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Biochimica et Biophysica Acta 1758 (2006) 1184–1202

BIOCHIMICA ET BIOPHYSICA ACTA

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Review

Tryptophan- and arginine-rich antimicrobial peptides: Structures and mechanisms of action

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Received 23 January 2006; received in revised form 3 April 2006; accepted 7 April 2006
Available online 21 April 2006

Abstract

Antimicrobial peptides encompass a number of different classes, including those that are rich in a particular amino acid. An important subset are peptides rich in Arg and Trp residues, such as indolicidin and tritrpticin, that have broad and potent antimicrobial activity. The importance of these two amino acids for antimicrobial activity was highlighted through the screening of a complete combinatorial library of hexapeptides. These residues possess some crucial chemical properties that make them suitable components of antimicrobial peptides. Trp has a distinct preference for the interfacial region of lipid bilayers, while Arg residues endow the peptides with cationic charges and hydrogen bonding properties necessary for interaction with the abundant anionic components of bacterial membranes. In combination, these two residues are capable of participating in cation–π interactions, thereby facilitating enhanced peptide–membrane interactions. Trp sidechains are also implicated in peptide and protein folding in aqueous solution, where they contribute by maintaining native and nonnative hydrophobic contacts. This has been observed for the antimicrobial peptide from human lactoferrin, possibly restraining the peptide structure in a suitable conformation to interact with the bacterial membrane. These unique properties make the Arg- and Trp-rich antimicrobial peptides highly active even at very short peptide lengths. Moreover, they lead to structures for membrane-mimetic bound peptides that go far beyond regular α-helices and β-sheet structures. In this review, the structures of a number of different Trp- and Arg-rich antimicrobial peptides are examined and some of the major mechanistic studies are presented.

Keywords: Antimicrobial peptide; Structure; Tryptophan; Arginine; Cation–π interaction

Contents

Abbreviations: BLM, black lipid membranes; CD, circular dichroism; cLZ, human c-type lysozyme; combi-1 (or-2), combinatorial peptide-1 (or-2); CPP, cell-permeable peptide; DiPoPE, dipalmitoleoyl PE; DOPC (or DOPE or DOPG), 1,2-dioleoyl-*sn*-glycero-3-PC (or PE, PG); DPC, dodecylphosphocholine; DPPC (or DPPE or DPPG), 1,2-dipalmitoyl-*sn*-glycero-3-PC (or PE or PG); DSC, differential scanning calorimetry; HEWL, hen egg white lysozyme; hLZ, human lysozyme; IC_{50}, inhibitory concentration at which 50% inhibition is achieved; ITC, isothermal titration calorimetry; Lfcin, lactoferricin; LfcinB, bovine Lfcin; LPS, lipopolysaccharide; LUV, large unilamellar vesicle; LysC, HEWL peptide, residues 98–112 in HEWL; LysH, residues 107–113 in hLZ; MD, molecular dynamics; MIC, minimal inhibitory concentration; MIP-3α, macrophage inflammatory protein-3α; MRSA, methicillin resistant *Staphylococcus aureus*; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PDB, Protein Data Bank; PIN-a (or-b), puroindoline A (or B); puroA (or B), puroindoline A (or B) peptide; SDS, sodium dodecyl sulfate; SMH, Shai–Matsuzaki–Huang; VRE, vancomycin resistant enterococci

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doi:10.1016/j.bbamem.2006.04.006

1. Introduction

Evolution and natural selection have endowed all organisms with a vast array of tools for survival. This includes ways to take advantage of the environment around us, as well as defensive mechanisms to prevent organisms from exploiting us. One of the systems present as a first line of defence in the innate immune system are antimicrobial peptides. These small and diverse peptides were initially isolated in the 1980s, from frogs and insects and in the former example, have been found to play a vital role in their survival in bacteria infested swamps [1,2]. Since then, a large number of additional antimicrobial peptides have been found virtually everywhere in nature and there are presently over 800 that have been identified (see http://www.aps.unmc.edu/AP/main.html). Several of these peptides possess functions that cover a broad range of activities. Initially it was observed that antimicrobial peptides are bactericidal but in the meantime it has been established that many of them can also possess antiviral [3], antifungal [4,5], antitumor [6], and immunomodulatory [7,8] activities. In fact, it has been observed that a single antimicrobial peptide can exhibit all of these functions [9]. One example of immunomodulatory peptides are the β-defensins, which are part of both the innate and adaptive immune response. They display direct antimicrobial activity, but they can also bind to chemokine receptors, stimulating dendritic cells and T cells [10]. This has led to the discovery that proteins involved in the immune response are indeed antimicrobial as well. The human chemokine MIP-3α has higher antimicrobial activities against both Gram-negative and Gram-positive bacteria than the β-defensins that bind to the same chemokine receptor [11]. Clearly, antimicrobial peptides and antimicrobial proteins are more common than originally suspected and come from an abundance of sources.

