Antimicrobial Peptides: Their Role in Innate Immune System and Usage in Future Drug Development

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Abstract
Antimicrobial peptides naturally play a role in the innate immune system of any living organisms. These small molecules are known as "ancient weapon" and also recognized as natural antibiotics. Antimicrobial peptides have distinct characteristics including their unique structures which are strongly correlated to their antimicrobial activities. Recently the development of antimicrobial peptides as future antimicrobial agents has become a major issue in the pharmaceutical business. This article will discuss the natural role of antimicrobial peptides in host defence system and the relationship of their structure with antimicrobial activity. This article will also discuss the challenge in developing antimicrobial peptides as future antimicrobial agents.
Key words: antimicrobial peptide, drug development, antimicrobial activity

Introduction

Over the last few decades, the world has been in war with microbial resistance. This phenomenon has caused several problems in health sector such as prolonged illnesses and increased mortality of patients which result in increased treatment cost. The progress of antimicrobial resistance is a part of natural process in microorganisms, and is accelerated by external selective pressures exerted by use and misuse of antimicrobial agents in humans and animals. On the other hand, the development of new antimicrobial agents to replace the existing ineffective drugs has showed a decelerated progress(WHO, 2014).

To overcome the microbial resistance, WHO has encouraged the global commitments to improve the finding and develop the new antimicrobial agents from any available sources(WHO 2015). One of the promising new antimicrobial agents is antimicrobial peptides. Antimicrobial peptides play an important role in innate immunity of host defence system and are defined as “ancient weapon”. These molecules mostly expressed in invertebrates since these organism do not produce specific antibodies and completely rely on the innate immunity mechanism(Cho et al, 1998).To date, many studies have explored the antimicrobial peptides including their structures and modes of actions which initiated the development of AMPs in pharmaceutical industries. This paper will bring the closer look on AMPs, including their natural behaviour in innate immune system, their unique structures and antimicrobial activities, their promising features as antimicrobial agents and also challenges in future drug development.

AMPs Action in Innate Immunity

The initial contact of host and any fastidious microorganism usually occurs at the inner or outer surface of the body and that is where the infection primarily takes place.Innate immunity as the first line of defence acts within a few hours after microbial exposure to a mucosal surface (Marshall, 2003).In both plants and animals, innate immunity is triggered by the recognition of microbe-associated molecular patterns (or pathogen-associated molecular patterns) by the pattern recognition receptors. This recognition is then followed by the local and systemic responses which are very specific to different taxa. However the expression of AMPs after infections of microbes in innate immunity is conserved in all types of organisms (Zasloff, 2002). Several effects of AMPs are enhancing phagocytosis, stimulating the prostaglandin release, neutralizing the septic effect of Lipopolysaccharides (LPS) and promoting recruitment and accumulation of various immune cells at inflammatory sites (Sánchez et al, 2013). Other effects of AMPs which namely as immunomodulatoryeffects are inducing the proliferation of fibroblast and epithelial cells in the airway (airway remodelling process), inducing the phenotypic and functional changes in dendritic cells and inhibiting pro-inflammatory cytokines and molecules, such as TNF-α, nitrite-oxide and tissue factors (Guaní-Guerra et al., 2010).

Antimicrobial peptides in animals are produced by the epithelial cells due to the direct contact with the environment and can be secreted to the circulation (through blood stream or lymphoid drainage). This circulation delivers the AMPs to the site of infections. In plants, AMPs are probably not circulated,

butthey areexpressed constitutively in specific sensitive organs or induced by local and systemic microbe infections (Sels et al., 2008).Recent studies have identified a large number of AMP-like genes in eukaryotic organisms. These findings emphasize the importance of AMPs in eukaryotic immune system, particularly in plants that do not have acquired immunity (Silverstein et al. 2007).

There are several AMPs produced in mammalian cells, such as defensins and cathelicidins. Most of AMPs in intestines are produced by paneth cells, including alpha and beta defensins. The expression of certain peptides encoded by AMP genes influences the tissue's susceptibility to infections from several pathogenic bacteria. Cathelicidins protects mice skin from *streptococcus* and pulmonary tissue from *Pseudomonas aeruginosa*. These examples indicate that the effects of AMPs in mammals are tissue-specific and strongly correlated to the expression pattern of AMP genes (Maróti et al., 2011).

Structure-Antimicrobial Activity Relationship of AMPs
Classically, all peptides can adopt the membrane-bound amphipathic conformation although their lengths, amino acids composition and secondary structures are extremely varied. Recent studies demonstrate that AMPs have multiple mechanisms of antimicrobial activities. In order to design the rational antimicrobial agents, it is very important to comprehend the structure-activity relationship of AMPs (Nguyen et al., 2011).

