CERTIFICATE

This is to certify that the review entitled **"ANTIMICROBIAL PEPTIDES AND THEIR THERAPEUTIC USE IN HUMAN PATHOGENIC INFECTIONS"** is the bonified work of Mr. Rehan Noor Ahmed Shaikh [Examination Seat No: 20P0460011], submitted to Biochemistry department, School of Chemical Sciences, Goa University in partial fulfilment for the Degree of Master of Science in Biochemistry, Goa University under the guidance of Ms. Snigdha Mayenkar, Assistant Professor, SCS, Goa University.

Date:

Place: Goa University

Mr. Rehan Noor Ahmed Shaikh

[20P0460011]

Ms. Snigdha Mayenkar

Guide,
Assistant Professor,
Biochemistry,
SCS, Goa University,
Taleigao, Goa

DECLARATION

I, Rehan Shaikh, declare that this written submission represents the concept of **"ANTIMICROBIAL PEPTIDES AND THEIR THERAPEUTIC USE IN HUMAN PATHOGENIC INFECTIONS"** with no plagiarism. I have adequately cited and referenced the original sources wherever required. This work was done under the guidance of Ms. Snigdha Mayenkar, Assistant Professor, School of Chemical Sciences [SCS], Goa University. This dissertation has only been submitted to SCS, School of Chemical Sciences, Goa University and not to any other institutions.

Date:

Place: Goa University

Mr. Rehan Noor Ahmed Shaikh
[20P0460011]

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Rehan Noor Ahmed Shaikh

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Abstract

Antimicrobial peptides (AMP) are small amphipathic peptides, with a size range of over 10 kDa, roughly made up of 9 to 50 amino acid residues, they display potent antimicrobial activity against many invasive pathogens. AMPs are part of the bodies innate immune system that are produced by nearly all living organisms, from unicellular bacteria to multicellular humans. They are classified on the basis of their structure that they acquire in the solvent, namely 1) α-helical, 2) β-sheet,3) peptides with uncommon amino acids 4) rich in a particular amino acid such as proline-rich peptides, tryptophan-rich peptides, arginine-rich peptides and 5) peptides with loop structures. Amps are isolated from many biogenic sources that shows great diversity with respect to its targets, with a broad spectrum activity against many pathogenic microorganisms such as Gram-negative bacteria, Gram-positive bacteria, fungi, parasites, protozoans and viruses, Some also display cytotoxic activity. Most AMPs inhibit pathogenic growth by disrupting their cellular membrane leading them to lyse, whereas some enter the cells of the pathogens and inhibit their metabolism. Due to AMP's broad spectrum activity against many pathogens, at a very low concentration and the growing problem of drug-resistance in microbes, AMPs have drawn quite the attention in the past few years. That makes AMPs a new class of potential antimicrobial drug agent and opens a board field of possible application in the field of pharmaceuticals. AMPs can be designed or existing ones can be modified to produce a synthetic antimicrobial peptide that has a broad spectrum activity and these AMPs can be designed using software and produced by using DNA recombination technology.

1. Introduction

Ever since the discovery of the first antibiotic in 1928 by Alexander Fleming, by an accident that went on to save lives of millions, antimicrobial medications have been extraordinarily successful at controlling bacterial infection, Antibiotics are extensively used to cure infectious diseases, to carryout surgery, organ transplant without worrying for risk of infection and for chemotherapy etc. Antibiotics are chemical compounds produced by microorganism as a secondary metabolite that has the ability to prevent the growth of microorganisms like bacteria and fungi, Unfortunately some bacteria have grown to be resistant to the currently available antibiotics, followed by decrease in the number of finding of novel antibiotics. By the end of 1980's no new antibiotic was discovered and the once which were discovered had a very narrow spectrum than the convention, that had broad spectrum targeting many types of bacteria. In the late 50's when drug resistance was seen scientist tackled this problem with the discovery of new antibiotics aminoglycosides, macrolides etc. which had narrow range of which bacteria soon grew resistance to. At present time the discovery of new drugs is not been able to keep pace with the growing resistance of existing drugs. Antibiotics were heavily prescribed even for infections that didn't require it like viral infections that they had no effect on, leading to increase of exposure of bacteria giving them opportunity to adapt to its effects by modifying its DNA through random mutations to produce counter gene products like proteins and enzymes that help to deactivate, destroy and flush out the drug. Even bacteria that were once non-virulent or suppressed are known to developing deadly infection. An example of common bacteria resistant to well-known antibiotics is *Staphylococcus aureus* or *S. aureus* is resistant to penicillin by producing beta lactamase an enzyme that has the ability to break the beta lactam ring, the function chemical component of penicillin class of antibiotics and Methicillin. Bacteria can gain resistance even by a single mutation that does not affect the virulence and viability of the microbial cell seen in Streptomycin resistance in *Mycobacterium tuberculosis*. However, administering combination of different antibiotic that work against the bacteria seems to solve the issue for now. Bacteria has the ability to share its plasmid DNA (non-chromosomal) too other strains and species who are in close proximity through the process of conjugation and gene transfer by transformation means like phages. (Gold and Moellering, 1996). Increase in antibiotic- resistant pathogens and spread of life threatening infection and little or no progress in development of new and potent antimicrobial drug open an

opportunity to explore other option to tackle such situation. AMP or antimicrobial peptides can be used in therapeutics as they are minimal to non-toxic to the host administering has a good bactericidal to inhibitory effect and can engineered in vitro.

Antimicrobial peptides or AMPs, are an emerging class of therapeutic agents in the field of medical science and pharmacology, they are basically small protein molecules usually in the size range of below 50 amino acids. They cationic (positively charged) and amphipathic (hydrophobic as well as hydrophilic) in nature, this amphipathic property allows them to interface directly with the microbial membrane, which they can permeate more quickly rather than interacting with specific receptors on the microbial surface to enter the cell through endocytosis. AMPs are part of the innate immune system of the organism that actively participate in the initial immune response by interacting with the membrane of the invading microbial cell and facilitating its lysing. As AMPs are positively charged, they can directly bind and interact with negatively charged bacterial cell membrane, causing changes in the electrochemical gradient around the cell and changing the electrochemical potential of the cell membrane leading to the loss of membrane integrity, resulting in pore formation and changing the cell permeability allowing the entry of bigger molecules like proteins and external fluid into the cell, leading to damage to its shape and eventually causing cell death by lysing. They are structurally very diverse and are found in different organisms like plants, fungi, algae, bacteria, insects and animals contributing to their defences and displaying antibiotic properties against a broad range of organisms. AMPs can be classified as antifungal, antibacterial, anti-viral and anti-parasitic. In addition to their direct antimicrobial action they play a variety of roles as mediators of inflammatory responses, affecting epithelial and inflammatory cells, which influence cell proliferation, immunological induction, wound healing, cytokine releases, chemotaxis and the protease-antiprotease balance. (Koczulla & Bals, 2003). Apart from its innate response, AMPs also actively participate in the adaptive immune response by acting as a chemotactic factor for monocytes and T cells chemotaxis (T-Lymphocytes), as well as an adjuvant and polarizing factor in dendritic cell maturation.

AMPs first came into the limelight when they were discovered in 1939 by Dubos, who went on to isolate them from a soil *Bacillus* strain. This newly discovered soluble agent showed bactericidal properties by inactivating the glucose dehydrogenases of all Gram-positive bacteria studied so far. When such cultures were grown with this agent, they seem to have limited growth

and appeared lysed (Dubos, 1939). Further studies on Animal subject, a mouse showed protection against Pneumococci infection by simply incorporating this extract, though it showed no/minimal effectiveness against Gram-negative bacilli. (Dubos, 1939). Later on the extract was fractioned and the agent was identified as an AMP, which was later named as gramicidin by Hotchkiss and Dubos (Dubos and Hotchkiss, 1941). Until now over 750 AMPs have been discovered from both prokaryotes (Bacteria and Archaea) and eukaryotes (plants, fungi, protozoa, insects, animals, etc.) as they are constantly under an attack from microbial pathogens host has developed defense mechanisms this also include AMPs (Reddy et al., 2004)

In animals, they are predominantly found in nasal epithelial cells as these tissues are constantly exposed to airborne pathogens and as AMPs act as the first line of defense (innate response), other regions include epithelial cells of the gastrointestinal, urinary and genital tract. In animals however the first known AMP was discovered in rabbit serum in the year 1956, the macrophages and other defense cells (leukocytes) were lysed and the extract was observed for to have soluble elements that showed bactericidal effects on gram- negative enteric bacilli, on further characterization (dialysis, salt fractionation) and exposure to proteolytic enzymes the structure was depicted to be globulin, these peptide was named phagocytin, later known as defensin. (Hirsch, 1956), similarly a red protein, called lacto-transferrin or simply LF an iron binding protein was discovered in bovine milk that displayed antimicrobial properties in the year 1965 (Groves *et al.,* 1965). In humans, AMPs are found in leukocytes a study done by examining the lysosomal fraction by exposing it to electrophoresis, showing presence of small basic proteins having bactericidal properties (Zeya and Spitznagel, 1963).