Ever since their discovery, researchers have been hopeful that antimicrobial peptides will help solve the problem of bacteria becoming resistant towards common antibiotics at an alarming rate. Vancomycin resistant enterococci (VRE) and methicillin resistant *Staphylococcus aureus* (MRSA) are occurring with increasing frequency and already are of great concern in hospitals. In the last 25 years, MRSA incidence has risen by more than 10 times, while VRE has done the same within the last 15 years (source: Center for Disease Control, 2005). Many of today's antibiotics are rendered ineffective because they perform their function on a very specific part of the bacteria. As a result, minute changes to the antibiotic's target are sufficient to prevent the drug from being effective. For example, a single amino acid mutation in the S12 ribosomal subunit of *M. tuberculosis* results in resistance against streptomycin [12]. Consequently, it has never been more crucial than now for new antimicrobial agents to be discovered. That is a problem in itself, though, as the number of newly introduced antibiotics has been declining steadily, from 16 new compounds during the period of 1983–1987 to only 7 between 1998 and 2002 [13]. Antimicrobial peptides may one day be able to reverse some of these trends. Considering that they have been used successfully in nature for millions of years and are still highly effective today, they hold great promise. Furthermore, because many of them have a broad range of effectiveness and perform their action on the entire cellular membrane, it is believed bacteria will only develop resistance with great difficulty. Although some mechanisms of resistance have already been described, they either involve a major change in membrane composition to alter the charge and therefore interfere with peptide–target association or they are highly specific mechanisms that are not applicable to a large number of antimicrobial peptides [14]. Thus, it remains likely that antimicrobial peptides will be able to contribute as therapeutic agents. A number of studies have shown that antimicrobial peptides act in synergy with today's commonly used antibiotics, and do so against multi-drug resistant bacteria [15,16]. Therefore, even if the peptides cannot be used on their own, they can be administered in combination with today's antibiotics and improve their potency. Another intriguing aspect about antimicrobial peptides is the fact that some of them are derived from food proteins [17]. Because they are already part of our natural diet and known to be tolerated well, this alleviates the question of toxicity to some extent. Despite these beneficial aspects, there remain a number of problems that must still be addressed. Chemical synthesis of peptides leads to high production costs, which is compounded by the fact that linear peptides are often easily proteolytically degraded [18]. In addition, bacterial killing by antimicrobial peptides has been shown to be able to release endotoxin from the bacteria, which can lead to septic shock [19]. Nevertheless, some antimicrobial peptides such as polymyxin and gramicidin are already in use as topical agents today, proving that such obstacles may be overcome or circumvented and justifying the efforts currently under way to develop antimicrobial peptides for medicinal use.

Antimicrobial peptides have been proposed to achieve their bactericidal effect in a number of different fashions. In all cases, an initial interaction with the outer and/or inner membranes of bacteria is necessary. This first point of interaction between the peptides and their targets is often also the site of antimicrobial action, as they interfere with membrane function to cause bacterial cell death. Membrane disruption can occur by a number of different mechanisms. Certain models such as the barrel-stave, toroidal pore, and

