Magainin[73]
	Birds	*Gallus gallus* *Gallus gallus* *Struthio camelus*	Gallinacins[74] Fowlicidin[75] Ostricacins[76]
	Mammals	*Bos taurus* *Homo sapiens*	LfcinB[77] Kinocidines[78] Defensins[79] Cathelidicins[79]

As shown in Table 3, AMPs represent a universal feature of defence systems existing in all living forms and wide existence on a long evolutionary scale proves their extreme effectiveness and significance to combating invading pathogens. Antimicrobial peptides are promptly synthesized and readily available shortly after an infection to rapidly neutralize a broad range of microbes. The ability to produce antimicrobial peptides is well preserved in almost all living organism and cell types. They can be synthesized at a low metabolic cost, easily stored in large amounts and they recognise common characteristics, instead of unique and specific tags particular to a pathogen, which is safe and efficient [2]. Even though AMPs have a certain degree of similarity in their biophysical properties, their sequence is never identical nor/or the same peptide sequences found in two different species of animals, even among those that are closely related. Total similarity is never found, and the only identity found is often in the pro region or a conserved region or conservation pattern of amino acids only. This phenomenon could probably reflect the species adaptation to the unique microbial environments that characterize the niche occupied by the species[11].

It is predicted that each species could have more than two dozen AMPs [80], and describing all them is beyond the scope of this thesis. Therefore, I would like to

give an introduction about AMPs found in humans and a small discussion about the unique, widely abundant AMPs in other animals. In humans, defensins and cathelicidins represent two large groups of AMPs in addition to other groups of molecules, which are synthesized at specific sites or formed on proteolytic degradation of proteins involved in immune functions by enzymes derived from the host or pathogens.

Defensins

Defensins are 28-44 amino acid long peptides with six conserved cysteines and 3 disulfide bonds without glycosyl- or acyl- side-chain modifications. Defensins were first discovered in rabbit and guinea pig granulocytes as small cationic molecules[81]. Until now defensins have been discovered in mammals only and distantly related forms appear in insects[82] and plants[83]. To date, in humans, defensins have been identified in the granules of neutrophils, paneth cells, monocytes, macrophages, keratinocytes or mucosal epithelial cells of the respiratory, digestive, urinary and reproductive systems[40, 84]. Defensins are synthesized as 93-96 amino acids pro-peptides consisting of a signal region, an anionic pre-segment and C-terminal cationic region. Release of the C-terminal region from the pro-segment by elastase, metallo proteinase, or other proteolytic cleavage activates the antimicrobial activity. The occurrence of disulphide-bridged defensins in a wide variety of organisms underscores that stabilized structure is of utmost importance for activity. *Zhoa H* reported that replacement of cysteine residues by certain amino acids like alanine, aspartic acids and leucine leads to a loss of activity whereas replacement with hydrophobic or aromatic amino acids retains the activity[85]. However, disulphide bonds in defensins are not necessary for the activity, but are of utmost importance for protease resistance[86] and chemotactic activity[87]. Defensins have been shown to have a broad-spectrum antimicrobial activity against bacteria, fungi and enveloped viruses. The mode of action of these peptides is quite simple, peptides oligomers assemble and form channels in the microbial membranes leading to ion gradient loss and death of the microbe[88]. Defensins link the innate and adaptive immunity by chemotactic mobilization of immunocompetent leukocytes[89], and induction of cytokine production[90] etc.

Based on the site of expression, size, structure and pattern of disulphide bridges, defensins are classified into 3 types

α-Defensins

α-defensins are 29-35 residues long with a disulfide alignment pattern of 1-6, 2-4, and 3-5. α- Defensins are either stored as propeptides (in Paneth cells) or as active processed matured peptides (in neutrophils). To date, in humans, more than 30 α-defensin genes have been predicted using bioinformatic approach[91], however at the protein level only 6 defensins have been discovered. Of these, 4 are expressed in neutrophils and called as human neutrophil peptides (HNPs) and 2 (HD5 and HD6) are expressed in path cells[92] and epithelial cells[93]. Interestingly, HNP-1, -2, -3 constitutes half of the total protein found in neutrophils[94].

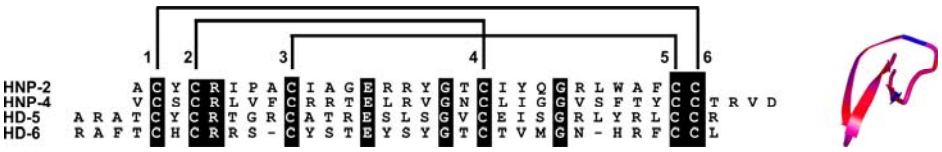

Figure 2: Schematic disulfide bond pattern of α-defensins

β-Defensins

β-defensins are 36-42 amino acids long with 1-5, 2-4, 3-6 disulfide alignment pattern and a longer N-terminal region, in comparison to α-defensins. More than 90 types of β-defensins have been isolated from various birds, reptiles and mammals. In humans only 4 different types of β- defensins (HBD) have been discovered in plasma, testis, gastric antrum[95], epithelial cells and neutrophils[96] and excluding HBD1, all are expressed only on inflammatory or infectious stimuli[79, 97]. At a concentration above 2 μM they can kill a vast spectrum of microorganisms under low salt concentration and serum free conditions. It has also been demonstrated that defensins of this class can stimulate host adaptive immunity[98].

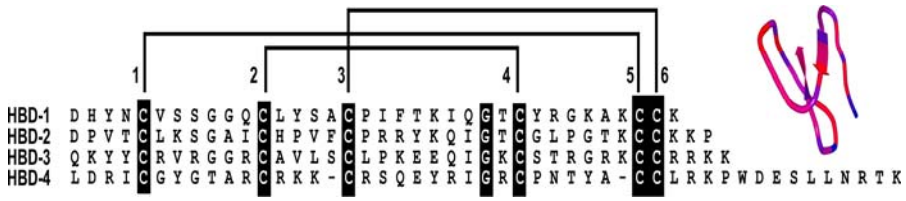

Figure 3: Schematic disulfide bond pattern of β-defensins

θ-Defensins

θ-Defensins are formed by post translation ligation of two 9-residue sequences derived by heterodimeric splicing of α-defensin-related precursors. The mature θ-defensin peptide is a circular two-stranded beta-sheet that is stabilized by three disulfides. However, the parallel orientation of the θ-defensin disulfide arrangement allows substantial flexibility around its short axis. θ - Defensins have been isolated from rhesus monkey, (*Rhesus macaque*) neutrophils[99] and the olive baboon, (*Papio Anubis*) leukocytes only[100]. Humans don't produce θ - defensins due to a premature termination codon in the signal peptide[101] and no data exists about the presence of these molecules in other animals. This molecule is of interest due to the lack of amphipathic nature and salt independent function. More interestingly θ - defensins have been shown to possess more antiviral properties, especially against HIV and HSV, than antibacterial and antifungal effects[102]

Cathelicidins

Cathelicidins are the second largest group of antimicrobial peptides produced by mammals and are characterized by far N-terminal end, a very unique conserved pre-proregion in the middle and a variable C-terminal region[103, 104]. The central conserved proregion is known as cathelin like region due to high sequence similarity with pig cathelin-like region. Like defensins, cathelicidins are also synthesized as propeptides, which are cleaved in two-step process to release the active peptide. The cathelin domain is highly conserved among different varieties of the peptides in both inter and intraspecies, indicating a common origin for this group[40]. To date cathelicidins have been found only in fish[105], birds[106], snakes[71, 107] and mammals[104]. There is only one type of cathelicidin in humans (hCAP18) and mice (CRAMP), whereas in pigs (PGs), cattle and sheep there are different types of cathelicidins with varied C-terminal and conserved N-terminal.

In humans, the hCAP18 propeptide is processed by a serine proteinase 3 in neutrophils to release the active fragment LL-37[108]. LL-37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) is a 37 amino acid long peptide with a highly hydrophobic N-terminal region and C-terminal region that adapt α-helical conformation in the presence of negatively charged lipids. Among all cathelicidins, LL-37, is highly investigated due to its unique structure, function, and composition[109]. It has been shown that LL-37, due to the amphipathic helical nature, is antimicrobial and binds to LPS [110, 111]. LL-37 has been shown to be toxic to normal eukaryotic cells at higher concentrations

[112, 113]. It is highly degradable by various enzymes[110, 113-115] and possess broad spectrum antimicrobial activity and synergistic action with other host derived

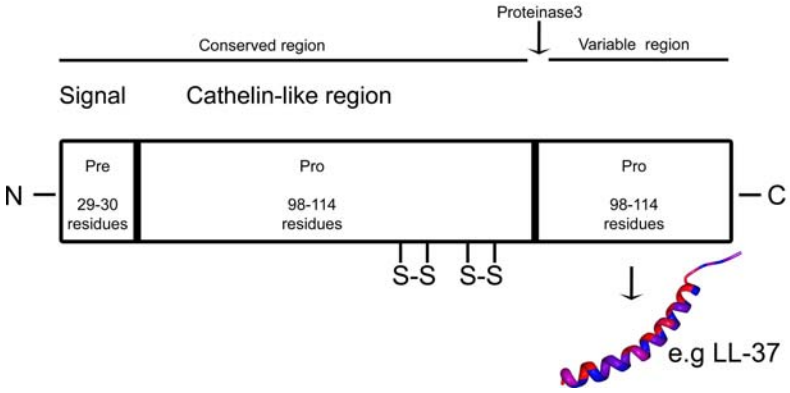

Figure 4: Schematic drawing of human cathelicidin hCAP18

peptides[109]. It can coordinate with other components of the innate immunity, such as recruiting neutrophils to the site of infections[116-118]. In humans, cathelicidins are expressed in macrophages[119], monocytes, B-cells, T-cells[120] and in most types of epithelial cells such as lung[121], skin[121], seminal plasma[122], epididymis[91] etc, whereas in other mammals, it is exclusively found only in the peroxidase negative granules[91].

Histatins

Histatins are a group of histidine rich cationic peptides found in humans and higher primates with broad spectrum antimicrobial activity[79]. Oppenheim first identified histatins in 1988, as antimicrobial peptides in human parotid and submandibular-sublingual gland secretions[123]. Basically there are 3 types of gene-encoded histatins, which undergo cleavage by proteases to generate 12 different types of histatins[123-126]. Of them all, histatins 5 is widely studied due to its α-helical structure stabilized by Zn^{+2} ions. Histatins mode of action is not by membrane permeabilization; instead it targets the mitochondria causing efflux of ATP, resulting in depletion of intracellular ATP contents and ultimately death [124, 127, 128]. Like most AMPs, histatins are not only antimicrobial, but also have other function such as inhibiting hemagglutination, co-aggregation and neutralisation of lipopolysaccharides by binding to lipid A[128-130], and acting as binding proteins for tannins[131, 132] .

Proline-arginine rich antimicrobial peptides

Mammalians, in addition to cathelicidins and defensins, produce a family of

antibacterial peptides known as proline-arginine rich peptides, which are rich (~ 60%) in proline and arginine amino acids.
To date three peptides belonging to this class have been isolated from bovine (BAC-5, Bac-7)[133] and porcine (PR39)[134]. In addition to a structurally identical N-terminal region, all the peptides have a highly repetitive sequence of Arg-Pro-Pro or Pro-Arg-Pro. All this similarity suggests that, proline–arginine rich peptides might have originated from the common origin or cathelicidins[6].