Plants are known to possess many antimicrobial agents in the form of secondary metabolites as they are constantly under attack by bacteria, fungi, viruses and pests due to their stagnant nature, apart from secondary metabolites they seem to have antimicrobial peptides that work intermutually with secondary metabolites much more effectively. A peculiar feature has been seen with plant AMPs; the polypeptide chain seems to bear many cysteine residues. This helps in formation of disulfide linkages giving it a stable structure that is resistant to a higher degree of heat, mechanical strain and structural and functional stability to other physical and chemical changes in the environment, making it more suitable to use as a therapeutic agent (Benko-Iseppon et al., 2010).

Bacteria has an arsenal of weapons when it comes to defend itself from viruses (phages), archaea and other bacteria or even the external environment, being unicellular they lack immune defenses but make up by adapting faster and producing antimicrobial agents that includes antibiotics, lytic agents, toxins, metabolic byproducts (organic acids), hydrolytic enzymes and peptide that have antimicrobial properties known as bacteriocins. Bacteriocins are produced by nearly all known bacteria including Halobacteria having evolutionary modification in its structure for greater stability at to counter its extreme conditions known as 'Halocins'. A place where bacteriocins and antibiotics differ is with respect to its spectrum, Bacteriocins have a very narrow-spectrum of inhibition, in comparison to antibiotics. As we know nearly all known bacteria and archaea produce antimicrobial peptides in one form or another here are some examples of some AMP produced by bacteria are:- Firstly, we have a well-known Gram-negative, model organism *Escherichia coli* also known as *E. coli* that are present in guts of warm blooded organisms, colicin gene cluster that is present in the extra chromosomal plasmid encodes for three genes: one for a toxin, a lysing gene and gene that codes for a microbial protein called 'Colicin'.

Archaea also produces their own antimicrobial peptides similar to bacteriocins, known as 'Archaeocins'. One of the known examplesis 'Halocin' produced by Halobacteria. Archaea produces archaeocins predominantly in the stationary phase when there is a depletion of nutrients, these are released in the environment and lyse other cells to decrease competition for nutrients and also to replenish nutrient content in the environment by lysing neighboring cells. As Archaeocins are very stable they remain in the environment for quite long, lysing many cells and aiding in Archaeal survival (Riley and Wertz, 2002).

Fungi are not well known to produce antimicrobial peptides but a recent study in 2005, witnessed an antimicrobial peptide similar to defensin, a cysteine-rich AMP present in mostly plants and animal known as Plectasin, isolated from saprophytic fungus of ascomycete class *Pseudoplectania nigrella*. Plectasin in a low quantity showed antimicrobial activity against *Streptococcus pneumonia*. Including strains that were resistant to antibiotics, giving a promising antibiotic agents. The gene could be transferred to other species and strains and the Recombinant Plectasin was been able to produce in high concentration with minimal nutrient and environment conditions with high purity. Plectasin showed extremely low toxicity in mice infected with

Streptococcus pneumonia and cured it the same efficacy as other antibiotics like vancomycin and penicillin. (Mygind et al., 2005)

AMPs does not only target bacteria but also other human pathogen such as fungi and virus, which are quite resilient to many therapeutic drugs including antibiotics. Over the years, invasive fungal infection has increase with majority of cases showing resistance to the antibiotic, especially in immunocompromised patients and infants. Which requires a higher dose which leads to major problem with regard to toxicity. Therefore, there is an imminent need to find an agent that is nontoxic to the mammalian cell but has inhibitory and cidal effect on fungi. Naturally occurring antimicrobial peptides have shown to be an effective solution as they are nontoxic to mammalian cell and attack only the fungal membrane as they are specific to chitin rich layer and not phospholipid present in mammalian cells. These kind of AMPs are called 'Antifungal' peptides or simply AFPs similar to bacteriocins they have the ability to lyse the microbial cell using different mechanisms.

As viruses are non-living and merely just inactivated particles without a host that lacks a basic cellular structure there aren't any antibiotic agents against viral infection. Treatment is entirely dependent on the host's immune system and the increase rate of mutation after its replication cycle, makes viral infection deadly, killing many people every year. As similar viruses do infect other mammal cells there are various antiviral peptides isolated from lymphatic cells. Over 60 antiviral peptides have been isolated and used for treatment of severe viral infections like HIV, influenza virus and hepatitis virus infection. They are authorized in many countries and make a good market in pharmaceuticals. Antiviral peptides have the ability either integrate its self into the viral envelope or block the entry of virus or viral genome by adhering to the host cell membrane receptors used by virus to enter host cell. It does by disrupting the envelope of the virus both enveloped DNA and RNA viruses. Research carried using rhesus θ defensins isolated from disrupting rhesus macaque leukocytes and isolating the contents along with human θ-defensin showing effects against type 1 and type 2 herpes-simplex virus. Retrocycins 1, 2 and 3 showed promising results against HPV, human papilloma virus that causes cervical cancer in humans, θ defensins also shown to block/ inhibit the entry of HIV-1 by binding to gp120 protein of HIV-1 (Yasin et al., 2004). Apart from naturally available antiviral peptides synthetic or designed peptides have shown to promising against viral infections

Peptides can be designed or existing ones can be modified to produce synthetic antimicrobial peptides having a broad spectrum activity and these AMPs can be designed using software and produced by using DNA recombination technology where the desired gene of the AMP is introduced into a transgenic organism to utilize its machinery to produce desired peptides. β-sheet peptides are example of synthetically synthesized peptides can be manipulated to adopt different conformation to mimics active antimicrobial peptide like cecropin and magainin that target the cell membrane causing it to lyse. Say suppose bacteria gains resistance to the AMPs modification can be done to the exiting peptides which will prevent it from being destroyed by bacterial cell and still carry its inhibitory activity (Liu and DeGrado, 2001). In this review we aim to analyze the sources, the structural characteristics and their mode of action in brief, and study their antimicrobial activity against pathogenic microorganisms.

2. Sources of Antimicrobial peptides-

All living organisms are found to possesss at least one type of Antimicrobial Peptide, from tiny single cellular bacteria to multicellular creatures (Reddy et al., 2004). Multicellular organisms and microbes live together in nature in harmony, despite of being directly in- contact with microbes most multicellular organism remain unharmed, like the cornea of eye in animals are directly exposed to various air-borne microbes, bugs do not have a well-defined immune system, they lack lymphocytes and antibodies. Plants also do not possess any of the adaptive immune system, seeds germinate without any hindrance of microbial pathogen, these organism despite being in constant contact with bacteria, fungi, viruses and protozoa manage to survive by producing broad-spectrum antimicrobial peptides. These make hotspots for extraction of novel antimicrobial agents (Zasloff, 2002), Up to this point over 750 distinct AMPs have been discovered in eukaryotic species, including plants, insects and mammals (Reddy and Aranha, 2004), prokaryotes like bacteria also seems to produce AMP's, over 50 have been known so far having bacterial origin, most typically found in gram positive bacteria, commonly known as Bacteriocins derived from lactic acid bacteria (LAB), includes nisin and lacticin (Lüders et al., 2003).

2.1 AMPs from Bacteria

Bacteriocins are widely used as bio preservatives, providing a great alternative to chemicals that can cause health problems. As they are basically proteinous in nature and only exert an effect on bacteria that makes it a good candidate to extend their shelf life of food and improve their safety (Settani and Corsetti, 2008). Nisin is obtained from *Lactococcus lactis* (Schleifer et al.,1985), being one of the first bacteriocins to ever be discovered in lactic acid bacterium by Rogers (Rogers, 1928). Although nisin is profoundly famous in over 45 countries and commercially synthesized on a large scale, another commercially produced bacteriocin, which is gaining a lot of attention as bio-preservative is Pediocin PA-1, obtain from *Pediococcus acidilact*ici by fermentation process using sucrose as substrate (Gonzalez and Kunka, 1987). Apart from safe bio preservatives, another type of bacteriocin, produced by *Escherichia coli*, which is toxic for other organism including other strains of *Escherichia coli* is colicin, colicins are only generated from *E. coli* that possess the colicinogenic plasmid pCo1. Production of colicin is mediated by the SOS regulon and only activates when the cell is under a stress or

shock. It is a last resort as the toxin produced along with colicin is lethal not only to the neighboring bacteria but also to the host cell itself colicin is a heat labile lipopolypepetide. Colicin is a large protein ranging in size up to 600 hundred amino acids that binds to specific receptors on the targeted microbial membrane surface inducing pores in the membrane or producing nuclease activity against its DNA, rRNA, and tRNA, It was first observed by Gratia in 1925, later it was revealed that *Shigella* and *Citrobacter* a group of enteric bacteria also possessed the plasmid and produced colicin (Cascales et al., 2007). Some examples and sources of colicin include, pyocins acquired from *Pseudomonas pyogenes* and *Pseudomonas aeruginosa* strains, 'cloacins' obtained from *Enterobacter cloacae,*' marcescins' from *Serratin marcescens,* megacins produced by *Bacillus megaterium,* etc., (Cascales et al., 2007). Gram-positive bacterium species also produces bacteriocin which quite differ with respect to gram-negative, for instance it isn't lethal for the host cell, i.e. the host need not be in stress situation to release it. This evolutionary event has to do with the pathway or transport mechanism as in case of gram-negative which encodes toxin along with bacteriocin, gram-positive have evolved to use specific pathway for bacteriocin or employ the *sec*-dependent export pathway that doesn't involve toxin production in any manner.