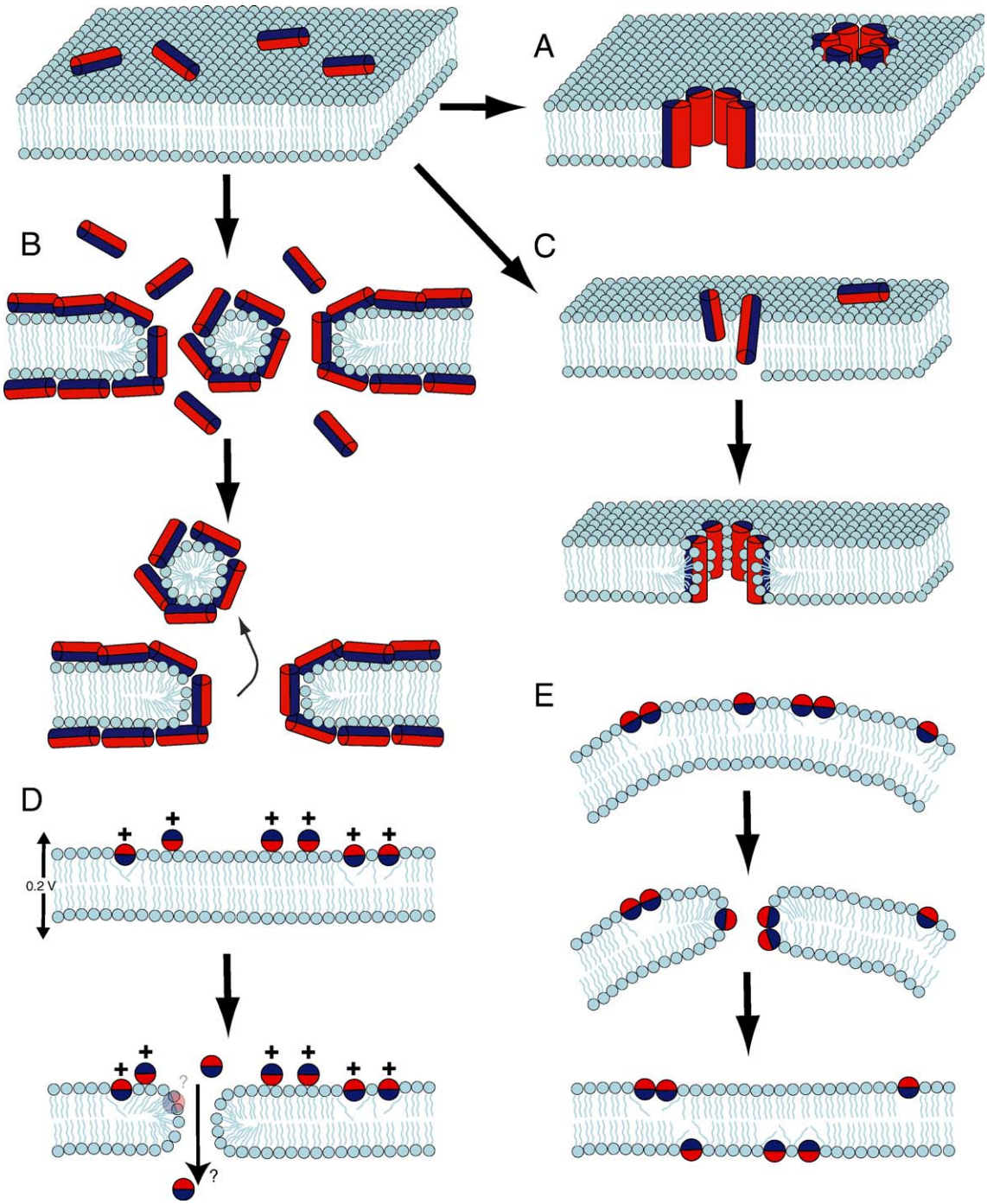

Fig. 1. Various models of antimicrobial peptide activity. A, B, and C all start from the same conformation, with the peptides associating with the bacterial membrane (top left). The red part of the peptide represents a hydrophilic surface, while blue is hydrophobic. (A) In the barrel-stave model, the peptides span the membrane and form a pore lined with peptides. (B) The carpet model is characterized by the peptides lining up parallel to the membrane surface and forming a peptide carpet, as the name implies. This is followed by a detergent-like action induced by the peptides that causes pore formation. (C) The toroidal pore model creates pores that contain peptides as well as lipid molecules that are curved inwards towards the pore in a continuous fashion from the surface of the membrane. The lifetime of these pores is believed to vary. After transient pore formation, the peptides end up in both leaflets of the bilayer, which presents a mechanism of shuttling the peptides inside. Longer-lived toroidal pores may have a lethal effect similar to barrel-stave pores; i.e., they dissipate proton gradients, etc. (A, B, and C reproduced with permission from Nature Reviews Microbiology, [14], copyright 2005 Macmillan Magazines Ltd.). (D) In the molecular electroporation model the cationic peptides associate with the bacterial membrane and generate an electrical potential difference across the membrane. When the potential difference reaches 0.2 V, it is thought that pores will be generated through electroporation [20,134]. (E) The sinking raft model proposes that binding of the amphipathic peptides causes a mass imbalance and consequently, an increase in local membrane curvature. As the peptides self-associate, they sink into the membrane, creating transient pores which result in the peptides residing in both leaflets after their resolution. (Adapted with permission from [22]. Copyright 2005 American Chemical Society).