Bactenecins
Bactenecins are 5 - 7 kDa cationic bactericidal polypeptides with a high proline and arginine content in addition to 4 - 6 hydrophobic resides with disulfide bonds between cysteines. They are found in PMN cells and have only been isolated from cows to date. Bactenecins work in the range of 10^{-5}- 10^{-6} M and have been shown to be antimicrobial and antiviral [135].

Antimicrobial proteins and polypeptides
In order to defend the host from infection, innate immunity is not only equipped with short cationic peptides that are synthesized prior to or after infection, but also with a large number of proteins which are on constant surveillance in the system. The most important are indicated below

Bactericidal permeability increasing protein (BPI)
BPI is a 55 kDa protein with 2 distinct functional domains; the N-terminal 25 kDa fragment is antimicrobial where as the C-terminal fragment is LPS binding and antiangiogenic [136]. To date, BPI has been isolated only from human and rabbit PMN cells, which are very similar in structure and function. BPI inhibits only Gram-negative bacteria and is not active against Gram-positive bacteria or eukaryotic cells due to its high binding affinity for the outer membrane [137]. BPI in a sense is a unique molecule, as it acts in synergy with defensins, membrane attack complex of the complement system, and acts at sites of inflammation.

Heparin –binding protein (HBP)
Human heparin-binding protein (HBP) or CAP37 or azurocidin is 37 kDa basic, proteolytically inactive neutrophil elastase homologue, with heparin binding and antimicrobial activity [138]. HBP activity is mostly active against Gram-negative bacteria and the activity increases at low pH conditions. The basic amino acids in the HBP are responsible for the activity[139].

Histidine-rich glycoprotein (HRG)

HRG is a 67 kDa heparin-binding histidine-rich plasma protein, which was first isolated in 1972 by Heimburger *et al.*[140, 141]. This protein is synthesized in liver and is present in human plasma at high concentration (1.5–2 μM)[142, 143]. HRG contains two cystatin-like domains, a variable C-terminal region and a central histidine-rich region (HRR) with highly conserved GHHPH tandem repeats flanked by proline-rich regions [142, 143]. HRG can acquire positive net charge either by incorporation of Zn^{2+}, or by protonation of histidine residues (~13%) in the HRR domain at acidic conditions[142-144] as a result of which it acts as an antimicrobial[144-146]. Recently, various novel roles have been discovered for HRG derived peptides, involving antiangiogenesis[147], antitumor activity[148], as well as multiple interactions involving ligands such as heparin, plasminogen, fibrinogen, thrombospondin, heme, IgG, FcγR, and C1q[146].

Lactoferrin

Lactoferrin (80 kDa), is a major epdidymal globular multifunctional secretory protein found abundantly mainly at mucosa, secreted fluids, like semen, tears and breast milk with a potent activity against bacteria, fungi and viruses. Like cathelicidins and defensins, proteolysis of lactoferricin generates two different antimicrobial peptides, the N-terminal derived lactoferricins[149] and the kaliocins derived from an interior sequence[150]. Lactoferrin can permeablise membranes and disperse lipopolysaccharides through cation-mediated process especially chelating $Fe3^{+}$ (ferric state) ions [151] .

Lysozyme

Lysozyme is a 14 kDa cell wall degrading enzyme that is widely disturbed in biological fluids and tissues. Lysozyme damages bacterial cell walls by catalysing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan. The absence of lysozyme in some animal species is not associated with decreased resistance against infection and suggests that it is not a major protein playing a role in defence[137]. It usually functions in synergy with other AMPs such as lactoferrin, as it can't pass through the cytoplasm. It probably weakens the membranes so that AMPs can deliver their deadly punch more effectively [152].

Major basic protein (MBP)

MBP is 13 kD small basic protein, which is rich in arginine and cystine-rich

amino acids and comprises almost half of the protein content in the large specific granules of mammalian eosinophils. Even though this protein is toxic to human cell, host tissue damage is negligible due to the delivery of the protein locally at the target site. At high concentrations, MBP has been shown to be antibacterial, antihelminthic, cytotoxic and involved in immune hypersensitivity reactions, but the exact mode of antihelminthic action of this protein is unkown[153].

AMPs generated by proteolysis

Innate immunity, in addition to classical AMPs, contains a large number of antimicrobial proteins and/or polypeptides that are generated by proteolysis of different proteins[139, 154, 155]. Our group has been a pioneer in showing that AMPs are not only synthesized or stored as inactive forms, but can also be generated by enzymatic cleavage of proteins such as such as complement[155], and kininogen[156], or found as epitopes in growth factors[157] and matrix proteins[158]. AMPs generated by this method are usually larger in size then classical AMPs, but the active domain of the peptide is cationic and in many cases has an amphipathic structure. Most of the peptides used for the study in this thesis are derived by modifications of parent peptides that are generated by proteolysis of host proteins involved in the innate immune system.

Anaphylatoxin

Anaphylatoxins are small molecules (9 kDa) that are generated as a result of the activation of the complement proteins[159]. These molecules play an important role in inflammation and are responsible for the activation of various components of the innate and adaptive immune system[160]. The anaphylatoxin C3a, generated by activation of complement factor C3 is 77 amino acids long (molecular weight of 9 kDa), contains α-helical cationic regions stabilized by 3 disufide bonds and has a net charge of +2 (pI 11.3). The C4a anaphylatoxin is derived from complement factor C4 by action of protease C1s, and is a cationic polypeptide with 77 residues and devoid of histidine, tryptophan, and carbohydrate[29]. C5a anaphylatoxin is 74 amino acids long with four helices connected by loops and is released from C5 by the action of C5 convertase. Furthermore, C3a, C4a, but not C5a, have been shown to be antimicrobial and antifungal [28, 155]. It was concluded from the sequence comparison that C3a, C4a, and C5a are a family of bioactive factors derived from precursor molecules that share a common genetic origin. However, alignment and homology (dN/dS) studies between C3a, C4a, and C5a indicate only a 30% homology between C3a and C5a; 36% homology between C5a and C4a [28, 29,

[160]. It is a noteworthy point that, even though the primary sequences of C3a, C4a and C5a from various animals differ significantly, the crucial elements required for the stability and integrity of molecules are conserved, especially the cysteines for disulfide bonds, and arginines.

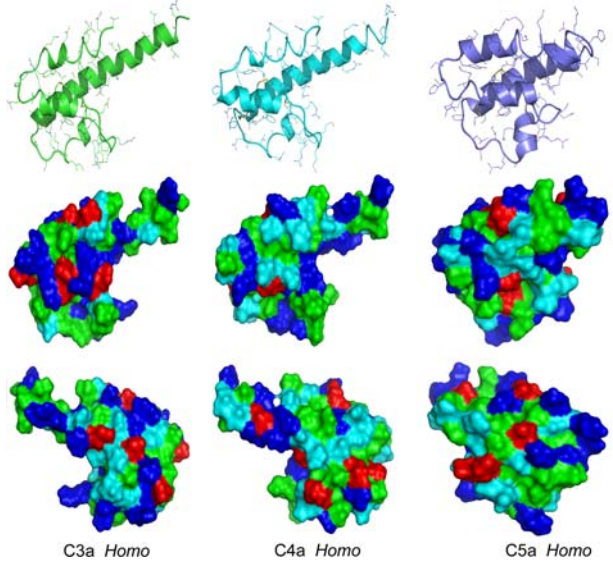

Figure 5: Molecular models of human anaphylatoxins

Kininogen

Kininogen is a 120 kDa, multifunctional glycoprotein found in plasma and in mast cell α-granules. It consists of 5 different domains, each with different biological functions. It is a parent protein for bradykinin and serves as a cofactor for coagulation factor XI and prekallikrein assembly on biologic membranes. Unlike, the complement system, controlled cleavage of kininogen releases different peptides with potent vasoactive, proinflammatory, heparin-binding, cell-binding and antiangiogenic properties[161]. It has been shown that kininogen when cleaved by mast cell tryptase and neutrophil elastase enzymes releases a domain 5 fragment, which is antimicrobial in nature[139]. Furthermore, cleavage by plasma kallikreins during contact system activation releases antimicrobial domain 4 peptides[156] as well as the bradykinin sequence.

AMP classification

All AMPs share roughly similar basic characters like positive net charge,

amphipathicity and hydrophobicity, thus it not possible to classify them based on it. In addition, AMPs are present in all living forms and sequence diversity among them is so large, that it is difficult to classify them broadly except on the basis of their secondary structure[162, 163]. Strictly speaking, peptides dissolved in water assume a random coil conformation whereby the hydrophobic motifs/amino acids are buried inside, but on contact with lipid membranes or a different solvent interface e.g TCA, acetonitrile, they assume either an α or β-sheet configuration. Therefore, taking antimicrobial function into account, the shape taken by a peptide when it interacts with bacterial membranes is considered during classification.

Group I (Helical peptides)

e. g: Magainins, LL-37

Helical peptides are the most abundantly distributed and widely studied groups of antimicrobial peptides[8, 28, 112, 164]. Among all AMPs with known secondary structures, 27% of them belong to this group. These peptides are totally unstructured in an aqueous environment, but adopt a helical conformation upon encountering hydrophobic solvents or lipid surfaces with a slight bend in the center[165]. Helix induction capability and flexibility is an important parameter for selective discrimination between the microbial and eukaryotic membranes[112]. From various structure-function studies, it has been confirmed that the first 3 amino acids in the helix at the N-terminal region are not important for activity, whereas truncation of the first 4 amino acids reduces the activity and further deletion abolishes activity and toxicity[110, 112, 166]. Capping at the N- and C-terminus stabilizes the helix further and results in salt insensitive antimicrobial peptides[167]. Peptides belonging to this group usually kill the microbes by creating channels in the membranes, leading to a loss of ion gradient. One of the best-studied antimicrobial, amphipathic peptide of this class is LL-37, as previously mentioned the first amphipathic α-helical peptide to be isolated from humans. Furthermore, not only cationic peptides, but even hydrophobic and anionic peptides and α-helical peptides exist in this class. However the later class exhibit a lower selectivity towards microbes compared to that towards mammalian cells.

Group II (Beta sheet containing peptides)

e. g: *Defensins*

In contrast to the helical peptides, β-sheet peptides are semi or cyclic molecules constrained by an intramolecular disulfide bridges. The best-studied peptides in this group are defensins and very little is known regarding how these peptides,

which have a constrained β-sheet structure, can permeablise membranes. Based upon the results obtained with model membranes, some researchers believe that most β-sheet peptides act on intracellular targets, as they are very effective in inducing lipid flip-flop movement and undergoing membrane translocation[168]. It is widely accepted that for antimicrobial activity, cyclization, a low degree of amphipathic nature, and maintenance of certain degree of a hydrophobic and hydrophilic balance is important for this group of peptides [169-171].