A lot bacteriocins are isolated and produced commercially on a large scale using simple and vegetable and other organic waste as raw material from various bacterial species, some examples include.,

- Subtilisin produced by BFE 5301 and BFE 5372 strains of *Bacillus subtilis* species. Isolated from okpehe fermentation, a traditional fermented vegetable product (Ogutoyinbo et al., 2007)
- Mundticin, A33 and Enterocin P isolated from T33, 4, A33 strains of *Enterococcal faecium*, showed a good antimicrobial activity on characterization. Isolated using raw barley and sorghum as substrate (Hartnett et al., 2002).
- Plantaricin D, a very heat stable (even at 121° C) antimicrobial peptide was obtained from BFE 905 strain of *Lactobacillus plantarum* species found in Waldorf salad (Franz et al., 1996)
- Amylovorim L471 a thermostable and strongly hydrophobic bacteriocin, produced by *Lactobacillus amylovorus* by culturing it in corn steep liquor, which maximum production

observed at pH of 5.0-5.4.the product was extracted using a mixture of organic solvent (chloroform/methanol) (De Vuyst et al., 1996)

- Buchnericin LB is a thermostable (upto 121° C for 15 minutes) peptide and retain its activity even at a very alkaline or acidic pH, (2.0-9.0) and can be stored at a very low temperature without losing it biological activity, it is derived from *Lactobacillus bunchneri* LB by fermentation of vegetables (Yildirim and Yildirim, 2001)
- Lueconocin J a promising bacteriocin showing inhibitory activity against many food-borne pathogens derived from *Leuconostoc sp.* Found in the naturally fermented Kimchi, a Korean dish (Choi et al., 1999)
- Cleucocin C, Leucocin A and BC2 were extracted from *Lactobacillus mesenteroides* isolated from different types of malted beer (Vaughan et al., 2001)
- Mesentericin ST99 acquired from a Lactic Acid Bacteria, *Leuconostoc mesenteroides* that is obtained from a fermented cereal beverage from Bulgaria called Boza (Todorov and Dicks, 2005)

2.2 AMPs from Plants

Cysteine-rich AMPs originated from plants include defensins, thionins, hevein-like peptides, knottin-type peptides (linear & cyclic), lipid transfer protiens, hairpinin, and the snakins. (Tam et al., 2015). PR or Pathogenesis related proteins are one of the most common sources of allergens, first discovered and isolated from tobacco plant leaves which seem to be released in response to viral infection, the Tobacco mosaic virus in 1970. A total of 17 families of PR proteins have been found originating from plants in stress situations such as microbial and insect infections, wounding, exposure to harsh chemicals and toxins, and climatic conditions. Plants release PR as a defense response. However, plants that are under constant exposure to harsh conditions like UV radiations, insect/ pest infections or fungal infections seems to constantly produce PR on a regular basis. (Sinha et al., 2014).

2.3 AMPs from insects and invertebrates

Since insects lack a well-defined circulatory system and an adaptive immune system, that has immunoglobulin and lymphocytes, they counter the deficiency by having a very effective humoral response that houses lysozymes, which display a broad spectrum antimicrobial effect. Eukaryote produces defensins, which participate in a wide spectra of antimicrobial activity, found in mammals, insects and plants

One very well-known example was seen in the pupae of a giant silkmoth *Samia cynthia*. when it was injected with a live non-pathogenic Gram-negative bacterium, the humoral immune system of the pupae responded by secreting a large number protein, some which showed antimicrobial activity. But the activity was lowered if Actinomycin D or Cycloheximide were given at earlier stage, indicating that the humoral system of the insect was able to recognize the intruding microbe, thus activating gene response by removing the suppressing components from messenger ribonucleic acid and producing the respective proteins. (H.G. Boman et al., 1974). A study conducted using the Hemolymph of another silkmoth, *Hyalophora cecropia* by immunizing it by injecting it with live bacteria, saw a similar response as seen in the previous case, the bacterial humoral system responded by producing ten different types of immune proteins, namely (P1-P9A, P9B) among which four displayed bactericidal activity. P9A and P9B showed strong antimicrobial activity against *Escherichia coli* becoming an emerging class of antibacterial agent called cecropins (cecropin A and B) (H.G. Boman et al., 1981)

An enhanced understanding of the AMPs can be done using the innate system of *Drosophila melanogaster*, a model organism commonly known as fruit fly. When a septic injury occurs to the organism several AMPs are released into the hemolymph in its response produced by the fat body, that shows a strong antimycotic activity against fungi. When a drosomycin-green fluorescent protein (GFP) reporter gene was used for detection, it showed that when a part of the fat body other than the epithelial tissue is in direct contact with object that comes from the external environment like digestive, reproductive and respiratory tract also express antimycotic peptides thus showing that the epithelial cells act more than just a physical barrier (Ferrandon et al., 1998).

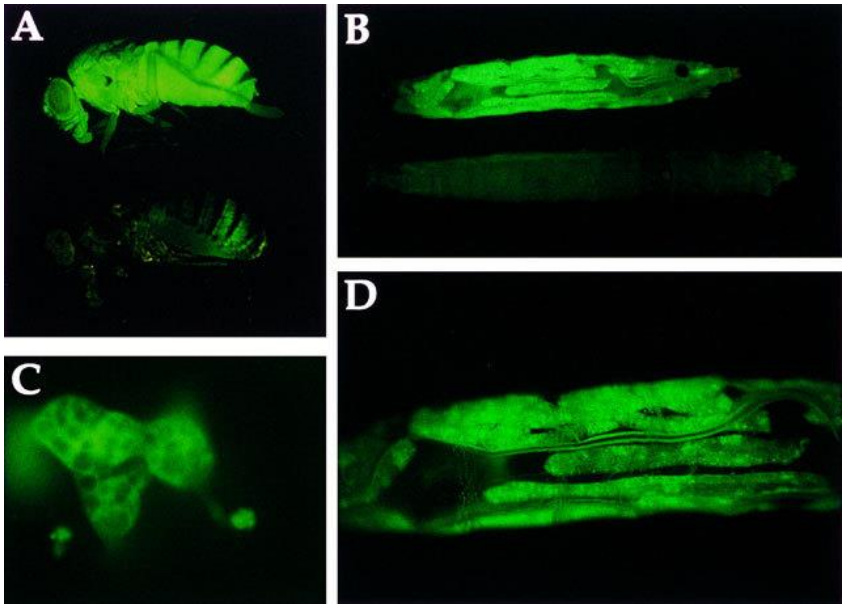

Figure 1: Fluoroscopic image of drosomycin-GFP reporter gene, expressed in the fat body during systemic response. A) An adult transgenic fly (*Drosophila melanogaster)*, that was immunized 48 hours before observing. B) Two transgenic larvae of the fly, one was immunized 12 hours before testing (above) and the other was unimmunized (below). C) A dissected fat body of an adult *Drosophila melanogaster*, undergone immunization. D) An enlarged image of B) that is illuminated neighboring fat body (Ferrandon et al., 1998).

Some of these AMPs found in hemolyph of *Drosophila melanogaster* are: 'Cecropin', a 9 kDa antimicrobial peptide, the gene coding for cecropin, CecC is greatly expressed in both larval stage as well as in adult stage when encountered with infection in *Drosophila (*Kylsten et al., 1990; Tryselius et al., 1992), Another is 'Drosocin', a 19 amino acid residue peptide having a disaccharide group attached to a threonine residue present in the middle of the peptide chain. It showed potential antimicrobial activity against most of Gram-negative bacteria with an exception to *Micrococcus luteus* (Bulet., 1996). Insect defensin, a cationic AMP of size 4-kDa, rich in cysteine residue in the entire polypeptide chain and have three disulfide bridges, give the molecule a structural integrity and stability. It is quite similar in structure to the mammalian defensin present in the neutrophils and macrophages in mammals (Dimarcq.,

1994). Metchnikowin, a 26 residue proline rich antimicrobial peptide transcribed and found in fat body and can also be induced in tumorous blood cell line, it shows activity against Gram-positive bacteria as well against some fungal species, with a minimum inhibition concentration of less than 1 microliter (Levashina et al., 1995). Attacin a 20 kDa antibacterial peptide, first studied in *Hyalophora cecropia*, shows promising bactericidal effect against Gram-negative bacteria but unlike cecropin, it directly stimulates the disruption of the bacterial membrane (Boman et al., 1991). Attacin appears to be interfer and inhibit the protein synthesis of the other membrane proteins, which was specially observed in *Escherichia coli* (Asling et al., 1995; Carlsson et al., 1991) and 'Drosomycin', is a major antimycotic peptide characterized among the broad range of peptides and protein displaying response towards infection and septic injury in insects, It a small 44 amino acid peptide, having a beta sheet structure that is stabilized by four disulfide linkages (Fehlbaum et al ., 1995; Landon., et al 1997). All these peptides are synthesized in the fat body and released into the hemolymph as the humoral response in *Drosophila melanogaster* (Ferrandon et al., 1998)