carpet models have received the most attention, however, other lesser known models such as the molecular electroporation [20] or the sinking raft model [21,22] may prove to be important to explain the mechanisms of antimicrobial peptides as well (Fig. 1). Not all antimicrobial peptides are thought to exert their major action on membranes. An increasing number of peptides are being described that act on intracellular targets in bacteria, inhibiting protein-, or cell-wall synthesis, interact with DNA or RNA or inhibit some sort of enzymatic activity [14]. Such a breadth of activity stems from a wide range of antimicrobial peptides that can be classified into seven different groups (see [23]). One of these classes encompasses peptides with an unusual composition of regular amino acids. Of particular interest to this review are peptides with a high content of Trp and Arg residues, as these residues represent a recurring theme among many antimicrobial peptides and almost always play important roles for activity [24,25]. Not only are Trp residues important in antimicrobial peptides, they play a crucial role in membrane spanning proteins as well, as Trp has a strong preference for the interfacial regions of lipid bilayers [26–28]. In addition, Trp residues are involved in protein folding, forming both native and nonnative hydrophobic contacts even in denatured proteins to ensure their proper folding [29]. Further testament to the significance of Arg and Trp residues is the fact that upon screening of a complete combinatorial library of hexapeptides, peptides rich in these two amino acids were found to possess the highest antimicrobial activities [30]. This review will focus on a selection of Trp- and Arg-rich antimicrobial peptides and examine studies addressing important findings for indolicidin, tritrpticin, puroA, combi-1 and combi-2, as well as the antimicrobial peptides derived from the food proteins lactoferrin and lysozyme.

2. Role of tryptophan and arginine residues

Arg and Trp residues are found in unusually high proportion in many antimicrobial peptides. Their roles have been investigated thoroughly but it is not entirely clear what specific properties they bring to antimicrobial peptides, apart from their positive charge and hydrophobic bulk, respectively. For example, Trp residues have been placed at many different and contrasting positions on hydrophobicity scales of amino acids [31]. In certain cases, Trp is considered hydrophobic due to its uncharged sidechain, while on the other hand, it is observed that Trp residues do not reside in the hydrocarbon region of lipid bilayers and consequently it is placed towards the more hydrophilic side of the scale. Another important factor is the extensive π–electron system of the aromatic indole sidechain that gives rise to a significant quadrupole moment (Fig. 2) [32]. A quadrupole moment can be envisioned as two dipole moments extending perpendicularly out of either side of the ring plane. That is, their positively charged tails reside close to the plane of the ring, while the negative charges make up the ends of the dumbbell-type shape that is formed (Fig. 2). Because the Trp sidechains do not have a dipole moment and no other obvious charges, it is often considered as essentially hydrophobic, when in fact it has a significant quadrupole moment that cannot be ignored. The π–electron system of Trp results in negatively charged clouds that can participate in cation–π interactions that occur in proteins between the negatively charged electron cloud of any aromatic residue and various cationic species, such as ions or sidechains of positively charged amino acids (Fig. 2) [32]. Cation–π interactions are important in proteins for substrate binding, catalysis, as well as ion channel activity [33]. For example, human chromodomains use cation–π interactions between Trp sidechains and methylated lysine residues for histone recognition [34,35]. An inspection of high resolution structures in the Protein Data Bank (PDB) shows that there is a large number of cation–π interactions [36]. Although many interactions take place between cationic amino acids and aromatic residues, the interactions between Arg and Trp are of particular interest to us. The cation–π interaction can take place in either a parallel or a perpendicular, T-shaped orientation. The structures in the PDB reveal that the parallel (i.e. stacked) arrangement between Arg and Trp residues is preferred and has been shown to be energetically favorable in aqueous solution (Fig. 2) [36]. Interestingly, in the stacked conformation, the Arg sidechain