Group III (Over representation of one or more amino acids)

e. g Tritrpticin, Indolicidin, Histatin

Not all AMPs belong to the above-mentioned classes, some AMPs lack general classical secondary structure due to their unusual amino acid composition. Usually peptides grouped in this class are rich in proline and/or glycine or tryptophan or histidine amino acids. Interestingly, proline rich peptides can't form amphipathic structures but adapt a polyproline helical structure[172] and form hydrogen bonds and Vander-waals interactions with membrane lipids instead of intermolecular bonds[169]. Indolicidin is the best-studied AMP in this class due to its high tryptophan and proline content. Indolicidin is found in bovine neutrophils[173] and is 13 amino acids long, comprising 5 tryptophans, with a C-terminal amidation. The exact mode of action of Indolicidin is still controversial. *Falla et. al* have shown that it creates a voltage induced channel [174] in the membranes, whereas *Subbalaxmi et. al* reported that it prevents DNA replication[175]. Based upon the contradictory results obtained with either live bacteria or model membranes, although controversial, it is widely agreed that indolicidin first forms informal aggregate channels in the membranes that are short lived and on collapse, the peptides are translocated into cytoplasm where they execute their final functions[174-178]. At present various studies are now been directed to delineate the role of multiple tryptophan residues in its biological activity as well as interactions with model membranes.

Group IV (Looped peptides with single bond)

e. g:Thanatin, Lantibiotics

These groups of peptides are characterized by their looped structure imparted by the presence of a single bond (disulfide or amide or isopeptide bond). This group differs from the group II peptides in having only single disulphide bond and anti parallel β-sheet orientation. Lantibiotics belonging to this class are widely studied due to their unique biochemistry, genetic regulation, and a range of biological functions. Lantibiotics are small (19–38 amino acids) peptides

that undergo extensive posttranslational modification, especially dehydration of Ser and Thr residues in the propeptide to yield 2,3-didehydroalanine (Dha) and (*Z*)-2,3-didehydrobutyrine (Dhb), respectively. Later a lanthionine (Lan) or methyllanthionine (MeLan) bridge is created by addition of a stereospecific intramolecular Cys residue onto Dha or Dhb. This class of peptides, holds considerable potential in fighting existing and emerging infectious diseases because they are short in size, easy to synthesize and proteolytically stable.

Biophysical parameters influence the antimicrobial activity of AMPs

Selective toxicity is crucial for any antimicrobial peptides and to achieve this, molecules must have a set of biophysical themes or constrains. Until now, various biophysical properties such as amphipathicity, hydrophobicity, charge, polar angles etc, are found to influence the interaction and insertion of AMPs into the membranes. It is most important to note that these constrains are interdependent and changing one will lead to changes in the other. It is noteworthy that many AMPs have two striking and unique biophysical features, i.e hydrophobicity and amphipathic nature, which is conserved in all the phyla and is roughly similiar in almost all the AMPs isolated from various sources.

Sequence

The most characteristic feature found in AMPs is conservation in function and no conservation in sequence and length. Interestingly, for many helical AMPs whatever might be the sequence, ~50% of amino acids are hydrophobic[179], and arranged in the pattern of i+3 or i+4[112]. The reason being, when the peptide assumes helical structures all the hydrophobic and hydrophilic amino acids are on two different planes forming a perfect amiphatic structure. Even though there is no sequence homology among the AMPs belonging to similar families or isolated from the same animals, there is some degree of conservation of specific amino acids at significant positions. In most of the AMPs, aspartic acid and glutamic acid are rarely seen; whereas cationic amino acids like lysine or arganine are highly represented. In addition, cysteines and tryptophan residues are involved in disulfide bonds and hydrophobic interactions, respectively. It has been concluded that the binding of peptides to the interface was mediated by lysine residues which formed H-bonds with either the phosphate oxygen atoms or the glycerol oxygen atoms on the lipid head groups[180]. In addition to strong electrostatic interactions, arginine and lysine have been shown to contribute more towards peptide-membrane interactions, due to their long side chains which penetrate deep in the membrane core[181]. It is quite usual to find glycine at the C- or N-terminal position as it is a good capping agent, helix

stabilizer and provides protection from amino or carboxypeptidases[112, 164]. The fundamental reason for the different sequence, but common antimicrobial function, is due to the immuno relativity problem i.e, necessity of the host immune system to adapt successfully to different environments by retaining its efficiency against specific microbial pathogens[164]. In addition, AMPs have evolved to act against distinct microbial targets that differ in their membrane characteristics, and in different physiological conditions

Net charge (Q)

Many AMPs characterized so far have been shown to have a net charge of +2 - +9 due to the presence of lysine and/or arginine in highly defined cationic domains[182] and the absence of aspartic or glutamic acid[183]in such regions. It is widely accepted that cationicity is primarily responsible for the initial electrostatic interaction of the antimicrobial peptide with the negatively charged membrane surface of the bacteria[183, 184]. Bacterial membranes have ~50 % higher membrane potential ($\Delta\Psi$) than mammalian cells due to the presence of LPS and teichoic or teichuronic acid, which impart the additional negative charge on the surface[182]. Recent studies with magainin[185] and other helical peptides[112, 179] have shown that a direct correlation exists between charge and potency[112], and increasing the charge enhances the activity and specificity, up to a limit. However, increasing the charge beyond +7 doesn't increase the activity due to strong interactions between the peptide and the phospholipid head groups, which prevents structuring[186] and translocation into the deeper layers of membranes [112, 182, 186-188].

Conformation or shape (χ)

Even though more than 1400 AMPs have been isolated from diverse phylogenetic sources and having different sequences, they can be grouped into three classes based upon the conserved structure and charge; like α-helical, β-sheet or extended helices and loops. The α-helical antimicrobial peptides are the most abundant form isolated from both lower and higher animals such as arthropods, amphibians and mammals[183]. Most AMPs are random coils in solution and their insertion into membrane drives transition of random coil to helical structure. This transition is a must for the interaction of hydrophobic amino acids with the non-polar residues in the lipid bilayer core. For an efficient antimicrobial action and low toxicity, α-helical peptides should be flexible, because rigid structures are found to increase the toxicity without increasing the antimicrobial activity [33, 112]. β-sheet classes of AMPs are a quite diverse group of molecules with several anti-parallel β strands stabilized by a

series of disulfide bonds. Less is known about the structures of extended helices and loops[183, 189]. Despite these structural differences, all AMPs share a unique topology such as positive net charge and amphipathicity with a distinct hydrophobic and hydrophilic surface.

Amphipathicity (A)

Amphipathicity is traditionally defined as a relative abundance and distribution of hydrophobic and hydrophilic residues or domains within a peptide[183, 184]. Peptides with a perfect amphipathic nature when plotted on a Schiffer – Edmundson helical wheel show that basic and polar residues are aligned on a portion of the helical cylinder whereas the lipophilic side chains occupy the remaining surface. The proportions of each surface varies from one peptide to another[190].

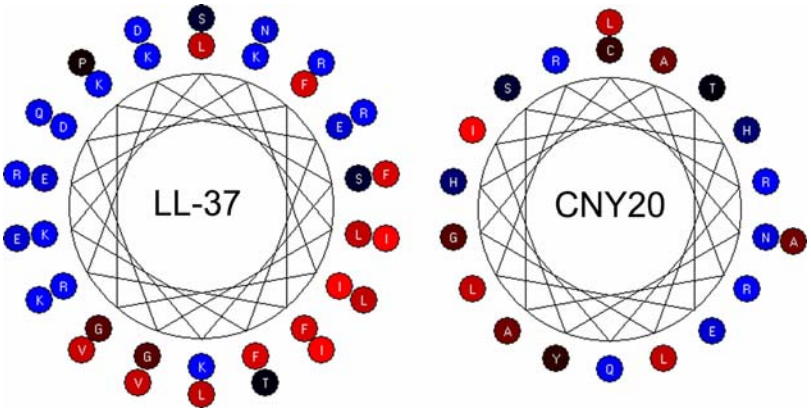

Figure 6: Schiffer – Edmundson helical wheel projections

Most α-helical peptides have a periodicity of 3-4 residues per turn, optimal for interaction with the amphipathic bilayered membranes[112]. All AMPs form an amphipathic structure upon interaction with the membranes in order to accommodate the inherently amphiphilic nature of the membrane lipid matrix. It is noteworthy that in most AMPs, the amino acids leucine, alanine lysine and/or cysteines are well represented. These amino acids have a strong propensity to stabilize helix or form a disulphide bond[28, 189]. The propensity of peptides to form helical structures is known to be influenced by N- capping agents such as glycine and helix stabilising amino acids like leucine, alanine and lysine, which are commonly found in most of the AMPs[112, 164]. Various

studies have shown that increasing helicity moderately influences the activity of peptides against negatively charged surfaces, but has a significant effect on cytotoxicity[112, 191-193]. Interestingly, α-helical peptides were found to be toxic towards eukaryotic cells when compared to β-sheet peptides with an identical composition, charge and hydrophobicity[194]. In helical peptides, amphiphilicity and hydrophobicity are closely linearly related because hydrophobic residues occur periodically in doublets or triplets alternating with similar patterns of polar amino acids[189]. The linear relationship between amphipathicity and hydrophobicity in AMPs indicate that there is a requirement for a characteristic balance between them.

One quantitative measure of amphipathicity, as introduced by Eisenberg[195], is hydrophobic moment (M_H). Hydrophobic moment is the hydrophobicity of a peptide measured for different angles of rotation per residue. The hydrophobic moment is calculated as the vectorial sum of individual amino acids hydrophobicities, normalized to an ideal helix[196]. In other words, it is a measure of the probability that the peptide at any particular position is located at the interface between the interior of the protein and the surface, or more exactly, that the peptide separate hydrophobic and hydrophilic regions[189, 197]. Moment helps one to recognise amphiphilic structures by identifying where the residues on one side of the structures are more hydrophobic than on the other. Studies with magainin as a model peptide have shown that increasing M_H doesn't increase the antimicrobial activity but in turn increases the toxicity[187, 198]. It is now widely accepted that amphipathicity, although not exclusively, is an important parameter involved in antimicrobial activity and toxicity[112, 180, 182, 183, 192, 194, 199, 200].

Hydrophobicity (H)

Hydrophobicity of a peptide is defined as the proportion of hydrophobic residues within a peptide and is typically around 50% for most antimicrobial peptides. Hydrophobicity is an important physico-chemical characteristic of AMPs, which is considered to be independent of other structural parameters[183]. Biophysical studies have shown that hydrophobicity can modulate the antimicrobial efficiency and specificity of individual α- helical AMPs, as they govern the extent to which a peptide can partition into the lipid bilayers[179, 201]. It is noteworthy that different strains and types of microbes respond differently to increasing hydrophobicity[202].

Although hydrophobicity is required for membrane permeabilization, above

optimum levels leads to a loss of antimicrobial activity[183] and an increase in mammalian toxicity because of peptide association [112, 180, 203, 204]. Eventhough peptide self association is an important parameter for antimicrobial activity, a strong association will decrease activity, because the peptides fail to pass through the capsule and cell wall to reach the inner membrane. The reason for increased toxicity in eukaryotic cells is that most anionic lipids are localized at the intracytoplasmic leaflet and peptides with higher hydrophobicity can enter easily and damage the eukaryotic membranes[189]. Interestingly, a strong correlation has been observed between cytotoxicity and hydrophobicity[112, 205-208]. There is no doubt in saying that cationicity is a primary determination factor, whereas amphiphilicity and hydrophobicity contribute to the structural features that govern the antimicrobial activity. It appears that there is a delicate and appropriate balance between them for selective toxicity against pathogens [112, 189].