Other insects like larvae of *Sarcophaga peregrine* or simply flesh fly produces three groups of antibacterial protein recognized as sacrotoxin I, II, and III, active protein from these groups include sacrotoxin IA, IB, IC, they are amphiphilic in nature and differ in their mobility rate when placed in an electrophoretic unit where IA not only act as defensin and participate in immunity but also act a growth factor and support in the development of the insect (Matsuyama and Natori., 1988). AMPs could also be extracted from spider's venom, example was seen in *Cupiennius salei* or Ctenidae, from which a new class of AMP was isolated known as Cupiennin 1, a small peptide with 35 amino residue having a hydrophobic N- terminal region and polar C terminal, protein isolated from the venom showcased membrane disrupting activity in both prokaryotic as well as eukaryotic. Lycotoxins are a another class of AMPs that were isolated from spider venom from *Lycosa carolinensis* (Schaller et al., 2002). Melittin is another peptide that is isolated from the venom of honey bee, a European honeybee *Apis mellifera*, it is a 26 amino acid peptide that perform lytic action by formation of pore in the lipid membrane (Sitaram and Nagaraj, 1999; van-den Bogaart et al., 2008). Due to its ability to disrupt cells and its stability due to being a very molecule, it has shown a great potential of being a pharmaceutical agent. It is also seen that Melittin showcases an anticancer effect due

to its cytotoxic nature and it is currently used and produced commercial by many Pharmaceutical companies in many parts of Asia, to treat arthritis (Ju Son et al, 2007).

Other than insects there are many invertebrates that have a diverse variety of AMP's that have showcased a broad spectrum of antibacterial activity, one of such example is seen in crab. 'Tachypleus', a cationic peptide was found in the acid extract of *Tachypleus tridentatus* commonly known as horseshoe crab. The peptide showed antibacterial activity against both Gram-positive bacteria as well as against Gram-negative bacteria at a very low concentration. Venom extract from various scorpion species seem to have a diverse variety of clinically important pharmacological functions, not just as an antibacterial agent but also as an antimycotic, antiparasitic and antiviral peptides. Some notable examples are;

- 'Scorpine', a 75 amino acid residue polypeptide having three disulfide linkages produced by *Pandinus imperator*, a black scorpion species originated from the savannas of west Africa. It produced antibacterial activity against many human pathogens like *B. subtillis* and *K. pneumonia* and showed a strong inhibitory effect on the gametes and ookinete of *Plasmodium berghei*, a protozoan parasite that Is known to cause malaria in many mammals (Conde et al., 2000)
- Defensins were isolated from the venom of an African *Opistophthalmus carinatus*, a total of four types of scorpine peptides were isolated and designated as opiscorpines 1-4 based on their structure, each displaying antimicrobial effect on different species of bacteria and different pathogenic organisms; these molecules showed antifungal activity against two species of yeast, *Fusarium oxysporum* and *Fusarium culmorium* when assayed. Opiscorpines also displayed antibacterial effects by inhibiting the growth of *E. coli*. It may show effects on other bacterial species on further testing (Zhu and Tytgat., 2004)
- Hadrurin,-is a 41 amino acid residue peptide with a molecular mass of 4.4 kDa having no disulfide linkages or cysteine residues, isolated from a Mexican *Hadrurus aztecus.* Hadrurin exhibited antibacterial against many pathogenic bacteria to humans such as *Salmonella typhi, Enterococcus cloacae, Klebsiella pneumonia, Pseudomonas aeruginosa, Escherichia coli and Serratia marscences* at a minimal inhibition

concentration of a very low micromolar concentration, also displaying cytolytic activity on human erythrocytes (Torres-Larios et al., 2000)

- Two closely related antimicrobial peptides were isolated from *Androctonus amoreuxi*, a scorpion species native to North America that displayed broad-spectrum antimicrobial and antimycotic effects on many yeast cultures. They were designated as AamP1 and AamP2, these peptides were similar in structure and amino acid composition with amidation in the carboxyl terminal of the amino acid chain and differing in amino acids at two sites. Antibacterial activity was seen on *S. aureus* (gram-positive bacteria) and *E. coli* (gram-negative bacteria) at a low concentration (20μM -150μM) (Almaaytah et al.,2012).

These were some of example of AMP's extracted from venom extract of scorpions, invertebrate habituated to water have also displayed to possess a variety of AMP's constituting their humoral immune system, example: freshwater shrimp produced AMP's, common one being 'Penaeidin', a class of polypeptides of size ranging from 5.48kDa to 6.62kDa, that combines a proline rich N-terminal and a C-terminal domain containing 6 cysteine residue forming three disulfide linkages, Penaeidins were produced in the hemolymph extract and plasma extract of *Penaeus vannamei*. it displayed both antibacterial towards Gram-positive bacteria and antifungal activity, it showed a far better bactericidal property against *Bacillus megaterium* with a minimal inhibition concentration of 2.5-5 μM and bacteriostatic properties against *Aercoccus viridans*. and a broad spectrum antimycotic activity against filamentous fungi with MIC below 10 μM, but was ill effective against yeast when tested on *Saccharomyces cerevisiae* and *Candida albicans* (Destoumieux.,1997; Destoumieux., 2000). Other examples of sea water invertebrates include mussels like *Mytilus edulis*, that produces mytilin 3.7kDa peptide and 'Mytimycin' a 6.2 kDa peptide, structurally very similar to insect defensins, indicating evolutionary linkage between molluscs and arthropods, mytilin displayed antibacterial activity against gram- positive bacteria and antimycotic activity was seen by mytimycin. These antimicrobial peptides are economically very beneficial as molluscs like mussels and oysters are frequently exposed to infections, thus using such antimicrobial agents can greatly reduce losses in their production and generate more revenue from better yield (Charlet etal.,1996).

2.4 Antimicrobial peptides from vertebrates

Defensins and cathelicidins constitute the majority of antimicrobial peptides in vertebrates, these are present in the tissues and cells that are constantly exposed to the external environment that encounters pathogenic organisms on a regular basis. Defensin is a family of abroad spectrum antimicrobial peptides that are present in most mammalian and other vertebral organisms. They are divided into two class; α-defensins and β-defensins (Ganz and Lehrer., 1998). Human produce both, six classes of α-defensins and two classes of β-defensins respectively. Four α-defensins, called HNP 1, 2, 3 and 4 are produced by the neutrophils, a leukocyte that constitute the humoral response system of body's defenses. Defensins are present in the granules that are released during the process of degranulation by the neutrophils (Ganz and Lehrer, 1997). The rest two α-defensins, HNP 5and 6 are released by the paneth cells, a type of stimulatory epithelial cell of the small intestine that releases lysozymes, α-defensins activate by secretory phospholipase mechanism upon cholinergic or bacterial stimulation caused by the presence of a foreign bacteria in the colons. (Mallow et al., 1996; Qu et al., 1996). Unlike α-defensins, β-defensins are not stored in a localized cytoplasmic granules and are secreted by various epithelial cells that make up the tissues that are constantly exposed to the incoming pathogens (Diamond et al.,1996), HBD1 and HBD2 are the types of β-defensins found in humans, they show a great resemblance in homology to β-defensins that are found in bovine tracheal and lingual epithelial cells (Harder et al.,1997; Bensch et al.,1995). Apart from humans nearly all mammals and vertebrates produce defensins like rabbits, guinea pigs, cows etc. Cathelicidins are short, amphiphilic antimicrobial peptide that are found in many mammalian species, of size genrally ranging around 3 to 5 kDa, they have a highly conserved and identical N-terminal preprosequences and a highly varied C-terminal, all of which have an identical sequence of protein named cathelin, these are stored as prepeptide and stored in granules of neutrophils of cows, pigs, rabbit, mice and humans (Zanetti et al.,2006), This is to prevent any indiscriminate proteolysis that would not only destroy the incoming microbes but may also cause premature proteolysis and damage the host cell (Ganz and Lehrer., 1998). It seems to have first been isolated from the porcine leukocytes (Ritonja et al., 1968). In humans two types of cathelicidins are produced, LL-37 and CAP-18 in the testis as well as in squamous epithelial cell, it is known to display a broad-spectrum antibacterial activity towards many bacteria such as *Escherichia coli, Bacillus megaterium* etc. (Agerberth et al.,1995; Agerberth et al., 2000).