There are two common physiochemical features of AMPs; a cationic charge and a substantial proportion of hydrophobic amino acids. Cationic charge promotes selectivity for negatively charged microbial cytoplasmic membranes over zwitterionic mammalian membranes. Hydrophobic features facilitate the interaction with fatty acyl chain (Lohner & Prenner, 1999). There are also anionic AMPs more likely to demonstrate other biological activities and more toxic to mammalian cells (Harris et al., 2009).

The most common antimicrobial activity of AMPs is the disruption of bacterial cytoplasmic membranes. The event starts when bacterial cytoplasmic membranes come in contact with AMPs through absorption process. This initial event is followed by several theoretical disruption mechanisms such as pore forming, carpet model, barrel stave, electroporation, non-lytic membrane depolarization, disordered toroidal pore, membrane thinning/thickening, charged lipid clustering, non-bilayer intermediate, anion carrier and oxidized lipid targeting (Giuliani et al., 2007; Nguyen et al., 2011). To date, there are other cellular targeting mechanisms that have been identified, such as disruption of DNA and protein synthesis, protein folding, enzymatic reactions and cell wall synthesis (Nikolas, 2009).There are three major structures of AMPs namely α-helical AMPs, β-sheet AMPs and extended AMPs. The relationship between each structure and the antimicrobial activity will be discussed below:

α-helical AMPs
α-helical AMPs form a membrane-bound α helices containing large hydrophobic surfaces which are correlated to cytotoxic effects to mammalian cells. AMPs with low toxicity can be formed in a membranous environment to induce the proper folding. Most of α-helical AMPs with low cytotoxicity often have proline or glycine induced kink in the middle of α-helix formation. The peptides can lie parallel to the membrane plane during the initial interaction with the charged side facing outward toward the phospholipid head groups and the hydrophobic sites inserted into the acyl tail core, which represent the amphipaticity along the axis of α-helix. The α-helix tail is a very important structure which influences the depth of insertion to membrane, thus also determining the antimicrobial activity (Nguyen et al. 2011). Amphipathic helical conformation influences the antimicrobial activity. In the study of magainin, the replacement of several amino acids in AMPs by their D-isomer resulting in the loss in microbe-killing activities (Epand & Vogel, 1999).

During the insertion, the AMPs undergo partial unfolding; therefore the α-helix structure is not always maintained. There are a number of polar or charged amino acids in the hydrophobic face of AMPs which pull the lipid head groups into the membrane interior, resulting in pore formation(Nguyen et al., 2011). The pore formation is a cooperative process; therefore, the membrane integrity is temporarily ruptured. Membrane-bound peptide threshold level plays a critical role in the microbial membrane disruption. Once it is reached, the membrane will solubilize in detergent-like manner(Nicolas, 2009).

Another membrane disruption mechanism of α-helix AMPs is lipid segregation. The ionic lipid in bacterial membranes will form aggregation thus resulting in slow leakage of intracellular components and membrane depolarization(Fleming et al., 2008). The release of ROS products during phagocytosis will increase the lipid oxidation. Oxidized lipids become the target of α-helical AMPs, such as temporinB and L, in order to intercalatemore efficiently to the membranes(Mattila et al., 2008). In

certain AMPs, membrane disruption mechanism is pH-dependent (Epand & Epand, 2009). Despite the membrane perturbation, several α-helical AMPs obtain the intracellular killing mechanism. A study in AMPs Buforin II shows that the AMPs accumulate in the cytoplasm after translocation and interact with the nucleic acids (Lan et al., 2010).

β-sheet AMPs
The β-sheet AMPs include several β-hairpin peptides in addition to the defensin mini-proteins. This structure forms oligomerictransmembrane β-barrel in anionic membranes and β-sheet aggregates in cholesterol-containing membranes. Many of these AMPs kill microbes by membrane toroidal pore forming. Cathepsin, one of β-sheet AMPs, is able to disturb the DNA-protein interaction, whereas β-hairpin AMP bovine lactoferin acts synergically with other AMPs in affecting transmembrane potential and proton-motive force which results in inhibition of ATP-dependent multi-drug efflux pump. Bovine lactoferininhibites DNA and protein synthesis during its presence in cytoplasm after translocation process. Inhibition of cell wall synthesis in *Staphylococcus* is conducted by AMP human defensins(Nguyen et al., 2011).

