Introduction

Prologue, Objectives and Rationale

Background information

In general, the living organisms are continuously exposed to potentially harmful pathogen through contact, ingestion and inhalation (1, 2). The endurance of such organisms in microbe-thriving surroundings depends mainly on self defence system i.e. immune system and also on external agent i.e. antibiotics (3). However, the current scenarios of developing resistant microbial strains against present antibiotic drugs endorse the development of new antimicrobial molecules, which can circumvent the emergence of resistant microbes. Antimicrobial peptides are considered as potential promising therapeutic candidates, since these peptides are important component of natural defense system of most living organisms and their mode of action often destroys the integrity of microbes. Though, in many cases the peptides possess multiple targets within the target microbial cells (4), the likelihood of emergence of resistance is thought to be considerably reduced as compared with that for many current antibiotics, which have more specific molecular targets. In order to develop resistance particularly against 'destroying integrity' action of antimicrobial peptides, microbes will need the transformation of membrane compositions, which is metabolically very costly for cellular system. Therefore, we can say that antimicrobial peptides could be a probable alternative antimicrobial molecule of future.

Antimicrobial peptides are generally classified into four structural classes which are α-helical, β-strand with disulphide bond i.e. having β-sheet, loop structured and extended peptides (5, 6). Most of the antimicrobial peptides adopt random structure in aqueous environment but adopt certain structural conformations when come in close proximity of hydrophobic milieu like phospholipid membrane or organic solvent, Trifloroethanol (TFE). Hitherto, and present literature reveal that, regardless of their actual target of action, all antibacterial cationic peptides must interact with the bacterial cytoplasmic membrane (4). It suggested that their early contact with the bacterial membranes happen via electrostatic interaction which is called self-promoted uptake and in the next phase they disrupt the organization of the membrane by carpet or barrel stave, or toroidal mode of pore formation, when primarily membrane damaging (7, 8). While, those peptides exert their effect on intracellular target, traverse plasma membrane by either of two proposed mechanism viz. spontaneous lipid-associated membrane translocation and other one is lipid phase boundary defect mechanism (9, 10). Despite tremendous work done to elucidate the mechanism of action, the exact mode of action of these antimicrobial peptides are yet to be work out.

Antimicrobial peptides exhibit broad spectrum of activity; for examples these peptides exhibit bactericidal (Gram +ve and Gram –ve both), fungicidal (11), anti-parasite (12), virucidal (13) and also tumoricidal activities (14). The versatile activity of antimicrobial peptides makes them attractive candidates for potential therapeutic application. However, major hurdles to convert these antimicrobial peptides into anti-infective drugs are their undesired toxicity, weaker stability and high production cost. The stability issue is being resolved subtly, by incorporation of non-natural amino acids or non-peptidic backbone or by formulation (e.g. liposome entrapment) etc, and high production cost issue through recombinant technology, but at commercial level this has not proven feasible to date (15-17). The cytotoxicity is one of major concern in antimicrobial peptide research field. In order to circumvent the cytotoxicity of AMPs or enhancing their cell selectivity extensive studies have been made by modulating different parameters like, charge, hydrophobicity etc (18). However, it has not been very much cleared that what could exactly endow cell selectivity to these peptides. In present study, we have also tried to investigate the role of structural parameter in cell selectivity, by designing de novo or analogues of natural antimicrobial peptides.

Objectives of the present work

A. To design and synthesis of novel antimicrobial peptides with modulated toxicity or novel analogues of naturally occurring antimicrobial peptides with reduced toxicity.

B. To examine the lytic activity of these peptides against different microorganisms and cytotoxic activity against human red blood cells and other normal human or mammalian cells.

C. To understand the basis of biological activity of the peptides under investigation by studying their interaction with phospholipid membrane as well as with different cell types by biophysical and cell biological techniques.

D. To determine the role of secondary structures of these selected anti-microbial peptides and their analogues in their anti-microbial and cytotoxic activities.