Defences as antifungal agents(AFP)

There are various forms of alpha-defensin isolated from rabbit neutrophil extract that has shown promising antifungal activity. Defensin adhere to the cell membrane and forms multimeric pore which disrupts the selective permeability of cell leading it leak out intracellular contents and increase protein influx causing lysing due to change in osmotic pressure in the cell. Another way in which it inhibits fungal growth is by causing membrane depolarization that causes change in the electrochemical gradient leading to decrease in ATP synthase activity resulting in decrease in ATP production and inhibiting cellular respiration that are essential for microbial growth. There are 6 types of Alpha defensin present in rabbit neutrophils among which three Np-1, Np-2 and Np-3a were highly effective against *Candida albicans*. (Selsted *et al*.,1985) whereas NP-1 showed fungistatic and fungicidal effect against strains of *Cryptococcus neoformans*, the minimum inhibition concentration was found to be 3.75 to 15 milligrams of NP-1 per 1 milliliter for encapsulated strains and a lower concentration of around 0.93 milligram for acapsular strains a similar result was seen with Fluconazole. (Alcouloumre et al., 1993), similarly Alpha-defensin subclasses seem to have lethal inhibitory effects against *Coccidiodes immitis* and *Candida albican* (De Lucca & Walsh, 1999) other antifungal peptides of bacterial origin includes: Schizotrin A obtain from cyanobacteria *Schizotrix*, shown to have antifungal activity against *Candida albicans* and *Candida tropicalis (*Pergament and Carmeli, 1994). Cepacidines are glycopeptides isolated from *Burkholderia cepacia* having two active compounds Cepacidines A1 and Cepacidines A2 which have shown antifungal effects on a wide variety of fungi like C. *neoformans*, many species of *Candida, Aspergillus niger, T. mentagrophytes, Trichorphyton rubrum, M. canis and F. oxysporum* . with Minimal inhibition concentration of Cepacidine A of 0.049 to 0.391 microgram per ml much better than the well-known fungal antibiotic Amphotericin B that has been proven to cause nephrotoxicity in humans (Lee et al., 1994).

3. Structure and classification of AMPs

Over thousands of antimicrobial peptides have been isolated and identified till date, many of these peptides have common structural properties, making it easier to categorize them into groups. AMPs can be classified into different groups based on their amino acid components, their structure and their biological function. (Hof et al.,2001; Andreu and Rivas.,2004), AMPs can also be classified on basis of their gross composition and 3D structure derived from using Nuclear magnetic resonance (NMR), an analytical technique used to analyse the structure and the chemical groups by observing the behavior of the atomic nuclei around a magnetic field. As most of the AMPs are short in length their structure can be analyzed using a 2D NMR spectroscopy (Wuthrich., 1986). Upon analysis AMPs were classified into five groups:

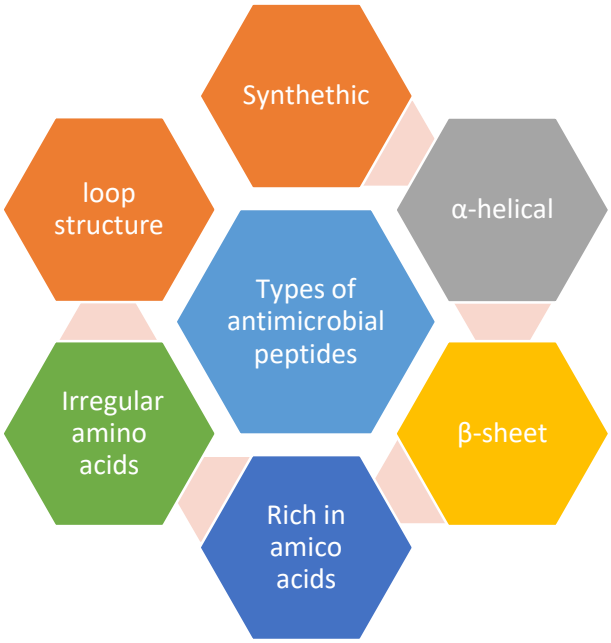

Fig. 2: Classes of Antimicrobial peptide based on their structure.

3.1 α-helical structure

The α-helical structure seen in AMPs is particularly widespread and abundant in nature, they have an α-helical domain and are amphipathic in nature, which allows them to interact directly with the surface microbial membrane (Tossi et al., 2000). the α-helical antimicrobial peptide family is very versatile and are found in the defenses of nearly all eukaryotic organism and are the ones that are extensively studied, well known example of such class of AMPs are ceropins,

protegrin, magainin, cyclin indolicin etc. (Huang et al., 2010). All class of cecropins form a helix in a certain organic solvent such as trifluoroethanol that was confirmed by a study done using NMR on cecropin-A isolated from *H. cercopia*, that displayed an α-helical structure in 15% hexafluoroisopropyl alcohol (Cammers-Goodwin et al.,1996; Holak et al.,1998). Magainin, a 23 amino acid peptide isolated from African clawed frogs, *Xenopus laevis* skin cells, that displays an α-helical structure in organic solvents (Matsuzaki, 1999). In 25% trifluoroethanol both cecropin and magainin form an aliphatic α-helical structure (Marion et al.,1998).

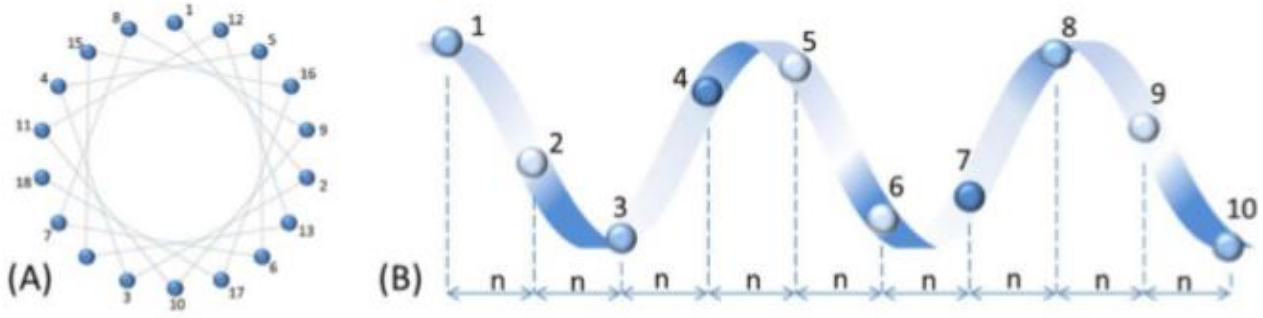

Figure 3: A schematic representation of an α-helical antimicrobial peptide, it is a general view taking inconsideration of all type of amino acid within a peptide A) the top view of helical wheel projection of peptide, where dotted lines show adjacent amino acid and the angle between the two consecutives is 100° B) the side view of the helical peptide, where "n" is the distance between the two adjacent amino, which is roughly 0.15nm (Bahar and Ren, 2013)

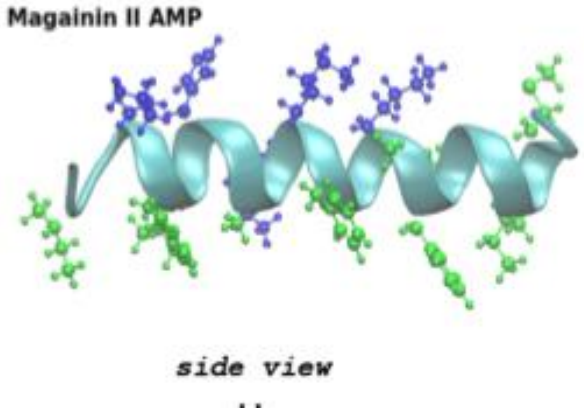

Figure 4: A side view of Magainin II AMP, that displays a helical and amphiphilic structure with a hydrophobic side (displayed in green colour) and a hydrophilic side (displayed as blue) (Lei et al.,2019)

3.2 β-sheet structure

β-sheet structure is rarely seen in known AMP's, peptides form a β-hairpin structure with around 20 amino acids stabilized by two disulfide linkages from cysteine residues. Plant and mammal defensins like α-defensin, β-defensin, insect defensins, protegrin, tachyplins, proline rich AMP's and polyphemusin II, are known to have β-hairpin motif which is stabilized by two disulfide linkages (Haney et al.,2019; Tamamura et al.,1993). Thanatin isolated from *P. maculiventris*, a hemipteran insect showed quite the homology with tachyplesin but displayed an antiparallel β-sheet structure maintained by a single disulfide linkage on studying with NMR. A 25 amino acid residue containing peptide, Lactoferrin B also displays a β-sheet structure that is stabilized by a single disulfide linkage in organic solvent (Hwang et al., 1983).

3.3 Peptide with rich in regular amino acid

Many AMP's have a large number of one type of amino acid in their peptide chain, making their structural conformation different from regular α-helical and β-sheet peptides. Common examples of such peptides are:

I. Proline-rich peptides-: simply known as PrAMP, as proline is non-polarin nature, the peptide (PrAMP) simply diffuses into the bacterial cytoplasm by using inner membrane transporters like SbmA, instead of binding to the cell membrane and disrupting like convention AMPs, they rather interfere with protein synthesis mechanism of bacteria (Mattiuzzo et al., 2007). Example: Tur1A a PrAMP, isolated from *Tursiops truncatus* was known to interfere with the protein synthesis by binding to the ribosomes of *E. coli, K. pneumonia etc*. (Mardirossian et al., 2019).