Polar angle (θ)

Polar angle is a measurement of the relative proportion of polar versus non-polar facets of a peptide conformed to an amphipathic helix[183]. For hypothetical helical peptides, composed solely of hydrophobic residues on one face and hydrophilic residues on the other side, the polar angle will be 180°. Most naturally occurring helical AMPs have a polar angle[164] of 140° - 180°. Unlike other biophysical characters, polar angle has been shown to influence the overall stability and half life of the peptide-induced membrane pores[209]. In numerous studies of, both natural and synthetic AMPs, peptides with a smaller polar angle i.,e greater hydrophobic surface, have been shown to induce more extensive membrane permeabilization, translocation and pore formation rates than peptides with higher polar angle[187, 192]. Interestingly, peptides with higher θ formed stable pores due to their large surface of charged and /or more peptide molecules per channel[210]. Strangely, increasing the polar angle decreases the antimicrobial activity whereas no or little effect was found on cytotoxicity[191]. Studies show that a direct proportionality exists between θ and pore stability, whereas pore formation is inversely proportional to it[183]. To put it in a nutshell, polar angle has no significant role in antimicrobial activity, but plays significant role in pore stability.

In conclusion, the activity of AMPs is not determined by a single factor but by a subtle combination of factors such as sequence, hydrophobicity and position of cationic residues. Beyond any doubt, amphipathicity, hydrophobicity, θ and conformation of a peptide play a role in the antimicrobial activity, however

there is no strict rule regarding the optimal number of charged and hydrophobic residues for maximum antimicrobial activity and minimum cytotoxicity as it varies widely among different peptides and within a given structural group[180]. At this moment, it is widely accepted that important factors for the antimicrobial activities are; (i) for linear helical peptides a lack of secondary structure with an inducible secondary structure in hydrophobic environment; (ii) presence of amphipathic surface with charged residues in the center; (iii) minor peptide self-association in an aqueous environment[203, 211-214].

Mode of action

Despite the great success in identifying novel AMPs from various sources, there are still some areas where there is a great dearth of information, especially with regard to the mode of action. Enhanced understanding of this will be of great use in peptide-based drug designing [215-217]. Even though AMPs belong to innate immunity, the mechanism by which they kill the microbes is quite different from that of cytokines and phagocytes.

Before we look at the mode of action, we have to understand the membrane biology of bacteria, fungi and eukaryotic membranes, which are the primary target for most AMPs. Universally, all cell membranes are fluid mosaics of proteins and phospholipids, which are arranged as bilayers with hydrophobic and hydrophilic domains. However, there exists a significant lipid compositional difference between the prokaryotic and eukaryotic membranes as well as among cell types. Bacterial membranes are made up of negatively charged phospholipids such as phosphatidylglycerol (PG), cardiolipin (CL), or phosphatidylserine (PS)[218], which are stabilized by the divalent cations such as Mg^{+2} or/and Ca^{+2}. Even though there is not much difference in lipid composition, Gram-negative bacteria differ from Gram-positive bacteria. They have a smaller peptidoglycan layer and an outer membrane, in addition to a cytoplasmic membrane containing lipopolysaccharides (LPS), which acts as permeability barrier[219]. In contrast to bacteria, fungal membranes are rich in phosphomannans and other related constituents such as negatively charged phosphatidylinositol (PI), phosphatidylserine (PS), and diphosphatidylglycerol (DPG), which give a higher negative charge surface for the membranes [184, 202, 220].

On the other hand, mammalian membranes are rich in sterols and zwitterionic phospholipids with neutral net charge including phosphatidylethanolamine (PE), phosphatidylcholine (PC), or sphingomyelin (SM). Moreover, cholesterol

is present in significant amounts in mammalian membranes and can reduce the activity of AMPs by affecting the fluidity and dipole potential of phospholipids, in addition to stabilizing the lipid bilayers and delaying the binding of peptides to the membranes [221, 222]. Therefore, sterols in the mammalian membranes are thought to be involved in differentiating mammalian and fungal cells from prokaryotes[222]. However, cholesterol in the membrane is not the sole molecule that influences the specificity because *Fusiarum moliniforme*, fungi contain cholesterol and yet are sensitive to cecropin as the ergosterol containing *Fusiarum oxysporium* and *Aspergillus sp* [163, 223, 224]. These studies point out that in addition to cholesterol, membrane potential and asymmetric distribution of phospholipids in eukaryotic membranes contributes to prevention AMPs binding [40, 221]. Thus, a higher proportion of negatively charged lipids on the surface monolayer of the microbial cytoplasmic membrane plays an important role in the selectivity and binding of antimicrobial peptides for bacterial cells over eukaryotic cells. In other words, composition difference likely provides an important determinant by which AMPs selectively targets microbial versus host membranes.

A widely accepted notion is that, electrostatic interaction between the positively charged amino acids and negatively charged lipopolysacharides /phospholipid head group of the target cell is involved in the binding and accumulation of the peptides on the surface of the membrane. Thereafter bound peptide lies on the membrane with its long helix axis parallel to the membrane surface until a threshold concentration is reached. Threshold concentration is defined as the concentration at which peptides assembled on the surface of the membrane undergo a second round of reorganization[183]. Parameters that are likely to influence the threshold concentrations are the propensity of self-assembly or oligomerization, fluidity, biochemical properties of peptides (amphipathicity, hydrophobicity, hydrophobic moment, and polar angle), phospholipids composition etc[184, 225]. Once the threshold concentration of peptides are accumulated on the surface of the membrane, peptide-peptide and peptide–lipid interactions will create a complex structure, which is associated with the specific antimicrobial action.

Virtually peptides are inactive at lower concentrations or until the threshold concentration is reached on the surface of membrane. Once it is reached, most peptides undergo a final conformational transition leading to formation of patches of hydrophobic and charged residues that permit the peptides to interact strongly with the membrane. These interactions can be relatively selective for

bacterial membranes due to their high concentration of anionic lipids at the surface. After the final change, peptides enter into the interfacial (hydrophobic and hydrophilic) region of the bacterial membrane surface by electrostatic and hydrophobic interactions. However, peptide association with membranes is quite strong and devoid of any covalent association; therefore killing curves may reflect the time required for microbiocidal activity, as indicated by loss of colony forming units in viable count assays[219].

Unfortunately, there is no clear-cut evidence or proof to distinguish the peptides as either membrane disrupters or non membrane disrupters, due to the fact that a peptide may act as membrane disruptive against one strain and metabolic inhibitor for another[215]. Moreover, the result depends upon the membrane characteristics and metabolic state of the target cell, and this will determine the mechanism of action, concentration used and potency of the peptide[33, 226]. However, the exact mode of action of AMPs is considerably debated. Based upon the evidence available three models have been proposed, and its generally agreed that peptides either follow one or both of the methods to kill the microbe [33, 183].

Barrel stave model

Barrel-stave model, also known as helical bundle model, was first proposed by Ehrenstein and Lecar in 1977[227]. According to the barrel stave model, a variable number of individual peptide molecules, known as "stave" are arranged in ring like structure to form a barrel like pore or channel, thus the name describe the overall topology of the channel. In this mechanism, peptide hydrophobic surfaces interact with the acyl chains of lipid in the membrane and their hydrophilic surfaces point inward producing an aqueous pore consisting of more than four peptides [227, 228]. A crucial step in this model is that peptides have to recognise each other in the membrane bound state. It is highly energetically unfavorable for a single peptide to traverse the membrane, hence the peptides aggregate on the surface until the threshold concentration is reached and then insert into the hydrophobic core of the membrane by undergoing a conformational phase transition, forcing polar–phospholipids head groups aside to induce localized membrane thinning[182]. This event is followed, by additional recruitment of peptides around/in the channel, leading to an increase in pore size and stabilization, thus killing the microbe by leakage of intracellular components. Interestingly, a large number of membrane conductance studies with Alamethcin[229], Pardaxin[230] and α-5 segment of the Cry delta-endotoxin family[231] have shown that transmembrane pore formation

is not a single step process but involves multiple steps, and this needs to be further investigated [182, 229].

However, it is very clear that not all peptides follow this mechanism. Theoretically, it is not possible for peptides with high charge and hydrophobicity to use this mechanism. Peptides that follow use method should adopt amphipathic structures, with their net charge localized along the backbone should be near neutral, inorder to avoid intramolecular repulsion of positively charged side chains in the peptides and collapse of the pore [221, 232]. Therefore, peptides with large numbers of lysines or arginines spread across the peptide chain cannot permeate the bacteria or eukaryotic membrane using the barrel stove model.

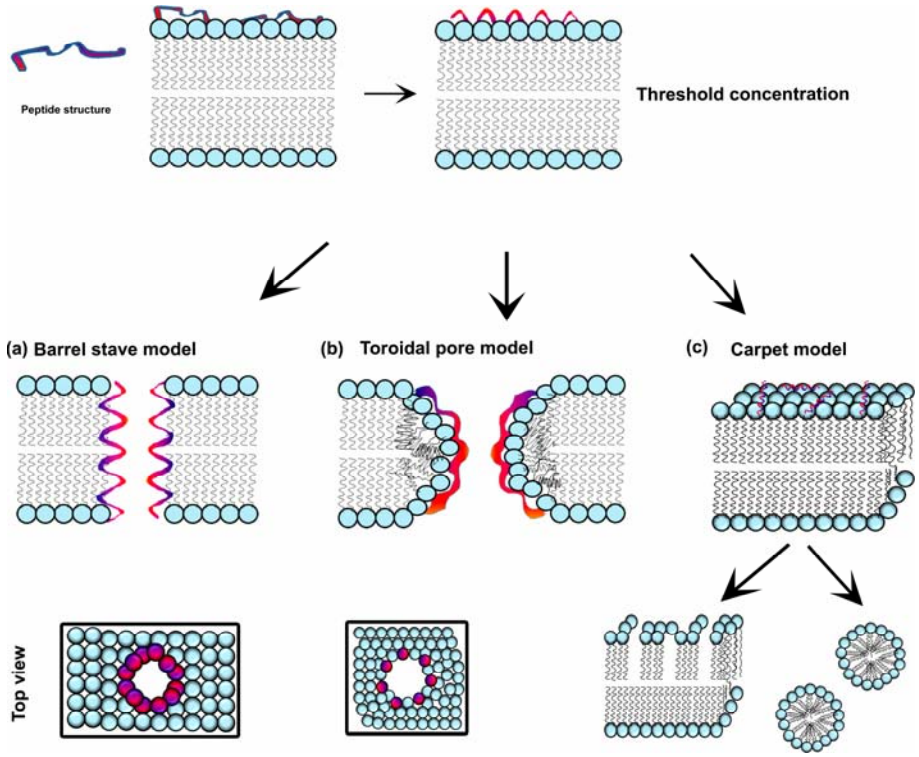

Figure 7: Mode of action of membrane active AMPs

Toroidal pore model

The toroidal pore model, also known as as wormhole mechanism, is one of the well-characterized peptide membrane interactions[182] Many peptides use this mechanism e.g dermaseptin[233], magainin[210, 225, 234], protegrin[235] and melittin[236]. Even though it appears that the barrel stave (Figure 7a) and the toroidal pore (Figure 7b) model are the same, there is primary difference in the architecture and composition of the pore formed. In later model, peptides are always associated with the lipid head group even when they are inserted perpendicular into the lipid bilayers (Figure 7b).