E. To determine the role of oligomerization of these peptides in their anti-microbial and cytotoxic activity.

Rationale of the present work

A major hurdle to convert antimicrobial peptides into potential and promising therapeutic candidates is toxicity or lack of defined cell-selectivity. To convert these molecules into future antibiotics, researchers need to find out the parameter which control cytotoxicity of these peptides. In literature, structure-function relationship has provided great insight regarding structural parameters and cell selectivity. In the present study, we have characterized the importance of heptadic sequences of leucine (leucine zipper) and other different hydrophobic amino acids on cytotoxicity and antimicrobial activity in de novo designed and naturally occurring antimicrobial peptides. Characteristically, a leucine zipper motif comprises the repeat of seven amino acids, and every 7^{th} amino acid is typically leucine, which is called as 'a' position and residues in between are designated as 'b' 'c' 'd' 'e' 'f' and 'g', and in many cases the 'd' position is too occupied by leucine or isoleucine residues. Firstly this motif recognized in DNA binding protein GCN4 and later in membrane associated viral fusion protein and many other structural and functional proteins (19-21). In antimicrobial peptides, firstly Asthana *et. al* recognized this motif in bee venom peptide Melittin. They observed that the selective substitution of 'a' position leucine with alanine drastically reduces the cytotoxicity of Melittin (22). Later on, Ahmad *et. al.* have demonstrated its importance in totally novel antimicrobial peptides, designed on the basis of leucine zipper sequence (23)._Furthermore, significance of this motif in cytotoxicity has been validated in other naturally occurring antimicrobial peptides, BMAP-27 and BMAP-28 (24, 25). Recently, Pandey *et. al.* have demonstrated that just by mere introduction of a leucine zipper like motif in an appreciably less-toxic antimicrobial peptide, magainin-2, with minor rearrangement of its sequence of hydrophobic amino acids and without changing its amino acid composition can enhance toxicity of the peptide significantly (26).

Encouraged from the above observations, we have designed short 15-residue peptides by incorporating leucine, phenyalanine, valine and alanine at all the 'a' and'd' positions of the heptads named as LRP, FRP, VRP and ARP respectively._It is to be mentioned that leucine, phenylalanine and valine have comparable hydrophobicity while alanine possesses lower hydrophobicity than the other three amino acids. Though leucine, phenylalanine and valine have similar hydrophobicity values they differ in participating helical assembly of the peptide molecules (27). Therefore, the purpose of the present design was to investigate how the amino acids with varying hydrophobicity at these 'a' and 'd' positions influence the bactericidal and cytotoxic activity of the peptides. Besides,

it was addressed whether similar hydrophobicity at these positions yields the similar microbicidal and cytotoxicity to these novel antimicrobial peptides. Detailed studies on these peptides have been described in Chapter-4. Many antimicrobial peptides exhibit immunomodulatory properties. Therefore, we examined whether any of these designed peptides can neutralize LPS-induced pro-inflammatory response in macrophage cells. Results of this study have been described in chapter-5.

Melittin: The major toxic component of European honey bee (*Apis mellifera*) venom (28). Along with very good antimicrobial activity melittin is a prominent cytolytic antimicrobial peptide (29, 30). There have been great efforts made by several research groups to design analogues with modulated toxicity. Firstly, Blonde *et al* designed by deletion of some amino acids, later on by substitution of few amino acids with their D-isomer (29, 31) or by hybridising (consist of melittin and cercopinA) (32) or by truncation. Later on, asthana *et al* recognized leucine zipper sequence within it, and found that it plays crucial role in maintaining haemolytic activity by substitution of heptadic leucine with alanine (22). It was of interest to study the effect of substitution of hydrophobic leucine residues at the 'a' and 'd' positions of leucine zipper sequence of melittin by similarly hydrophobic valine residues towards the properties of melittin. The detailed characterization of this analogue along with the parent molecule melittin has been described in chapter-6.

BMAP-28, bovine neutrophile derived potent antimicrobial peptide with high toxicity against mammalian cells. Earlier, Zeniti *et. al.* have reported that the C-terminus (upto nine residues from C-terminus) of this peptide solely responsible for cytotoxicity over mammalian cells (33), and this was validated by designing truncated analogue of 19 residues (33). In order to get further insight in the structure-function relationships of BMAP-28 an analogue of BMAP-28 was designed by exchange of proline at 19^{th} position with isoleucine at 20^{th} position. The impact of interchange of the position of proline with adjacent isoleucine in the biological, functional and structural properties of BMAP-28 was studied in detail and described in chapter-7.