The β sheet and β barrel protein conformation is often stabilized by disulphide bonds between Cystein residues (Cézard et al, 2011). This covalent bonds do not alter the antimicrobial activity. The rearrangement of Cystein residues in AMP chain does not abolish the antimicrobial activity nor the secondary structures. Although antimicrobial activity may be unaffected, the analogue of AMPs without disulphide linkage shows the lower chemotactic activity (Nguyen et al., 2011).

A study on garamicin S shows that the peptides with high amphipathicity perform high haemolytic activity but low antimicrobial activity. Low-amphipathicity peptides, at some point, can pass the threshold and perform the higher antimicrobial activity. This study suggests that the ionic interaction between positive charges on peptides and negative charges on microbial membranes is very fundamental to antimicrobial activity (Midura-Noeaczek & Markowska, 2014).

Extended AMPs
Extended AMPs do not fold into regular secondary structures. The sequence is rich by one or two amino acids, such as Arginin, Prolin or Tryptophan (Epand & Vogel, 1999). In insect-derived Prolin-rich AMPs, drosocins and apidaecins, the antimicrobial activity can be performed by penetration to membranes and interaction with the intracellular properties, resulting in inhibition of DnaK ATPase activity and Chaperone assisted protein folding (Brogden & Brogden, 2011). Other extended AMPs act by stopping the replication fork, thus preventing recombination and DNA repair (Nguyen et al., 2011).

The Proline and Arginin-rich composition in porcine PR-39 plays an important role in bacteria killing mechanism through its interaction with cytoplasmic proteins. Indolicins is an AMP analogue which successfully shows its antimicrobial activities such as activating the phospholipaseA which hydrolyses the anionic membranes, disrupting membranes through lipid segregation of oxidized phospholipids, acting as small anion carriers across the membranes and interfering DNA binding Enzymes in *Escherichia coli* (Marchand et al., 2006).

Challenges in Future Drug Development
AMPs have a number of desirable features as novel antimicrobial agents or as a complement to conventional antibiotic therapy (Giuliani et al., 2007). They are ubiquitous in nature, typically show a rapid and broad spectrum antimicrobial activity against pathogenic bacteria and fungi. They are also able to destroy enveloped viruses, parasites and even cancerous cells (Nguyen et al., 2011). In accordance with theirnatural roles in innate immunity, AMPs also exhibit immunomodulatory effects (Guaní-Guerra et al., 2010). These features are the underlying idea for the development of AMPs as antimicrobial agents. Another interesting feature of AMPs are their ability to reduce biofilm and persister cells (Chen et al., 2011), thus expanding the use of AMPs as the antimicrobial coating agents for medical devices (Onaizi & Leong, 2011).

The most important feature of AMPs, especially eukaryotic AMPs, as future antimicrobial agents is the low susceptibility to microbial resistance due to gene mutation. Many eukaryotic AMPs perform more than one killing mechanism. They can act on the microbial membranes and other generalized targets whereas the conventional antibiotics act only in specific protein targets (Peschel & Sahl, 2006). A study by Fedders et al reveals that Ci-MAM-A24, the synthetic AMP deriving from the immune cells of a marine invertebrate, *Cionaintestinalis,* shows significant antimicrobial activity against multidrug resistant bacteria and pathogenic anaerobe bacteria in human (Fedders et al., 2010). The bacterial resistance mechanisms to AMPs have been identified and reported, but the number is considerably

lower than the resistance to conventional antibiotics (Peschel & Sahl, 2006).Despite their desirable features, AMPs have several shortcomings which can limit their characteristics in clinical applications, such as haemolytic activity, rapid turnover in the human body, reduced activity due to salt sensitivity, high cost of production and broad spectrum antimicrobial activity (Aoki & Ueda, 2013).

Haemolitic activity
The haemolytic activity can be measured by a therapeutic index, which represents the ratio of antimicrobial activity to haemolytic activity. The high therapeutic index of AMPs is essential for host cells to avoid the haemolysis. In most AMPs, their haemolytic activity is equal to antimicrobial activity (Lee et al., 2011). The increase of hydrophobicity leads to a more effective non-specific interaction with membranes of both of bacteria and erythrocytes (Findlay et al., 2012). Amphiphilicity also contributes to higher haemolytic activity. That is why changes in hydrophobicity and amphiphilicity can be considered in order to minimize the undesirable effects (Aoki & Ueda, 2013). Haemolytic activity can be avoided by using non-haemolytic seeds of AMPs (Aoki & Ueda 2013), such as Lumbricin-1, annelids-derived AMPs (Cho et al. 1998). A study by Strandberg et al concludes that the haemolytic activity can be reduced while maintaining the antimicrobial activity by conducting C-terminal deamidation of AMPs (Strandberg et al., 2007).