II. Tryptophan and Arginine-rich peptides, these AMP's are known inflict damage on bacteria by interacting with their membrane, as the peptide is rich in tryptophan that is non-polar in nature facilitating interaction with bacterial membrane, whereas Arginine is basic in nature and has a net charge which helps in hydrogen bond interaction and combine with anionic components of bacterial membrane, apart from it tryptophan also act as an activator of Arg-

rich AMPs through ion-pair π interactions. (Walrant et al., 2020). Some example of such AMP's are indolicidin and triptrpticin, Octa 2 another tryptophan and arginine rich peptide has shown antibacterial activity against Gram-negative *Pseudomonas aeruginosa* and *Escherichia coli* and Gram-positive *Staphylococcus aureus* (Strem et al.,2002).

III. Histidine-rich peptides-: Histidine is a basic amino acid, it gives high permeability properties to the peptide towards the bacterial membrane, A common example is HV_2, which is known to increase permeability of bacterial cellular membrane thus causing it rupture due to disturbance in its osmolarity and eventually lyse to death. (Dong et al., 2019).

IV. Glycine-rich peptides-: Glycine residue in peptides has an important effect on its tertiary structure due to its non-polar highly reactive R group, examples of glycine rich peptide are diptericin, attacins etc. (Lee et al., 2001; Kwon et al., 2008). glycine rich AMP GG3 is known to display a potent antibacterial activity against Gram-negative bacteria making it an ideal candidate for use in commercial drugs (Wang et al., 2015).

3.4 Peptides with irregular/uncommon type of amino acids

Some Antimicrobial peptides have unusual amino acid than regular, these amino acids have slight modifications with respect to their structure that differentiates them from the regular amino acids, it is seen that AMPs originated from bacteria show such unusual characteristics, known example is nisin, a lantibiotic AMP isolated from *Lactococcus lactis*, that has an amino acid like lanthionine, dehydroalanine, dehydrobutyrine and 3-methyl lanthionine in its peptide chain. It shows activity against Gram-positive bacteria (deVos et al. 1993; Hooven et al., 1996). These AMPs do not show a defined structural conformation unless if it is put in an organic solvent. Leucocin A is another example of AMP with an unusual amino acid derived from *Leuconostoc gelidum* (Gallagher et al., 1997). These unusual amino acid are results of post-translation modification that add groups on the amino acids giving them additional properties, gramicidine is another example that has a dihydroamino acid in its peptide backbone that allows it to form a very unusual cyclic β-hairpin structure (Gibbs et al., 1998).

3.5. Peptides with loop structures

Some antimicrobial peptides have loop structures that are stabilized by disulfide linkages between cysteine residues, amide bonds or isopeptide bonds. An Example of such peptide is thanatin, a 21 amino acid peptide derived from insect called *Podisus maculiventris*. Has loop structure that is stabilized by a single disulfide linkage between 11 and 18 cysteine s the peptide chain (Power and Hancock, 2003).

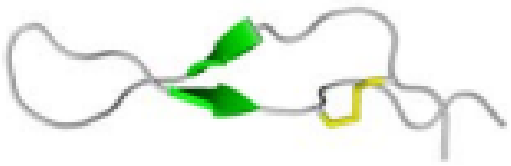

Figure 5. Lactoferricin B, displaying loop structure (Ahmed and Hammami, 2018).

4. Physiological and chemical properties of AMPs

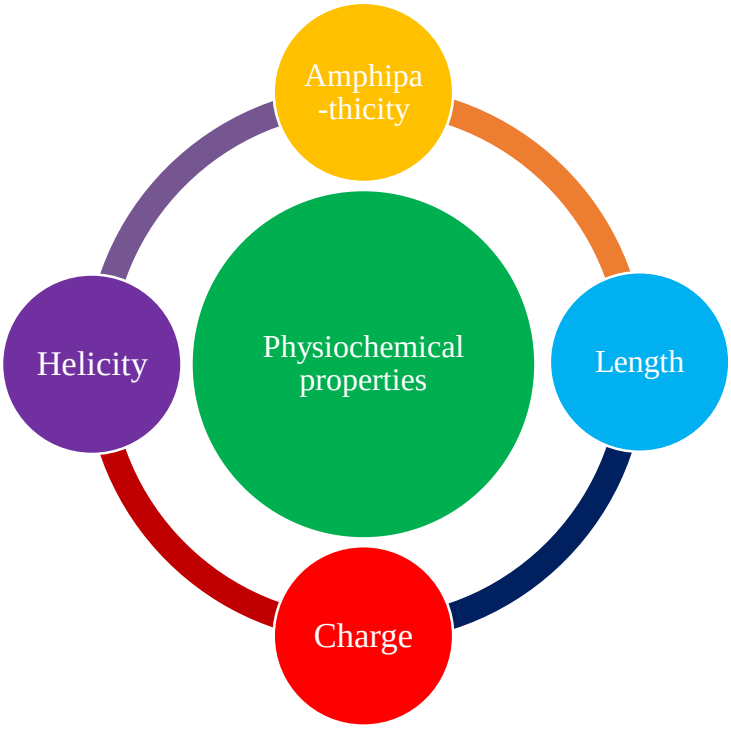

Fig 6: Important Physicochemical properties of AMPs

4.1 Length,

The length of the peptide with respect to its amino acid residues is an important factor governing AMPs, It is seen that atleast 7-8 amino acid are required to form a basic aliphatic structure aligned to form a beta- sheet structure, for helical AMPs, at least 22 amino acid residues in a peptide chain is required to cross the lipid bilayer of the bacterial cell, while sheet AMPs require 8 to 9 amino acid (Weaterhoff et al., 1989).

4.2 Net charge,

The net charge is the sum of all the ionizable group present in the peptide contributed by the amino acids, giving the peptide a particular charge either negative or positive, which is very important in order to interact with the negatively charged cell membrane of the microbial cell, charge can be manipulated by adding, removing or blocking these ionizable groups (Jiang et al., 2008).

4.3 Helicity

It is basically the ability of an AMP to form a helical structure in a solution, it's not a very important characteristic that governs the antimicrobial activity but it is an essential feature to determine whether or not its toxic to eukaryotic cells. Toxicity can be reduced by simply incorporating D-amino acid in the peptide backbone, not only does it reduce its hemolytic activity but also preserves its antimicrobial activity (Papo et al., 2002).

4.4 Amphipathicity,

Amphipathicity is an important character that determines the ability of the AMP to interact with the microbial cell membrane and the aqueous environment at the same time, this interaction is need to carryout membrane disruption activity of the AMP (Fernandez-Vidal et al., 2007).

5. Mode of action of AMPs

Effect on membrane: many AMP's inhibit the growth of microorganisms interfering with the working mechanism or structure of the cellular membrane, as the peptides are usually amphiphilic and cationic, positively charged it is attracted to the negatively charged cellular (phospholipid) membrane due to electrostatic attraction. After binding they quickly adopt its amphiphilic structure, acclimatizing to the specific conditions at the water-membrane interface. This interaction generally leads to increase in permeability of the cell membrane in a lethal way (Hof et al., 2001). Many models have been proposed to describe this event, interaction of peptide with cellular membrane of target cell some are described below.

5.1 Barrel-stave model

This model was originally described for nisin, lantibiotic peptide, wherein one the peptide binds to the outer leaflet of bacterial membrane by electrostatic attraction, the peptide forms a barrel-like cluster that leads to the formation of amphipathic pores (Boheim et al., 1974). Such that the hydrophobic surface of the peptide interacts with the lipid core of the bacterial membrane and it hydrophilic side chain of the peptide point towards the water filled pore producing an aqueous pore. The next step involves recruitment of more peptide monomers in order to increase the pore size, this causes the leakage of extracellular components of the bacterial cell through the pores leading to disruption of cell morphology eventually causing the cell to lyse. The pore size generally ranges approximately more than 18 A in diameter (Boheim et al., 1974; He et al., 1996).

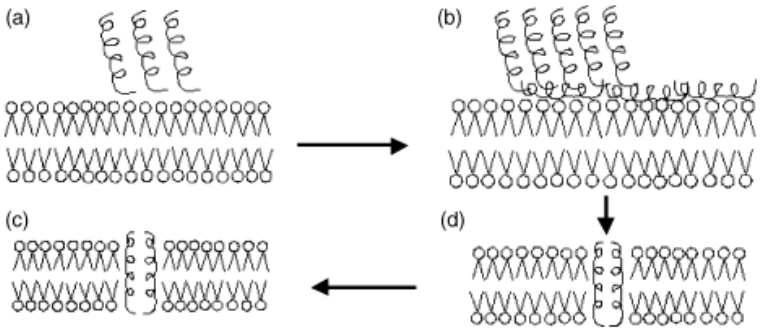

Figure 7. The barrel-stave model, (a) binding of peptide to the bacterial membrane (b) insertion of the helical peptide into the hydrophobic core of microbial membrane (c)

recruitment of additional monomers of helical peptide (d) death due to leakage of cytoplasmic materials (Reddy et al., 2004)

5.2 Carpet-like model

The barrel-stave model was limited to hydrophobic helical peptide, such as nisin and alamethicin, whereas membrane interaction of other amphipathic AMPs work according to the carpet-like model, In this model the microbial membrane surface is fully covered by a cluster of peptides resembling a carpet. When the concentration increases to a critical level the membrane collapses and holes are formed all over the membrane, which are quite larger than the pores seen by the alamethicin and nisin using the above mode of action, these leave lysis of the microbial cell due entry of fluid into the cell causing it to swell and burst. This mechanism was first proposed for magainins (Shai, 1999; Ludtke et al., 1996).