In this mechanism, peptides bind to the surface of the membrane and then undergo a conformational change. The hydrophobic residues of the bound peptides displace the polar head groups creating a breach in the hydrophobic region and inducing positive curvature strain in the membrane [237, 238]. At the threshold concentration, peptides aggregate to form complexes, reorient and transverse across the membrane, such that the polar residues are no longer exposed to the hydrocarbon chains of the membrane. The positively charged amino acid spread along the peptide chain are in contact with the phospholipids head group during the process of peptide transverse along the membrane thus leading to formation of composite dynamic peptide-lipid supra molecular pore[225].

Carpet model

The carpet model, is the first and best described model to explain the mode of action of dermaseptin. [239]. As per the model (Fig 7c), peptide monomers at high concentration are spread over the membrane as a carpet with the hydrophilic surface of peptides facing the phospholipids head groups. This binding displaces the phospholipids, leading to changes in the membrane fluidity, bilayer curvature and integrity. Once the peptide threshold concentration is reached, the membrane is subjected to unfavorable energetics leading to disruption and collapse [60, 232]. According to Shai and Oren, peptides with a large positive charge spread across the peptide chain and a moderate hydrophobicity will kill the microbes using the mode of action described by the carpet model [232]. The presence of negatively charged lipids is important for this model because, in their absence the peptides repel each other, due to electrostatic forces, and are not able to assemble on the surface of the membrane. Furthermore, peptides that permeablise the membrane by this model can adapt different structures such as cyclic or linear on binding to the membrane. Recently a number of peptides, including deremaseptin natural

analogues[240], cecropins[241], LL-37[242], caerin[243], trichogin GA[244], melittin[245], ovispirin[246] etc have been shown to use this mechanism.

Metabolic inhibitors

Historically, it is presumed that the primary targets for AMPs are lipid membranes and doesn't involve any stereoselective interaction with an enzyme or lipid or protein receptor because of any significant difference in the antimicrobial activity between D and L- forms of peptides [247, 248]. However, recent studies of the D- and L enantiomer variant of various peptides have shown differences in the spectrum and antimicrobial activity, thus suggesting existence of alternate methods/targets[249, 250]. Single monomers can be transferred across the membrane without being entrapped in the hydrophobic core due to their low dielectric constant and inability to establish hydrogen bonds. Strangely, some few studies carried out with intact bacteria showed that peptides can be translocated across the membrane, block the essential cellular processes and kill the bacteria without damaging the membrane extensively[251, 252]. Very recently, multiple cell targets or alternative mode of action have been demonstrated for AMPs. These include, inhibition of nucleic acid synthesis (Pleurocidin, Buforin–II)[175, 251], RNA synthesis (Bac5,Bac 7)[253], Protein synthesis (Indolicidin , PR-39, Attacins)[172, 175, 254] enzymatic activity (Pyrrhocidin, Apidaecin and Drosocin)[255, 256], ATP efflux (histatins)[79, 124, 127, 128] and cell wall synthesis (Nisin)[257].

Interestingly, whatever might be the target site or mode of action, every peptide has to primarly interact with the membrane [258]. It is likely that the mode of action of individual peptides may vary in accordance with the bacteria targeted, concentrations at which they are assayed and the physical properties of the interacting membranes[33]. Due to technical difficulties, mode of action studies have been carried out with artificial model membrane systems or with membrane potential sensitive dyes and fluorescent labeled peptides[103] consisting of one or two components of microbial origin e.g LPS, LTA[28, 112, 114, 157, 204, 259-261]. But in the true sense, model membranes don't resemble biological membranes, since they lack the lipid heterogeneity, membrane proteins and efflux pumps controlling the flow of ions and other molecules (polyanionic molecules such as DNA, RNA etc)[219]. Due to this inherent problem, it is difficult to establish a perfect correlation between the minimum concentrations needed to obtain activity against model membranes and microbes. Nevertheless, model membranes have been useful in defining the factors involved in peptide membrane interactions.

Various attempts to crystallise peptide in the lipid membranes have met with failure. Moreover each experimental method provides a slightly different view of the peptide activity and no single technique is capable of adequately determining the mechanism of action of the peptides. Hence a combinatorial approach is best, where a variety of methods are employed and the combined results are considered[182, 183]. Monitoring voltage dependent channels provides information about the stability and formation of peptide induced pores whereas secondary structure orientation of the bound peptide is given by CD spectroscopy[262-264]. Studying the interaction between AMPs and phospholipids in model membranes and electron microscopy, in addition to advanced techniques such as Fourier Transform Infrared (FTIR) Spectroscopy, Raman spectroscopy and fluorescence spectroscopy, CD optical spectroscopy, NMR, and X-ray crystallography provides more insight than using a single method[262].

Most cationic peptides that have been characterized as membrane permeablizers could probably have other modes of action on living cells, as these studies were carried out on model membranes in the presence of artificial medium, which do not reflect the true physiological environment and high concentration of peptides[33, 262]. Unfortunately, inclusion of a particular peptide into a structural group doesn't give any indication about its mode of action nor its spectrum of activity, as the molecular mechanism of AMPs depends upon a number of parameters such as amino acid sequence and arrangement, peptide concentration and degree of hydrophobicity. As per *Zhang et. al,* peptides with similar structures and/or with minor difference in sequence show a significant difference in mode of action[33, 265]. Therefore, every peptide has to be investigated thoroughly before confirming its mode of action.

Basis for antimicrobial activity vs toxicity

The important property of AMPs is their target specificity by which they kill microbes but not mammalian cells. Although a lot of information has been described about the peptides structure and activity, very little is known about the molecular basis of marked differences in peptide activity and structure in detail. Notably, basic rules governing the differences in selectivity and toxicity among peptides remains to be fully understood[266]. Although, this thesis tries to explain these factors, studies with other classes of peptides and using different methodologies will likely further add to our understanding. According to *Yount and Yeaman*[183], the major themes which are responsible for selective toxicity

are (i) difference in membrane composition (ii) conformational dynamic flexibility and peptide association, which allows peptides to reconfigure on the bacterial membrane, but not elsewhere (iii) target cell energetics, which retards or enhances the peptide attachment to the surface (iv) restricted access of the peptide to highly vulnerable areas or tissue such as blood, internal organs, etc.

It has been proposed that the negatively charged outer surface of bacteria and the higher negative transmembrane potential ($\Delta\Psi$) accounts for the preferential binding of AMPs, which have a net positive charge[221]. For example, normal pathogenic bacteria in the mid logphase have a $\Delta\Psi$ ranging from -130 to -150 mV. On the contrary eukaryotic cells have a $\Delta\Psi$ ranging from -90 to -110 mV. In addition, fluidity of bilayers, dipole moment, curvature and content of acidic phospholipids such as PG or PS in bilayers also plays a minor role in the activity. The significance of these factors is more prominently seen for the activity difference among the sub strains of the bacteria.[267-269]. Even though, no correlation has been found between LPS/LTA binding and antimicrobial activity, a clear and direct correlation has been found between the order of efficiency to permeate model membranes and potency of peptides[112, 261]. Therefore, a relative difference in the membrane architecture has been attributed to selectivity of AMPs action [191, 221, 270].

Under normal circumstances, human cells are resistant to AMPs, but some AMPs such as, LL-37 and DP1, show cytotoxicity at high concentrations. Therefore, cytotoxicity of the AMPs should be assessed carefully with an understanding of the limitation of the experiment. The hemolytic assay, is routinely used to assess cytotoxicity of peptides. Human erythrocytes are used as a safe, simple way to demonstrate toxic ability of peptide against human cells, with good noise to signal ratio. However, data derived from *in vitro* and *ex vivo* erythrocyte assays or hemolysis have limited utility of such assays, which are carried out in austere buffer rather than in complex biomatrices and physiological settings *in vivo*[271]. Most AMPs are devoid of any antimicrobial activity and or cytotoxicity in the presence of metals, pH and serum[112, 114, 204, 271, 272]. Thus the degree to which antimicrobial peptides permeabilise or lyse human erythrocytes may not reflect their relevant potential cytotoxicy *in vivo*.

The fundamental reason for a requirement of higher concentrations of AMPs for toxicity is due to the lower negative charge and membrane potential, in addition to the presence of cholesterol [222, 273]. Cholesterol has a condensing effect on the membrane by increasing lipid order while only marginally

reducing fluidity of the alkyl chains. Then condensing effect will increase the bilayer thickness and reduce the lateral density fluctuations, thus preventing or delaying the peptide from binding and reaching the threshold concentration. Even though normal eukaryotic cells can resist AMPs action, tumor cells can't. This is due to the partial loss of lipid symmetry and architecture which leads to an increase of negative membrane potential, thus facilitating helping in efficient binding of AMPs to membranes[274]. It is note worthy that the maximum activity of AMPs typically occurs in the low micromolar range, although there is significant variability in the ability of AMPs to kill specific bacteria. *In vitro* antimicrobial activity is rapid and occurs at low ionic strength, and low concentration of divalent cations and plasma proteins.

Optimization strategies to enhance the activity

Many of the naturally occurring AMPs are not optimised for efficient activity and need to be improved, before they could be used as therapeutics. Due to their ease, modifications in charge and hydrophobicity have been a favorite method for increasing the activity, however over increasing often leads to the development of toxic peptides in addition to loss of activity and/or spectrum[112, 114]. Recently various other methods were tested using native templates to generate more efficient AMPs, such as QSAR[112, 275, 276], altering structure by cyclisation[277, 278], introducing fluorine atoms or trifluromethyl groups[279], increasing positive charge or hydrophobicity by tagging [204, 270] etc.

Random mutagenesis

Random mutagenesis includes methods that modify natural peptide by addition/deletion/replacment of single or more residues or truncations at the N- or C- terminal or generation of chimeric peptides using a combination of both methods. Unfortunately, random mutagenesis is informative only in a few circumstances, and often raises more questions than the answers it provides[164]. Therefore this method is rarely used unless to answer a specific question or for generation of ultra short peptides[114].

Quantitative structure-activity relationships (QSAR)

QSAR is a mathematical relationship between biological activity of a molecular system and its geometric and chemical characteristics. Most structure-function studies provide a working conceptual model of bioactive models. In summary, QSAR studies attempt to find a consistent relationship between biological activity and molecular properties, so that these "rules" can be used to evaluate the activity of new compounds. Much like other chemical molecules, AMPs

can also be easily improved with appropriate amino acid substitutions, provided the structure-function relationship (SAR) and factors that govern the specificity and toxicity are known. AMP based QSAR studies involve limited sets of systemic modifications of residues in naturally occurring molecules to form optimum or amphipathic structures, which are then subjected to determination of biological activity range via a number of methods. In these studies, instead of using a pool of amino acids for analysis, only a few amino acids with specific characteristics, such as e.g basic (lysine or argnine) or hydrophobic amino acids (alanine, leucine, phenylalanine or tryptophan), are used in order to obtain a peptide with maximum activity and minimum toxicity towards the host. The major advantage of QSAR studies is that it reduces the number of peptides to be analysed and the experiments to be done in order to obtain meaningful data and a peptide with desired characteristics [164, 275]. Furthermore, in QSAR studies, due to the technical difficulties and experimentation limitations, mostly *in vitro* studies are carried out, such as RDA, MIC assay, bacterial membrane/ model membrane lysis, kinetics studies, time killing experiments etc, whereas extensive *in vivo* studies are very seldom performed [164, 280].