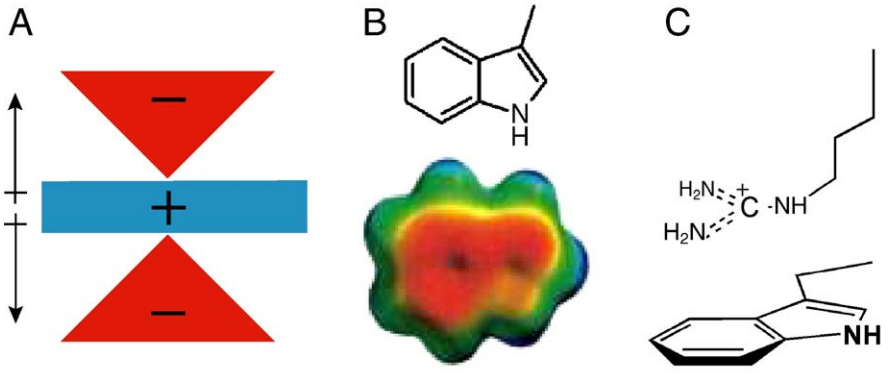

Fig. 2. (A) Schematic of the dumbbell shaped π-electron clouds above and below the aromatic ring structure. The arrows on the left indicate dipole moments. (B) Electrostatic surface of the Trp indole group (bottom) and the line-diagram above. (A and B excerpted with permission from [32]. Copyright 1996 AAAS.). (C) Most favorable, parallel cation–π interaction between an Arg and a Trp sidechain. Only in this orientation is the Arg still capable of hydrogen bonding with other species. (Reproduced with permission from [33]. Copyright 1997 American Chemical Society).

is able to form almost as many hydrogen bonds with the surrounding water molecules as when it is not involved in any cation–π interactions [37]. This is in contrast to lysines, which cannot form hydrogen bonds while engaged in cation–π interactions with an aromatic residue [37,38]. It has been suggested that this difference is responsible for the increased activity of Arg containing peptides over Lys substituted peptides. Cation–π interactions make the entry of Arg into the hydrophobic environment inside a lipid bilayer energetically more favorable. The Arg is effectively shielded from the highly hydrophobic nature of the bilayer by associating with a Trp residue [32,39].

The fact that Trp residues have a preference for the interfacial region of lipid bilayers has been well documented [26–28]. This behaviour is not only seen in membrane proteins but is also observed in many Trp containing antimicrobial peptides. An MD simulation of the short peptide dermaseptin S3 shows the N-terminal region inserting into the bilayer, with the Trp residue penetrating into the interfacial layer of the membrane [40]. Other simulations highlight the importance of Trp residues in membrane–peptide interactions as well. For example, simulations of pentapeptides show that the Trp residue can associate with the positively charged choline headgroups of the lipid bilayer [37]. Studies also show that Trps form hydrogen bonds with both water and components of the lipid bilayers when residing in the interfacial region [37,41]. These hydrogen-bonding partners are no longer available to the Trp residues when the peptide inserts further into the hydrocarbon core. Furthermore, the large, bulky, paddle-like shape of the indole sidechain disrupts the highly favorable and cohesive hydrophobic interactions of the lipid acyl chains when it buries deeper into the hydrocarbon core of a lipid bilayer, leading to high entropic energy costs [26]. Thus, it has been proposed that the complex electrostatic nature of the interfacial region of lipid bilayers is ideal to accommodate the equally complex nature of Trp residues [26].

Overall, the cationic nature and unique hydrogen bonding geometry of Arg and the complex properties of Trp seem to complement each other well for the purposes of antimicrobial peptides. The cationic charge of Arg provides an effective means of attracting the peptides to the target membranes and hydrogen bonding facilitates its interaction with negatively charged surfaces such as LPS, teichoic acid, or phosphatidyl glycerol phospholipid headgroups. Trp represents the most suitable amino acid to enable the peptide's prolonged association with the membrane. Depending on the peptide in question, different properties of these residues are utilized. For example, in puroA it appears that cation–π interactions between the Trp and Arg residues allow it to penetrate deeper into the membrane [39]. LfcinB on the other hand, uses Trp solely as a hydrophobic anchoring force, possibly to aid in threading the peptide through the membrane towards an intracellular target. This notion is substantiated by the observation that neither the hydrogen bonding potential of Trp, nor its amphipathic nature, are crucial for the activity of LfcinB, but rather its hydrophobic character [25].