5.3 Toroidal pores or Aggregate channel model

In this model the helices of the peptide insert themselves into the cellular membrane, which leads to bending of the lipid monolayer to form a pore in way that the water core Is lined by both lipid head group and the inserted peptide, This mechanism is seen in protegrins, melittin and in magainins (Matsuzaki et al., 1996). The toroidal pore models differ from the barrel-stave model based on the association of the peptide with the lipid head group is constant, even when the peptides are perpendicularly inserted into the lipid bilayer, as the presence of many monomers of peptide in the toroidal pore would simply result in a coulomb energy (a high charge between monomers that will cancel out/ repel each other) that is too high for pore formation. Therefore, the pore size of induced by alamethicin following the barrel-stave method is smaller in comparison to magainin-induced toroidal pore, having an outer diameter around 7 to 8.4 nm and internal diameter approximately 3 to 5 nm, even with a mild concentration of magainin monomers (rougly 4 to 7) (yang et al., 2001).

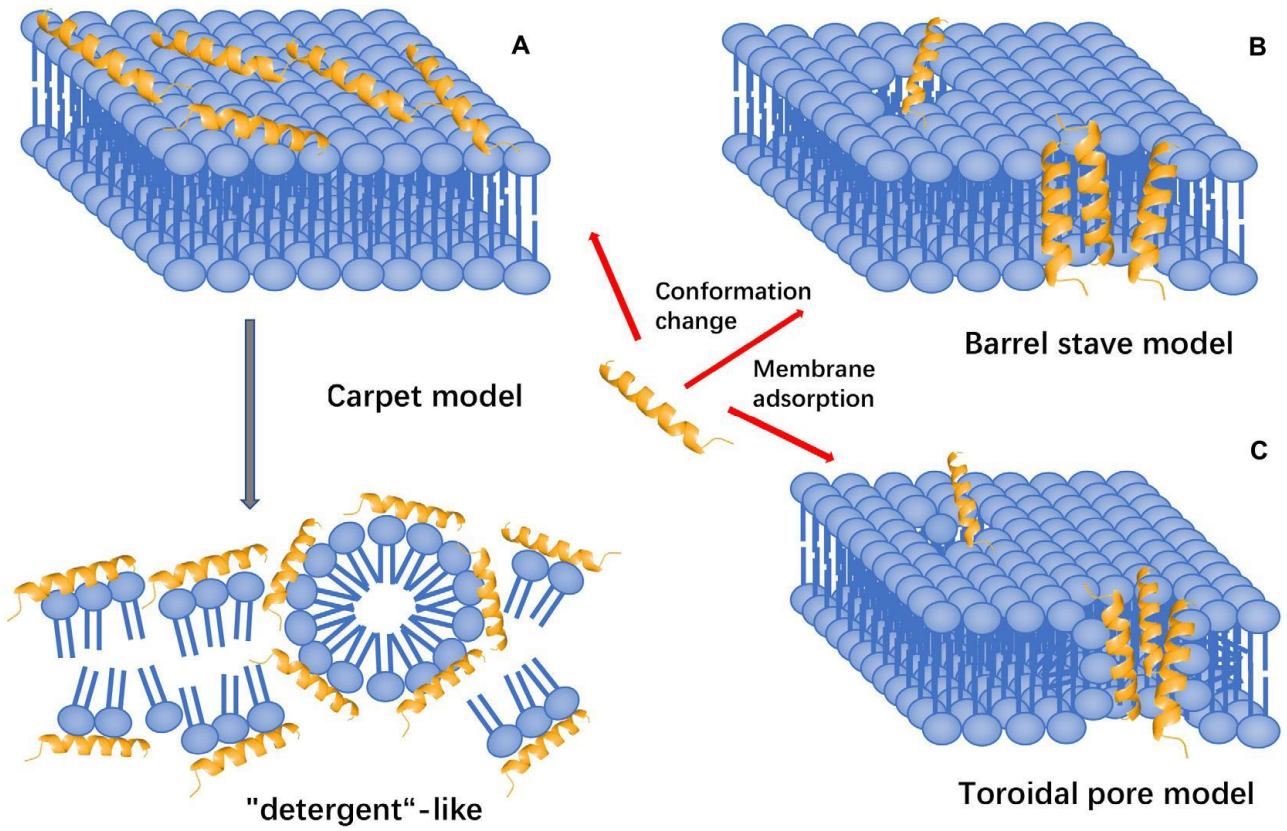

Figure 8. Pore formation models of AMPs

(A) The barrel-stave model: the peptide helical electrostatically bind on the bacterial membrane, monomers group tighter in a barrel-like cluster forming a line of amphipathic trans-membrane pores, the non-polar side chain of the peptide faces the hydrophobic tail of the fatty acid(phospholipid in bacterial membrane and inside, the hydrophilic side chain of peptide point inwards into the water filled pore (B) The carpet model: in this the microbial cell membrane is fully covered by a carpet-like cluster of peptides causing the integrity of the membrane to collapse due to difference in charge lead to formation of holes leading to lysis of the cell (C)Toroidal pore: peptide bind to the cellular membrane and aggregate to impose thinning of the bacterial cell membrane by exposing the polar head group, resulting in bending of the bilayer such that the upper and the lower leaflet of the membrane meet, forming a toroidal appearance to the formed pore (Huan et al., 2020).

Intracellular/ non-membrane targeting mechanism

Instead of binding to membrane and forming pores, some AMP's enter the cell by permeation (diffusion) or endocytosis, once entered the cell these AMP's identify and target cellular machinery to inhibit cellular growth, depending upon the target AMP's can be further divided into groups.

5.4 Inhibition of protein synthesis of microbial cell

Peptides enter the microbial cell through diffusion mechanism and inhibit its protein synthesis by affecting the transcription, translation mechanism and the assembly of proteins in the microbial cell by acting as molecular chaperones, folding and interfering with the enzymes involved in the protein synthesis pathway. Example of such peptides include Bac7-35, it inhibits protein translation process by targeting the microbial ribosome, Tur1A inhibits protein synthesis mechanism by inhibiting the binding of translation factors, thus halting the elongation phase in Escherichia *coli* and *Thermus thermopilus* (Mardirossian et al., 2014; Mardirossian et al., 2018). DM_3 affects many intracellular functions of protein synthesis (Le et al., 2016).

5.5 Inhibition of nucleic acid biosynthesis in Microbial cell

AMPs specifically target enzymes and other machinery involved in nucleic acid biosynthesis in microbial cells or it simply degrades the nucleic acid molecules. Well known AMP that uses this mechanism is Indolicidin, a cationic tryptophan rich Amp having its C-terminal amidated, targets the basic site of microbial DNA to crosslink, it affects both single stranded DNA as well as double stranded DNA through this mechanism, it also inhibits DNA topoisomerase I, an important enzyme in DNA replication that prevents any supercoiling (Subbalakshmi and Sitaram, 1998).

5.6 Inhibition of protease activity of Microbial cell

Protease are very important enzymes for microbial survival, as they are involved in many metabolic processes that takes place in the microbial cell as well as they aid in nutrient accumulation, some AMPs have the ability to inhibit the importance proteases secreted by the microbial cell. Example, 'histatin' an AMP that displays a strong inhibitory effect on

protease secreted by microbes like bacteria-, Indolicidin and eNAP-2 are known to inhibit microbial proteases like elastase, chymotrypsin and serine protease (Le et al., 2017).

5.7 Inhibition of microbial cell division

AMPs inhibit cell division in microbial cell through various mean such as inhibiting DNA replication, inhibition of DNA repair mechanism (SOS) that is necessary to repair damaged DNA and prevent cell apoptosis, preventing the chromosomes from separating during cell division or blocking cell growth at a particular stage in cell cycle (Lutkenhaus, 1990). Example: APP, a 20 amino acid peptide inhibits the growth of *Candida albicans* by simply binding to its DNA and arresting its cell growth in S-phase (Li L et al., 2016). Apart from this AMP's are also known to damage DNA indirectly, an example seen in Histintin 5, that interacts with fungal mitochondria, stimulating it to produce reactive oxygen species (ROS), which in turn damage the DNA causing cell death due to mutagenesis (Helmerhorst et a., 2001).

6. AMPs as drugs and it therapeutic potential

Due its broad spectrum activity against many pathogens, in a very low concentration and the growing problem of drug-resistance in microbes, AMPs have drawn quite the attention in the past few years. Their ability to form pore in selective microbial cell and interfere with the microbial metabolic machinery, make AMPs a new class of potential antimicrobial drug agent and opens a board field of possible application (Koczulla and Bals, 2003).