Various SAR studies indicate atleast seven parameters (size, sequence, charge, amphipathicity, hydrophobicity, helical content, distance between the hydrophobic and hydrophilic faces of the helix) that can influence the spectrum and activity range of helical peptides. However, preferentially it is wise to study the role of characteristics, such as charge, hydrophobicity, amphipathicity, aromatic amino acids rather than other factors, due to the specific conditions needed for the membrane binding. From the structure-function based studies with various AMPs, it has been learned that antimicrobial activity is essentially dependent upon hydrophobicity, charge and amphipathicity, and the extent of role played by these factors depends upon the bacteria. On the other hand, selective toxicity depends more upon the amphipathicity and environmental conditions [112, 275, 276]. Therefore, results of the SAR studies should be assessed with great care. Nevertheless, QSAR studies help in a greater understanding of the biophysical properties and in generating molecules with required activity in robust manner, which is required in an industrial setting.

Increasing the proteolysis resistance

The other widely used method is to reduce the protease sensitivity and increase the half-life of the peptide under *in vivo* conditions. In order to increase the

protease resistance, various strategies have been proposed such as cyclisation, use of non natural amino acids[103, 281, 282], amidation at the N-terminus[113], introduction of disulphide bonds[86], preventing the protease binding site by replacing amino acid[283], modifying the peptide bonds by alkylation of the nitrogen bonds[284] and mixing with a gel matrix such as PEG[83] to form complexes. The half-life of a peptide is highly enhanced by conjugation of the free amino groups of the peptides with PEG as this prevents the attack of proteases by steric hinderence[83]. Interestingly, reduced protease susceptibility and enhanced activities was obtained by introducing disulphide bonds or lactum bonds in indolicidin and cecropin-melittin hybrid peptide, respectively[33, 285, 286]. It is noteworthy, that in addition to increased antibacterial activity, introduction of disulphide bonds in the sakacin peptide leads to broadened spectrum of the peptides[287]. Indeed, these studies show the effectiveness of this method; strangely many people have not used these methods in their studies to enhance the antimicrobial activity of their native peptide, probably due to the difficulty of introducing the bond after peptide synthesis.

Significance and other functions of AMPs

The name "antimicrobial peptides or natural antibiotic" has strongly biased interpretation of the function of AMPs and delayed the discovery of other functions[40]. Increasing new evidence is indicating that the role of AMPs in host defence mechanism goes beyond the direct killing of microorganisms. AMPs, in addition to their bactericidal and fungicidal activities in host tissues, exhibit a plethora of activities such as stimulating cell proliferation, activating the immune system, and exhibiting cytotoxic effect on tumor cells[288]. Furthermore, most AMPs possess antiviral, antitumor[289], angiogenesis, and vasculogenesis[290, 291] properties and immuno-modulatory activity such as promotion of wound healing[219, 292-294], inhibition of LPS induced pro-inflammatory responses[295-297], recruiting leukocyte cells[298], chemokine production[89, 299-302], and anti inflammatory properties[303-305]. The primary reason for multiple functions of AMPs is their amphipathic and cationic charge, which gives them an ability to interact with a wide variety of receptors instead of a specific target. Based on these multiple roles of AMPs, many researchers are strongly arguing that there exists a certain degree of coupling between the innate and adaptive immunity, which influences the quantity of the immune response and damage/pathogen clearance effect [294, 306-308].

Problems and bottlenecks in use of AMPS as therapeutics

From the time of AMPs discovery, microbiologists have been tempted by the idea to develop them into 21th century novel antibiotics. The recent rise in antibiotic resistance has drawn the attention of researchers and companies towards these molecules. Most companies are trying to develop peptides as promising therapeutics due to their small size, wide-spread occurance in all living organisms, long evolutionary history, potential ability to overcome bacterial resistance and the added benefit of delivery routes other than intravenous injection [309]. However, the biggest hurdle is to develop these evolutionary conserved and interesting molecules into peptide therapeutics by overcoming the limitations imposed by nature from time immemorial.

In all living forms, the various biological processes are controlled by regulatory processes comprising of initiation or inhibition via specific protein-protein interactions. Thus, peptides and proteins are well suited to control complicated processes occurring in biological systems. This unique feature should put peptides on the top of a "probable future therapeutic molecule" list. But a look at the FDA approved list of peptide and protein based drugs shows that they form a very small group. Like in all drug developments, despite the promising results in animal disease models and pharmaceutical companies appreciation of the worth fullness of peptides, there are obstacles that remain to be solved before the wide-spread development of peptides as therapeutics kicks-off is achieved. The major issues that needs attention are susceptibility of peptides to proteolytic enzymes, lack of information regarding antigenicity, immunogenicity, potential toxicities of relatively large and highly charged peptides, ability to achieve high microbicidal activity under physiological conditions and comparatively high costs associated with peptide synthesis[33, 310].

Synthesis

Even though a huge source of literature has been generated to date, not a single molecule has been introduced into clinical use, due to the difficulty in synthesizing these molecules. Currently, there are three methods for the production of antimicrobial peptides: isolation from natural sources, chemical synthesis and expression using biological systems. Isolation from natural sources is important for novel peptide discovery, but may not always be practical to obtain a sufficient amount of materials required for detailed biophysical and biochemical characterization. For clinical trials and use, you should have a method whereby large quantities of the molecules can be

produced in a relatively simple and less expensive way. It is beyond doubt to say that that the use of a synthetic peptide approach, in comparison to the molecular cloning technique, has rapidly expanded this field. The typical standard peptide synthesis approach, Fmoc method, can allow the synthesis of peptides of maximum 40-50 amino acids due to the inefficiency of coupling of longer chains.

The other most commonly used protein expression systems include bacteria, yeast, insects, plants or cell-free systems, but in comparison to bacterial expression system, the cost involved in other systems may pose a problem. In spite of technical difficulties, very recently plectasin was isolated from the fungus *Pseudoplectania nigrella* and expressed in *Aspergillus sp* by using recombinant technology[42]. However, expression of AMPs using genetic engineering methods in bacteria is the best-advocated method, due to the practical importance of large-scale peptide production by fermentation technology. However, direct expression of antimicrobial peptides is usually difficult, since these peptides are toxic to the expression host. Therefore, these peptides are usually expressed as fusion proteins in protease-deficient bacterial strains such as *E. coli* BL21 in order to improve solubility and avoid degradation or toxicity to expressing cells[311, 312]. But the problem with expression of fusion proteins is the waste of energy/protein synthesis machinery, in addition to problems with cleavage and purification of the AMPs from the fusion molecules. If expressed individually, conditional toxicity is the major problem when synthesizing these molecules in bacteria, i.e, AMPs should not be toxic to the bacteria during the formation time, but should be active when used against the infectious bacteria. As a solution to this problem, Qing *et.al* developed pCold vectors which allow a high expression of the target gene by cold shock i.e, bacterial growth at 37°C and protein expression at 15°C[313]. But the wide utitlity of this method in large-scale production is quite controversial.

Unfortunately, most researchers and companies have experience in synthesizing large size proteins >50 kD, but very few people have experience of synthesizing molecules below 50 kD[314]. AMPs are less than 5 kD and they need to be active in specific conditions only. A literature review shows that not more than 8 papers have been generated which address this problem[314-318]. Production of novel AMPs in bacterial systems posess problems, which require new technological innovations to solve them. Solutions provided by this type of research have a wide applicability because nowdays the pharmaceutical

industries are looking for small molecules with therapeutic potentials[319], in addition to patents and publications.

Purification

Despite low yields, maximum ~500-1000 mg, the only current method of synthesizing the molecules is through chemical synthesis, which is quite expensive. The huge cost in peptide synthesis is due to the use of quite long, cumbersome and expensive synthesis procedures and the requirment of a purification step after each cycle. The other bottleneck is the purification step which follows the synthesis of each of the products used in the peptide synthesis [319]. Quite a lot of researchers are focusing in this area, but not much has been done particularly in the purification steps [320]. Most widely used step in purification is the use of HPLC alone or in combination with other methods. The main drawback of any single HPLC method is that it only provides negative identification i.e., it shows impurities that resolve from the main peak and not the impurities that co-elute. Thus a single product has to pass through round of separate purification systems, which adds to the cost of the final product[320] and a loss of peptides in significant amounts.

Cost aspects

The principal reason, in addition to technical hurdles, why most researchers and companies are reluctant to promote the use of AMPs as standard antibacterial therapeutics is the cost involved in the production of AMPs. For treating a single patient with an infection, one may need up to mg amounts per kg body weight per day, and producing such a quantity through chemical synthesis will cost around 400-3000 SEK, which is far more expensive than current costs for antibiotics [321]. The cost of AMPs should be near or below the cost of conventional antibiotics, in order to be a real alternative or to be used in the clinic as therapeutics. However, identifying the exact sequences with antimicrobial and/or immune modulator-functions will help in generating peptides with shorter length, thus reducing the cost and toxicity

Lack of specificity

The most important and essential property of any therapeutic drug is its target specificity and non-toxicity to other components of the host. An ideal drug is one, which is small, stable, easy and cheap to synthesize, has low or no toxicity towards the host, high activity at physiological conditions and synergy with other drugs. Despite the extensive research work carried out during past two decades, unfortunately no ideal AMPs have been made. The major problem

with AMPs is their lack of unique specific target site and loss of activity in physiological conditions. Out of hundreds of AMPs discovered at crucial sites of infection in various cell types and in concentrations well above the MIC values, only very few were found to be functional in physiologicallly relevant conditions[258, 322-324]. However, infection model studies in animals have convincingly shown that cationic peptides are able to limit or clear the infection, despite the loss of direct antimicrobial activity under physiological conditions[324].

By logic, peptides derived from human sources should not be toxic to humans, unfortunately this is not the case. Since AMPs share features with eukaryotic nuclear localization signal peptides, they are able to translocate into cells and cause apoptosis, mast cell degranulation; thus leading to cytotoxicity. However, the specific tissue distribution of an AMP indicated that AMPs toxicity depends more upon their environment. In my opinion, the loss of function in physiological conditions is probably a control mechanism to prevent the side effects of AMPs at unwanted sites. Furthermore, due to the rigid regulatory rules of various countries most of the pharmaceutical companies have devoted their attention to the development of AMPs more as topically applied agents than as internal drug agents[103].

Protease susceptibility

Sensitivity to proteases, unfavorable pharmokinetics and rapid clearance of the AMPs severely restricts their application as therapeutics. Interestingly, most pathogenic bacteria express specific and non-specific virulence factors such as endotoxins, proteases and peptidases in order to evade the host immune response. To circumvent these problems, various researchers have used the highly investigated method of introducing amidation at the N-terminus[113] and /or introducing D-amino acids in the place of the L-form at cleavage sites[113]. This approach works very well on a lab scale, but the synthesis of entirely D-from peptide or in combination with L-form adds extra cost as well as technical troubles. Also peptides consisting entirely L-form are potential immunogens and could lead to side effects, including hypersensitivity reactions. In addition, there is little information about the general immunogenicity of a peptide with a combination of D and L amino acids.