3. Cathelicidins

3.1. Indolicidin

Indolicidin belongs to the cathelicidin family of antimicrobial peptides. These peptides are produced with an N-terminal propiece similar to the proteinase inhibitor cathelin. While the N-terminal protein that is co-expressed is conserved, the antimicrobial peptide can vary greatly in sequence. It is thought that through natural selection, particular antimicrobial peptides were selected for, such that their bactericidal properties match the pathogens commonly present in the host's environment. Indolicidin was the first Trp-rich antimicrobial peptide discovered and has consequently been studied very thoroughly. It is a short, 13 amino acid peptide that is amidated at the C-terminus and contains the highest proportion of Trp residues ever seen with 39% (Table 1) [42]. In nature, the peptide is found in bovine neutrophils, from which it was first isolated [42]. Like many other antimicrobial peptides, it displays activity against a wide range of targets, such as Gram-positive as well as Gram-negative bacteria, fungi, and protozoa (Table 2) [43]. The structure of indolicidin was solved by homonuclear NMR in neutral DPC and negatively charged SDS micelles [44]. The peptide did not adopt any classical secondary structure nor did it display the characteristic amphipathic nature of antimicrobial peptides [44]. Rather, the peptide displayed an extended conformation in both micelle types with β-turns being the most prominent structural motifs that were found (Fig. 3) [44]. The peptide was observed to form a wedge-type shape, with hydrophobic Trp residues in a trough, flanked by positively charged regions of the peptide [44]. This way the peptide is thought to insert itself into the membrane with the hydrophobic core making contacts in the interfacial regions while the flanking parts interact more prominently with the lipid headgroups. The mechanism of action of indolicidin is still under debate. It is unlikely that indolicidin acts in a barrel-

Table 1
Amino acid sequences of Trp containing antimicrobial peptides

Name	Sequence [a]	PDB code [b]
Indolicidin	ILPWKWPWWPWRR-NH_2	1G89, 1G8C
CP-10A	ILAWKWAWWAWRR-NH_2	
CP-11	ILKKWPWWPWRRK-NH_2	1QXQ
CycloCP-11	IC_1LKKWPWWPWRRC_1K-NH_2	1QX9
Tritrpticin	VRRFPWWWPFLRR	1D6X
PuroA	FPVTWRWWKWWKG-NH_2	
Combi-1	Ac-RRWWRF-NH_2	
Combi-2	Ac-FRWWHR-NH2	
Cyclo-combi [c]	RRWWRF	1QVL, 1QVK
LfcinB	FKC_1RRWQWRMKKLGAPSITC$_1$VRRAF	1LFC
$LfcinB_{4-9}$	RRWQWR-NH_2	
LfcinH	GRRRRSVQWC$_1$AVSQPEATKC$_2$FQW-	
	-QRNMRKVRGPPVSC$_2$IKRDSPIQC$_1$IQA	1Z6W, 1Z6V
$Lfcin_{4-14}$	RRWQWRMKKLG	1Y5C
$Lfcin_{4-14Dis}$	C_1RRWQWRMKKLGC$_1$-NH_2	1Y58
LysH	RAWVAWR-NH_2	
LysC	IVSDGNGMNAWVAWR-NH_2	

[a] Disulfide bonded cysteines are denoted by subscript numbers.
[b] No PDB ID indicates there are no coordinates available.
[c] This peptide is cycylized end-to-end.

Table 2
Known activities of Trp- and Arg-rich antimicrobial peptides

Gram-negative	Gram-positive	Fungi	Ref.
Indolicidin			
E. coli	*S. aureus*	*C. albicans*	[128,129]
P. aeruginosa	*S. epidermidis*		
S. typhimurium			
Tritrpticin			
E. coli	*B. subtilis*	*C. albicans*	[128]
P. aeruginosa	*S. aureus*		
S. typhimurium	*S. epidermidis*		
PuroA			
E. coli	*S. aureus*		[39]
PIN-a, PIN-b			
A. tumefaciens	*C. michiganensis*	*A. brassicola*	[65]
E. coli	*S. aureus*	*A. pisi*	
E. carotovora carotovora		*F. culmorum*	
P. syringae phaseoli		*V. dahliae*	
Combi-1, combi-2			
E. coli	*S. aureus*	*C. albicans*	[73]
	S. sanguis		
LfcinB [a]			
E. coli	*B. cereus*	*C. albicans*	[89,130,131]
K. pneumoniae	*B. subtilis*	*C. tropicalis*	
L. monocytogenes	*C. perfringens*	*C. neoformans*	
P. aeruginosa	*L. monocytogenes*	*T. mentagrophytes*	
P. vulgaris	*S. aureus*	*T. rubrum*	
S. enteritidis	*S. bovis*		
Y. enterocolitica	*S. epidermidis*		
	S. haemolyticus		
	S. hominus		
LfcinH [b]			
Acinetobacter sp.	*S. aureus*	*C. albicans*	[106,132,133]
E. aerogenes			
E. coli			
Klebsiella sp.			
HEWL			
B. bronchiseptica	*B. subtilis*	*C. albicans*	[118]
E. coli	*M. luteus*		
K. pneumoniae	*S. aureus*		
P. aeruginosa	*S. epidermidis*		
S. marcescens	*S. lentus*		
S. typhimurium	*S. zooepidemicus*		
LysC [c]			
E. coli	*B. subtilis*		[86]
K. pneumoniae	*S. aureus*		
S. marcescens	*S. zooepidemicus*		
LysH [c]			
E. coli	*B. subtilis*		[86]
K. pneumoniae	*M. luteus*		
P. aeruginosa	*S. aureus*		
S. marcescens	*S. epidermidis*		
	S. lentus		
	S. zooepidemicus		