Rapid turnover in the human body
Rapid turnover in the human body is correlated with the protease activity which rapidly degrades the AMPs. The stability against protease is very essential for AMPs in clinical use. There are several strategies to overcome this stability issue namely D-isomerization, incorporation of chemical compounds, cyclization and peptidomimetic. D-isomerization turns to be the best strategy to obtain the best proteolytic stability (Aoki & Ueda, 2013), even though it is not cost-effective and cannot be used in certain AMPs (chirality-dependent AMPs). End capping shows that C-terminal deamidation provides lower proteolytic stability and higher antimicrobial activity meanwhile the N-terminal deamidation exerts higher stability and lower microbes killing ability. Peptide cyclization is able to maintain both proteolytic stability and antimicrobial activity (Nguyen et al., 2010). Incorporation of chemical compounds obtains steric occlusion which covers the protease active site on peptide sequence, thus resulting in resistance to protease degradation. (Matsuzaki 2009).The construction of peptide-mimetic, the polymer that mimics the complex structure of AMPs, shows an improvement in protease stability.The polymers do not have protease active site so they exhibit high stability against protease (Berhanu et al., 2012).

Reduced activity due to salt sensitivity
In order to form secondary structures, AMPs need electrostatic interaction with microbial membranes. This stage is salt-sensitive. The human body fluids contain high salt concentration which can deactivate the AMPs. Designing the salt-insensitive AMPs will produce the more stable secondary structures. The use of helix-capping motifs both in C-terminal and N-terminal of the peptides maintains the antimicrobial activity and obtains more stable helical AMPs in high concentration of NaCl compared to unmodified AMPs(Yu et al., 2011).

High cost of production
One of the major concerns in pharmaceutical industries in developing AMPs as novel antimicrobial agents is the high production cost. It is higher than production cost of conventional antibiotics. Another problem comes from the difficulties to produce heterologous AMPs in prokaryotic system since the AMPs are toxic to prokaryotic cells. The strategies to abolish the toxicity in the host cells and to increase the amount of AMPs are very essential to reduce the cost of production (Aoki & Ueda, 2013). Fusion expression using solubility-enhancing carriers obtains the high yield of AMPs. The use of thioredoxin(Bogomolovas et al., 2009) and Small Ubiquitous-related Modifier (SUMO) has produced non-toxic AMPs (Aoki & Ueda, 2013).

Broad spectrum antimicrobial activity
The human body has a symbiotic relationship with microbiota. The development of AMPs as antimicrobial agents must consider the presence of this important mucosal surface component so that the AMPs can kills the pathogenic microbes while sustaining the commensal microbiota(Aoki & Ueda, 2013). Therefore, it is very essential to construct AMPs which develop selectivity only to pathogenic microbes. Specifically targeted antimicrobial peptide (STAMP) technology has been introduced and generated a promising approach. STAMP technology is the construction of fusion peptide consisting of two functional independent domains (targeting domain and killing domain) using short flexible linker. The targeting domain is responsible for the interaction with membrane hydrophobicity charge, pheromone receptors, cell wall attributes and other virulence factors which are the specific

determinants on pathogen surface (Eckert et al., 2006). Selective killing mechanism can be accomplished by using environment sensing. This method is based on the environmental changes due to overgrowth and metabolic activities. One of the environmental indicators already studied is pH. The acid-activated AMPs can kill the pathogenic microbes such as *Candida albicans*in a low-pH environment (Aoki & Ueda, 2013). Another method to establish the selectivity is using protease-activated AMPs. This method develops protease as the target since the pathogenic microbes possess the specific proteases with substrate specificity characteristic (Aoki & Ueda, 2013).

Conclusion

Antimicrobial peptides have caught the attention of the research world and pharmaceutical business. As part of innate immunity, these molecules show interesting features related to the extermination of pathogenic microbes and possession of the immunomodulatory effects. AMP features are strongly correlated to their unique structures, such as α-helical, β-sheet and extended AMPs. Understanding the way the structures influence the activity is very important to facilitate the rational design of novel antimicrobial agents. Despite their desirable features, AMPs exhibit limitation related to their clinical application such as haemolytic activity, rapid turnover in the human body, salt sensitivity, high cost production, and broad spectrum antimicrobial activity harmful to commensal microbiota. These problems are challenges that need to be overcome. To date, there are a large number of studies conducted to modify the AMPs. These studies share the same objective that is to develop the AMPs as novel antimicrobial agents by eliminating the limitation and improving the desirable features.

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