Half -life of AMPs

Notably, a direct relationship is observed between the half-life and prolonged effect of peptide[325, 326]. Some researchers have used a novel approach, where

they fuse the peptides to albumin[327] or anti tissue factor Fab[309] by using a linker[327] or peptide addition[328]. The intention is to increase the half-life of the peptides. For unknown reasons, the half-life of albumin–peptide conjugates didn't increase significantly in comparison to albumin, which has a half life of 19 days[329]. In my personal opinion, it is quite dangerous to increase the stability of a peptide for a quite long time as it might lead to a host immune response against the peptide. Unfortunately, we have no complete information regarding the pharmacology and pharmacokinetics of AMPs, as very few studies have been done. Even with the minor information available there is urgent need to carry out additional studies on this issue, as it is needed to convince the investors/FDA authorities about its feasibility as therapeutics[33, 163].

Probability of development of resistance by microbes to AMPs

Cohabitation, in symbiosis or open warfare, over million of years resulted in host pathogen interactions and the key issues in pathogen survival are its host immune attack evasion. Successful pathogens have developed an array of inducible or constitutive counter measures to avoid multiple host defence mechanisms[183]. It has been proposed that microbes can't develop resistance to AMPs based on two points. Firstly, the membrane has to be modified and this is so finely tuned that modifications extensively in it are difficult and have more adverse reactions more than advantageous. Secondly, due to the presence of large numbers of AMPs in the host, it looks like microbes can't be resistant to all AMPs at the same time. However, we should not underestimate the power and magnitude of the microbial evolution. Individual cellular resistance mechanisms may drive the emergence and propagation of the antimicrobial resistance, as occurred with antibiotics[86]. Recently, reports have emerged showing that indeed *S. aureus* can resist various AMPs just by reducing the net charge of the membrane by introduction of cationic residues[330]. Interestingly, not all microbes are following the same strategy, e.g *Serratia sp* and *Morganella sp* express an outer membrane that lacks the acidic lipids for appropriate peptide binding[103], *Shigella* sp release plasmid DNA which inactivates AMPs by binding to them and *S. pyogenes*[331], *Pseudomonas sp*[332]add Schmidtchen et al., Mol Microbiol 2004, *S. aureus*[115], *S. epidermidis*[333] & *Porphyromonas gingivalis*[217] produces proteases which cleave AMPs. Interestingly, some bacteria such as *Pseudomonas sp* and *S. epidermidis,* in addition to protease production use other methods such as forming biofilms, changing hydrophobicity and permeability of outer membranes[334-338] to protect themselves from the AMPs. It is noteworthy, that

not all bacteria use a simple mechanism, *S. enterica*[339] , *S. aureus*[330, 340, 341] and *S. epidermidis*[341] use PhoP/PhoQ and/or analogous antimicrobial peptide sensing system consisting of complex two or three components to sense and block the activity of AMPs. Not all factors produced by microbes attack the host derived AMPs, but attack the control mechanism encrypted in the host to control a pathway. For example, *S. aureus* produces clumping factor A, which binds to C3a and enhances its degradation by factor I, which regulates the complement factor[342, 343]. In addition to this, some pathogens such as *Pseudomonas sp, E. faecalis, S. pyogenes* degrade host macromolecules e.g GAG, collagen, such that they interfere with AMP function[344-346]. A critical look at all the resistance mechanisms reveals that whatever might be the molecular strategy employed, one primary goal is to reduce the negative charge on the membrane surface[330, 337, 347].

In spite of conclusive evidence regarding resistance mechanisms shown by various pathogens *in vitro*, it is hard to believe that they similarly resist the AMPs under *in vivo* conditions, as strategies employed depend upon the physiological conditions[183]. It is noteworthy that, microbes which showed resistance to naturally derived AMPs such as defenisns, were found to be susceptible to synthetic AMPs[112, 337]. Nonetheless, advanced understanding of the mechanism of antimicrobial peptides action and resistance will reveal novel potentially vunerable targets for novel anti-infective agents. However, until the complete dynamics of the interaction of AMPs is clearly understood in physiological environments, it is most likely that AMPs may be initially used in therapeutics in synergy with antibiotics.

Why we need research on AMPs?

Without any doubt antibiotics are the most successful anti-infective agents used to control infectious diseases on the planet and the pharamaceutical industry has upgraded/modified the existing antibiotics and developed newer antibiotics in a timely fashion[12, 348]. Despite the unquestionable success of antibiotics, the major concern in the health care sector is increasing problem of antibiotic resistance, which rendered most current antibiotics useless against the pathogenic bacteria. If preventive measures such as antibiotic cycling are enforced antibiotic resistance might fall back[349], but will never reach zero. More over it leaves a mark in the microbe with every probability to rebound rapidly to become dominant on using the antibiotic. Resistance is a natural consequence of adaptation, an inherent factor in the evolution of organisms, hence it is impossible to stop antibiotic resistance altogether [350]. Bacteria's fast

growth facilitates rapid genetic changes and adaptation of traits to a constantly changing ecosystem for better survival. Therefore newer types of molecules are needed, which can replace the antibiotics in the long run.

Due to their broad-spectrum antimicrobial activity and multiple functions, AMPs are promising therapeutic agents and have every qualification required to be in the armamentarium of health care professionals in their fight against infectious disease. AMPs are versatile molecules with multiple functions in bridging the innate immunity response and adaptive immune response. Even though a substantial amount of work has been done, there are some areas, where concerted research efforts are needed, such as the role of AMPs in chronic diseases. The major drawback in identification of the true biological functions of AMPs is the lack of true representative models. Mice and humans have a different set up of AMPs, and thus don't serve adequately to represent the human condition. Furthermore, the condition is not so different with knockout mice due to the inherent problem of synergistic activity of AMPs, complicating interpretation of *in vivo* studies.

AMPs are present in almost all organisms across the phylogeny spectrum and are playing an active role in host defence from millions of years. It is somewhat enigmatic that microbes have failed to develop a complete defence mechanism against host defence peptides even after a constant exposure to them from millions of years[86]. Even though there are reports of AMPs resistance by certain microorganisms under *in vitro* test conditions, the likelihood of the emergence of resistance across the bacterial kingdom is considerably low, due to the fact that AMPs target unique and multiple target sites simultaneously, which are quite difficult to alter. Moreover, those strains that resist AMPs utilise complex machinery/pathways involving multiple genes and gene products and it is difficult to transfer the whole set of systems across the bacterial types in comparison to individual genes which cause antibiotics resistance[183]. The high degree of complexity of the mechanism is almost certainly the cause of the observation that it is extremely difficult to select cationic peptide-resistant mutants in nature. It is hard to believe literature referring to AMP resistance by a pathogen to a peptide under *in vitro* conditions, non-physiological environment, immune mechanism and synergistic molecules such as complement and other protein. Unlike antibiotics, which attack more specific molecular targets, AMP's act on general but fundamental structural characteristics such as the bacterial cell membrane. AMPs destabilize and/or destroy the membranes either as a final target or in a

process to reach their target site, and changing the membrane architecture altogether is an impossible task for microbes. There is a minimum chance that a microbe develops resistance against a given AMPs as they act in synergy with other AMPs[80] and host defence molecules, thus heterologous peptide interactions will lead to overall net antimicrobial efficacy, even if one peptide fails. Moreover it looks like antimicrobial peptide resistance is largely intrinsic, rather than acquired[183].

According to some researchers, innovative strategies and drugs to combat bacterial infection will emerge from the research in innate immunity, especially AMPs[351, 352], as they represent natural mechanisms of combating pathogenic challenge by rapid microbicidal activity and there is no little doubt about the efficiency of AMPs as novel therapeutics[103, 183, 309, 353-355]. It is noteworthy that conventional antibiotics and analogues forms have failed dramatically to reverse the disastrous trend of antibiotic resistance in human pathogens, which is rising at an alarming rate. Thus AMPs offer several potential advantages over currently available and widely used antibiotics.

Conclusion

Antimicrobial host defence peptides are widely distributed in animals and plants, as part of a strategy by innate immunity to control bacteria and prevent diseases. Even though AMPs were primarily discovered and thought to be natural antibiotics, the interest in AMPs has risen to a higher level, both in academic and industrial sector, with the discovery of immuno-modulatory function carried out by AMPs[356]. Even though there exists ambiguity regarding the primary role of AMPs in humans, to date, their primary role is to control the microbial infection, in addition to immunostimulatory and immunomodulatory functions related to both innate and adaptive immunity [118, 294]. Whatever the case, AMPs offer some hope in the search for novel therapeutic approaches due to their dual function as antimicrobial agents and immune modulators.

Antimicrobial peptides, described to date, are small and have marked cationic character as well as the propensity to form an amphipathic structure on encountering the lipid bilayers. Cationicity helps in initial binding of the peptides preferably to the bacterial surface and the amphipatic nature in combination with hydrophobicity helps in damaging the membranes. Unlike antibiotics, which kill every microbe, natural AMPs must spare the normal flora and kill only the pathogenic bacteria with some specificity. Futhermore they are optimised to function only in a given fluid composition and environment, thus avoiding cross-reactions. The primary task of AMPs is to prevent the colonisation and invasion of the host epithelia by potential pathogens.

Despite technical hurdles, several peptides have advanced to clinical trials showing wide spread interest and commitment to see peptides in daily clinical use. There is no doubt in saying that in an age where most pathogens have developed resistance to commonly used antibiotics, AMPs are attractive worthwhile candidates to pursue as therapeutic molecules owing to their small size, wide spread occurance among animals and plants, potent microbial and immune modulation functions. In addition, they target the membranes of microbes, which are difficult to change, thus microbes have limited resources to fight back.

Present investigation

Paper I: Preservation of antimicrobial properties of complement peptide C3a, from invertebrates to humans

Previously it has been demonstrated by our group that the human anaphylatoxin peptide C3a, generated during complement activation, exerts antimicrobial effects. In this work we used phylogenetic analysis, sequence analyses and structural modelling studies, paired with antimicrobial assays of peptides derived from various known C3a sequences to show that structural prerequisites governing antimicrobial activity can be traced from the human C3a molecule back to C3a molecules of invertebrates, such as those found in *C. rotundicauda* (the horse shoe crab; which is widely accepted as a "living fossil").

For the analysis, we selected all known animal C3a, C4a and C5a sequences and constructed a phylogenetic tree. We subsequently tested antimicrobial activity of these peptides in radial diffusion assays (RDA, measuring inhibition of growth) against *P. aeruginosa*. The results showed that crucial structural determinants governing antimicrobial activity have been conserved during evolution of C3a. Furthermore, C-terminal regions of the ancient C3a from *Carcinoscorpius rotundicauda*, as well as corresponding parts of human C3a, exhibited helical structures upon binding to bacterial lipopolysaccharide, permeabilised liposomes, and were antimicrobial against Gram-negative and Gram-positive bacteria.