6.1 Expression of AMPs with appropriate drug delivery system

In order for it to be an effective drug AMPs must be expressed with appropriate vectors at the specific sites to avoid cross reactivity or lose its effectiveness, there it has to be associated with an engineered probiotic as a vector to express itself. For instance, if it is engaged in wound healing and protection from susceptible infection, AMP's can be loaded in creams, gels, ointments, on nanoparticles or glutinous rice-paper capsule, this will not only deliver the drug effectively to the site but also maintain its integrity (Borro et al., 2020; Thapa et al., 2020).

An emerging class of drug delivering system which involves the use of nanoparticle such as nanotubes, quantum dots, metal nanoparticles, liposomes have seen to enhance drug delivery of AMP's, help to maintain a minimal dose or concentration of the agent, and minimize undesired side effects (Magana et al., 2020).

7. Designing AMPs

Natural AMPs isolated directly from organisms have great applicative prospects but are limited to certain factors such as: 1) AMPs are highly susceptible to proteases and some are unstable and have limited application at certain temperature and pH. 2) AMPs seems to possess cytotoxic activity wherein they damage the cell membrane of eukaryotic cell and may cause hemolytic side effects in host, although this property can be utilized to for immunosuppression and as an anticancer agent it still limits its therapeutic potentials. 3) The antimicrobial activity of AMP is seen to be reduced in the presence of metal ions such as iron. 4) The cost of production and maintenance of conditions on a large scale is relatively high. (Li et al 2017)

In order to be a good therapeutic agents following characteristic must be seen in an Ideal AMP:

- Have relatively very low toxicity towards mammalian cells
- Should be stable under certain environmental condition and resist protease activity
- High antimicrobial activity (against broad range of organisms) with a low MIC
- Low serum binding ability
- High yield with low cost of maintenance and production

Therefore, designing an ideal AMP to achieve desired effect has attracted interest from many scientists worldwide. For designing an AMP, the following aspects should be considered: amino acid chain length, its structure, net charge on the peptide, its hydrophobicity (in order to interact with membrane) and amphiphilicity (Li et al 2017).

Studies and research have been done focusing on a method to search and predict a peptide sequence, its folding and its properties by the use of softwares and data modeling tools. These technologies are constantly changing based on the peptide of desired features. Some methods have been identified to conduct research on known AMPs.

7.1 Template-based design method

By comparing structurally homologous peptide fragments of naturally obtained Antimicrobial peptides and studying their properties such as charge, polarity, hydrophobicity etc. template sequences can be obtained (Zelezetsky and Tossi, 2006) based on this data, modification can

be made to improvise parameters like the tendency to form helical structure in solution, cationic charge to interact with negatively charged membrane, amphiphilicity and hydrophobicity to improvise existing peptide. cecropin, magainin, protegrin, lactoferrin are some of the example of AMP's used as templates (Fjell et al., 2012).

7. 2 Based on self-assembly of peptides

Many peptides have the ability to self-associate into nanostructures likes micelles, vesicles and nanostructures like nanotubes, nanoparticles, nanofibers etc. this self-associating property can be utilized to enhance the antimicrobial activity of the AMP. Seen in KLD-12 a 12 amino acid residue peptide that has the ability to self-assemble into a nanostructure, which is well studied for its tissue engineering properties (Tripathi et al., 2015).

7.3 Computer based designing

Computer based designing of AMP's includes statistical modeling, machine learning, study relation between structure and its activity on a virtual scale, deep learning etc. (Abdel Monaim et al., 2018). Genetic algorithms are used to design α-helical amphipathic AMP with uncommon amino acids such as guavalin 2 (Porto et al., 2018). Many novel AMP's are identified and their expressed genetic sequences and structural-activity relation information is stored as a database by forming digital libraries (Juretic et al., 2011).

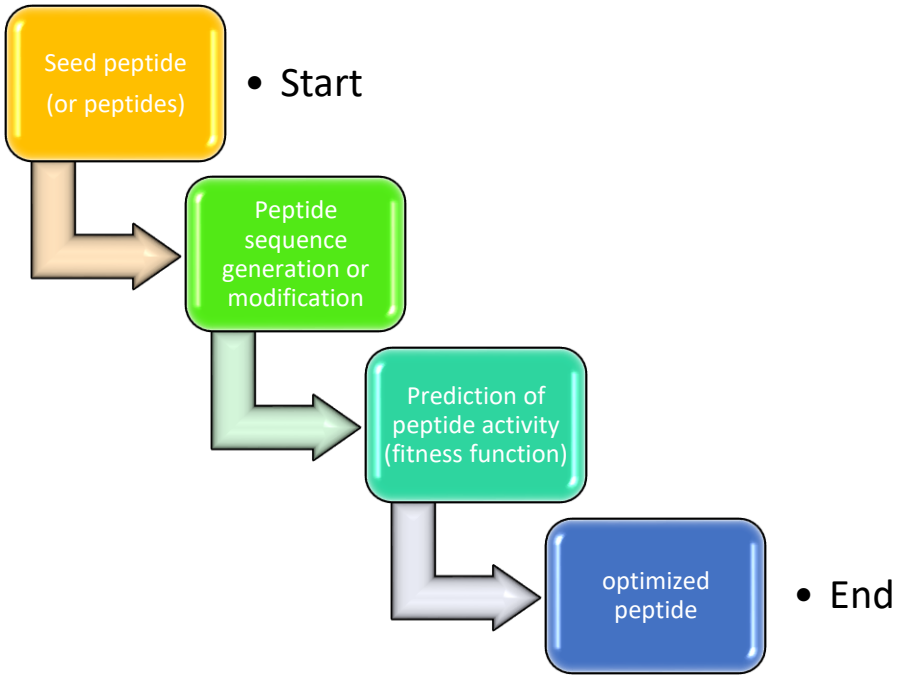

Fig. 9: Computer-assisted molecular design cycle.

7.4 Chemical modifications to AMPs

While designing AMPs, it is important to protect it from microbial proteases as it may disrupt structure and make it inoperable. AMPs can be protected from proteases and other forms of bacterial resistance by chemically modifying its structure in a way that it is immune to proteases and improving their stability (Zhong et al., 2020). Some examples of chemical modifications are as follows:

- Halogenation: is basically addition of halogen to the peptide chain can give more abilities to the AMP as well as give it resistance from microbial defenses, known examples: by introducing a halogen to jelleine-I, a short antimicrobial peptide isolated from jelly of *Apis mellifera* , the phenylalanine was replaced by halogenated phenylalanine in the peptide chain, exponentially increasing its stability against proteases by 100 times and also enhancing its antimicrobial and anti- biofilm activity (Jia et al., 2019).

- Cyclization: basically means forming a ring or cyclic structure of the molecules, there are many ways to cyclize AMP's, by including disulfide linkages, introducing internal bonds between side chain and by head to tail cyclisation, known example is arenicin-I, when

circularized increased its antimicrobial activity against many clinical drug-resistant isolates (Orlov et al., 2019).

- Addition of D-amino acid (stereoisomers) or unnatural amino acid to the peptide. It is effective against many stereospecific enzymes as incorporation of unnatural D-amino into the Amps will make unsusceptible against these enzymes and prevent from proteolysis, as most enzymes are stereospecific in nature. (Zhong et al., 2020).

Other modifications include amidation, acetylation, addition of non-amino acids that mimic amino acids to the peptide chain like Ornine, peptidomimetics etc. (Patch and Barron, 2002).

Examples of *De novo* AMP designs

In recent years a lot of amphiphilic AMP designs having been researched upon, a well-known example is GALA, an α-helical amphipathic AMP under mildly acidic pH, it is well studied for its pH-controlled membrane permeability and drug delivery mechanism (Li et al., 2004; Goormaghtigh et al., 1991). $L_1K_mW_2$ is another example of *De novo* AMP, has shown an excellent antibacterial activity against Methicillin-resistant *Staphylococcus aureus* (MRSA) (Lee et al., 2011).

8. Conclusion

Development of new antibiotics has slowed in recent years and growing microbial resistance to the existing ones is a matter of concern, making it a priority to develop a novel, safe and effective treatment. The past few years have seen an important progress in identifying novel antimicrobial agent in the form of antimicrobial peptides, their diverse nature, broad range of antimicrobial activity and an ease to change/ modify characteristic even with small modifications have made them an ideal candidate against rapidly adapting microorganisms. However, AMPs are still hindered by many challenges including low specificity, higher manufacturing cost, Instability to extreme conditions and enzymes, potential toxicity to mammalian cell and lack of robust guidelines for rational designs.

Some of these concerns can be easily countered by synthetic modification. Computational approach for further research in order to study and modify its physiochemical characteristics as well as its target spectrum can greatly enhance AMP's pharmacodynamics and pharmacokinetic properties, making it a vital therapeutic agent to treat human pathogenic infections. Since AMP's have the ability to directly attack the microbial cell membrane giving it no room to acquire resistance quickly, it may have potential in controlling persistent cells, activity against biofilms and broad application with respect to bio-preservation and agriculture (bio-pesticides), which makes it an attractive topic for future study.

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