During our phylogenetic analysis, we observed that both C4a and C5a formed separate clades from C3a. Given this, we proceeded to examine if this also reflected a functional difference. The results of the RDA experiments with human C3a, C4a, and C5a (the latter only available in des-Arg form) showed that C3a and C4a both exerted antibacterial effects, whereas the C5a-peptide was inactive against the panel of microbes tested. Molecular modelling of human anaphlatoxins showed that C5a lacked the typical C-terminal and antimicrobial protruding peptide. Thus, C5a displayed a significantly different structure when compared with both C4a and C3a,

Paper II: Rational design of antimicrobial C3a analogues with enhanced effects against Staphylococci using an integrated structure and function-based approach

The starting point for this work was our previous finding that C3a molecules are subjected to strong and precise selection forces aimed at maintaining the structure and antimicrobial function. Furthermore, the human natural C3a peptide, being active against several Gram-negative bacteria *in vitro*, displayed limited activity against the Gram-positive *Staphylococcus aureus* and *Candida albicans.* Therefore, in this work, we decided to design peptides that are active against *S. aureus* and the fungus *C. albicans* by a strategy based on mimicking the evolutionary changes and using sequence dependent QSAR modelling on the original human C3a peptide CNYITELRRQHARASHLGLA. The results showed that peptides with a relatively high net charge (+ 6-7) and a propensity to adopt an α-helical conformation with an amphipathic character were active against Gram-positive *Staphylococcus aureus.* Another novel observation was that peptides with perfect helices were found to be highly hemolytic and displayed no added increase in antimicrobial activity in comparison with peptides having imperfect or flexible helices. Viable count analysis (bacterial killing) in physiological concentrations showed that selected peptides lost their toxicity but retained antimicrobial activity. Membrane permeabilization experiments with FITC and liposome leakage revealed that, most likely, these peptides kill the microbes by acting on and destabilising their membranes.

In summary, we showed that antimicrobial activity is not governed by a single factor, but instead a combination of net charge, moderate amphipathicity and helicity are important for antimicrobial activity. By utilizing a low number of amino acid substitutions at strategical positions in the CNY20 peptide, we were able to develop peptides, which exert a significant activity on both *S. aureus* as well as *C. albicans* in contrast to the parent peptide.

Paper III: Boosting antimicrobial peptides by hydrophobic amino acid end-tags

Here we demonstrate a novel approach for boosting activity of antimicrobial peptides through end-tagging with hydrophobic oligopeptide stretches. *S. aureus* and some other common pathogens have relatively low electrostatic surface potential, which can be further reduced by modifications in the cell membrane. Additionally, electrostatic driven peptide binding is salt sensitive, and bactericidal potency of such peptides at physiological ionic strength is limited. Therefore we proceed to investigate whether we can increase the antimicrobial activity at physiological conditions by adding a hydrophobic tag to AMPs.

In the present study we focused on two peptides derived from kininogen, GKHKNKGKKNGKHNGWK (GKH17) and HKHGHGHGKHKNKGKKN (HKH17), tagged them with different hydrophobic amino acids at both N or C-terminals and tested them against *S. aureus* in the presence/absence of salt. We observed that microbicidal potency increased with tag length, also in serum, and was larger for W and F stretches than for aliphatic ones. Of all, those tested peptides with longer hydrophobic tags, such as those containing 4 or 5 Ws, showed an appreciable activity in physiological conditions.

Electron microscopy and FITC based membrane permeabilization studies showed that local perturbations and breaks were introduced in *S.aureus* after treatment with peptides. By using model phospholipid membranes, we could demonstrate that enhanced microbicidal effects correlated to a higher degree of bacterial wall rupture and the tagged peptide can very well differentiate between the bacterial (anionic and cholesterol-void) and eurakoytic membranes. In summary tagged peptides displayed low toxicity, particularly in the presence of serum, and resisted degradation by human leukocyte elastase, and staphylococcal aureolysin and V8 proteinase.

Paper IV: End-tagging of ultra-short antimicrobial peptides by W/F stretches to facilitate bacterial killing

This work is a continuation of paper III, where we investigated whether tagging by hydrophobic amino acid stretches can be employed to enhance bactericidal potency of ultrashort AMPs and still maintain limited toxicity. Considering this, peptides derived by hydrophobic tagging at the C-terminal region of kininogen-derived peptide KNK10 and truncations thereof were analysed in RDA assay against *S. aureus* and *E. coli* in the presence of salt. Results showed that through end-tagging, potency and salt resistance could be maintained down to 4–7 amino acids in the hydrophilic template peptide. Slot binding studies have shown that tagged peptides could still bind to the LPS and the binding is inhibited by heparin. Notably, experiments with tagged peptides in the presence of low ionic strengths showed increased eukaryotic cell permeabilization. However the toxic effects were lost at physiological ionic strength and in the presence of serum. Importantly, W-tagging resulted in peptides with maintained stability against proteolytic degradation by human leukocyte elastase, as well as staphylococcal aureolysin and V8 proteinase.

Finally the biological relevance of these findings was demonstrated *ex vivo* for pig skin infected by *S. aureus* and *E. coli*.

Main conclusions

- Antimicrobial domains of C3a are structurally and evolutionary conserved. C5a has evolved separately in higher organisms into a purely chemotactic molecule.
- Antimicrobial activity is not governed by a single factor. Indeed a combination of net charge, amphipathicity and helicity control the activity as well as spectrum. By utilizing a low number of amino acid substitutions at strategical positions in the C3a-derived CNY20 peptide, we were able to develop peptides, which exert a significant activity against both *S. aureus* as well as *C. albicans* in contrast to the parent peptide even in the presence of plasma.
- The antimicrobial activity of peptides can be boosted through end-tagging with hydrophobic oligopeptide stretches The tagging, which does not detrimentally affect the proteolytic stability of the peptides, promotes peptide binding to bacteria and subsequent wall rupture.
- Bactericidal potency of ultra-short AMPs can be enhanced by using end-tagging with hydrophobic amino acids while maintaining low toxicity.

Acknowledgements

There are many people whom I should acknowledge for making me to stand at the significant milestone of completing my doctorate degree.

I consider myself lucky to have Dr. Artur Schmidtchen as my doctoral supervisor. Four years of his guidance taught me the commitment to pursue ideas and art of high quality scientific research/publication. His working nature and passion for science is infectious and I hope; I never recover out of it. Working under him has been bountiful to me both financially and career-wise. Thank you, Artur, for your professionalism, large mindset, emotional & financial support and instilling the "I know and I can do it" optimism in me.

Next person whom a major share of acknowledgements goes is Mrs. Mina Davoudi. The loveliest adorable person I ever encountered in my life. I am sure that my thesis works would have gone at snail speed without you. Thanks, Mina for extending your hand to help me, both in professional and personnel problems, even during the worst and frustrating situations we have to pass in our career endeavours.

I would like to extend my thank to people who have become part and partsal of my professional career for last 4 years in alien snow land. First Praveen Papareddy, for keeping my sprits high during hardships times of the animal experiments and during the festival parties I hope that we could meet soon each other in India and start giving a shapes to our ideas. Good luck with your work Gopi and thanks for your instant sense of humour on cricket ground, dinner table and in our shopping adventures for fresh lamb meat. Thanks Andreas for sharing the cabin with me for the last 4 years and patiently listening to telugu songs with out a complaint. Thanks Victoria for helping me with experimental problems, critcal imput on thesis and the Swedish codes, due to which I could overcome the cultural shock problem easily. Thanks Emma for the initial work on C3a and also helping me with administrative work to be done before submission. Thanks Camillia Larsson for teaching various things about the advanced electrophoresis stuff. My deepest gratitude to students from Poland to our lab, firstly Mr.Lukaz Kacprzyk for giving company during the summer in lab and for tips about the photography at free time. Secondly Ania Chalupka, for the nice LPS blots and for being the co author in my papers.

I woe my sincerest thanks to Prof. Martin Malmsten for the valuable

suggestions and constructive discussion due to which my work has proceeded at unprecedented speeds. Thanks for your help at the every moment of need. Warmest thanks to all my co-authors on various manuscript Dr. Björn Walse for the nice molecular models, Dr. Bo Svensson for the statistics and QSAR, Lovisa Ringstad and Adam A Strömstedt for the biophysical analysis of peptide activity with model membranes. It was a really fun and joy to work with you people. I am grateful to Matthias Mörgelin for making nice EM pictures for my manuscripts and also for the fun in the B14 floor and kitchen, which relieved me from the stress of final days before submission.

A special thanks goes to various people in the B14 floor, Dr. Lars Björck, Dr. Mattias Collin, Dr. Ole E. Sörensen, Dr. Inga-Maria Frick, Dr. Bo Åkerstrom, Dr. Arne Egesten, Dr. Hans Tapper, Dr. Heiko Herwald for great ideas during my seminars on the floor and inspiring me what I should be in future. In addition to these people, there are some more whom I should thank personally for their help and cooperation rendered during my research work. I begin by thanking Maria Baumgarten for always welcoming me with beautiful smile how busy she is and helping me with EM stuff, Dr. Oonagh Shannon for every help given during last 4 years especially input on animal models, comments on the thesis and FACS, Anneli Edström for the nice intellectual discussion on science, religion, tourism etc in the bac lab, Markus Roupe for the help with prion stuff, Pontus Nordenfelt for the help with fluorescence microscopy, Maria Allhorn for the gel loading dye, Magnus G Olsson for the bug buster and column stuff. Thanks Martina Kalle, Marta Bober, Sonja Oehmcke, Axel Nelson, Dr. Ingrid Berkestedt, Dr. Tobba Sigurdardottir, Silla Rutardottir for your friendship and joy in the lab. Lastly, but not least, I sincerely thank Anita Berglund, Ing-Britt Gustafsson, Pia Andersson and Malgorzata Berlikowski for always being ready to help me even when I have asked for a stupid request.

I am highly indebted to my friends out side the lab, who never made me remember that I am in foreign country. Thanks Harish Reddy alias “cinnodu” for the nice tasty chicken and discussions on all stupid things in the world, Rajesh Babu Chunduri alias “Mr Tech guy” for the Europe trip and teaching me the basics of electronic stuffs shopping. My warmest thanks to Praveen Peetala and Koteswar Rao Cheetirala alias “Koti” for being with me at every moment of need during our stay together, Ramesh Tati for helping me to get things required for daily

prayer, pan cakes (dosa/prasadam), nice tasty dinners during crucial time of thesis writing. Thanks Sagar J for teaching me how to cook instant chicken and also for fun, discussions on the topics related to cinema, politics etc. Now it is time to say thanks for Vini Nagaraj for comments on the thesis and making my dump yard into a living room. Thanks Ram MR, shushu R, Rajesh Yfor all the fun in my swiss trips. Thanks Sunny V & Dhana V for adding a new spice to our routine research life with our neice Hansika V.

My warmest thanks to the people who were not with me personally here, but helped me in pursuing my dream with their good wishes especially my good friend and guide Azeem-ul-Hussan and Dr. S Bandhyopadya, whose suggestions have really improved my perspective of looking things. Thanks to my previous colleagues and best friends at my home town, Kranthi Chaitanya Y, Manoj Kumar Epur, Rajender Vaduluri for keeping me updated on the latest developments occurring around my home and other friends. At this juncture I would like to thank some good old friend Sarita K, ex-colleagues, Sridevi B and Samatha P for teaching me the basics of microbiology and transforming me from a molecular genetist to antimicrobial researcher.

As an international student, what I always missed the most are my parents, especially my mother's cooking, cleaning and washing activities which I took for granted all my life. It makes me happy to no end, to know of their pride for me having achieved my goal. I am grateful for their kind words, total support, unconditional love and their enthusiasm for everything that I do. I am what I am because of you both. You molded my personality, and my life is blessed because I have you.

Next I would like to thanks my alone brother in law Vasu B and sister Mrs. Kranthi B, for keeping me updated with the information occurring at home and also for the fun in the Las Vegas trip during my visit to USA. I always miss my sister shopping and packing skills, whenever I go out on conference or trip to home. I cannot express my appreciating love in words to my sweet and cute niece Dimpu, who always kept my spirits high on phone and warns me that " I should be serious, dignified and practical about life" with a single word "babai".

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