[a] Organisms shown here were susceptible to peptide fragments of the LysH sequence presented in Table 1.
[b] Only organisms susceptible to the sequence in Table 1 listed here, for other lysozyme peptides, see [115,118].
[c] LfcinB also possesses activities not listed here.

stave fashion, since it does not cause cell lysis at concentrations four times the MIC for indolicidin, indicating that indolicidin must possess another mode of action to kill bacteria [45]. These results were corroborated when it was shown that indolicidin causes DNA filamentation in *E. coli* cells and more recently, when gel retardation assays demonstrated indolicidin's ability to bind DNA [43,46]. Indolicidin is thought to cross the membranes into the cytoplasm at concentrations above the MIC but below the minimal lytic concentration [43]. Amphipathicity provides indolicidin with a hydrophobic surface important for protein–protein interactions, while the cationic surface is crucial for its interactions with both the negative charges on bacterial membranes as well as the negatively charged phosphate backbone of DNA.

Although indolicidin is an effective antimicrobial peptide, various attempts have been made to improve its potency. For example, peptides were synthesized with Trp analogues such as 3-(2-naphthyl)-Ala, which show that Trp can be replaced by non-natural amino acids to enhance the activity of indolicidin [47]. Another research group created dimers and tetramers of indolicidin by linking the peptides at their C-terminal ends via lysine residues [48]. Both the dimeric and tetrameric forms of indolicidin display increased inhibitory activity against HIV-1 integrase [48]. Another study examines modified versions of indolicidin including a Pro to Ala mutant, CP10A. This peptide has increased potency against Gram-positive bacteria and the mutation had a drastic effect on the peptide's structure. Instead of an extended structure, CP10A adopts a short amphipathic helix (Fig. 3) [49]. Interestingly, this mutant was also observed to influence DNA synthesis, suggesting it retains indolicidin's ability to penetrate into the cytoplasm. Another mutant, CP11, was created to enhance the overall charge of the peptide, which improved its activity against Gram-negative bacteria and made the peptide less hemolytic than indolicidin [50]. Membrane mimetic assays revealed that the presence of LPS enhances surface interactions with the indolicidin analog peptides and suggest that CP10A inserts deeper into the bilayer due to its increased helical content, while CP11 is less helical and has a greater positive charge and therefore inserts less (Fig. 3) [51]. The activity of CP11 was further enhanced through structure based design, after it was discovered that the peptide forms a U shaped molecule that can easily be cyclized by introducing a cysteine residue at either end [52]. Although both peptides are amphipathic in DPC micelles, cyclo-CP11 shows a more distinct separation into hydrophobic and hydrophilic regions [52]. While the antimicrobial activities were found to be largely similar between the cyclized and linear CP11, the most notable difference is seen in terms of their half-lives. In the presence of trypsin, linear CP11 has a half-life of 4 min, while the cyclic peptide has a half-life of 18 min, which allows cyclo-CP11 to remain active for 90 min even with the protease present [52].

3.2. Tritrpticin

Tritrpticin is another cathelicidin antimicrobial peptide. First described in pigs, it is 13 amino acids long and as the name implies, it has three consecutive Trp residues in its sequence (Table 1) [53]. This gives it an unusually high proportion of Trp residues (23%), much like indolicidin. Tritrpticin is highly cationic at neutral pH with four Arg residues. Its sequence is almost palindromic, containing two cationic residues close to

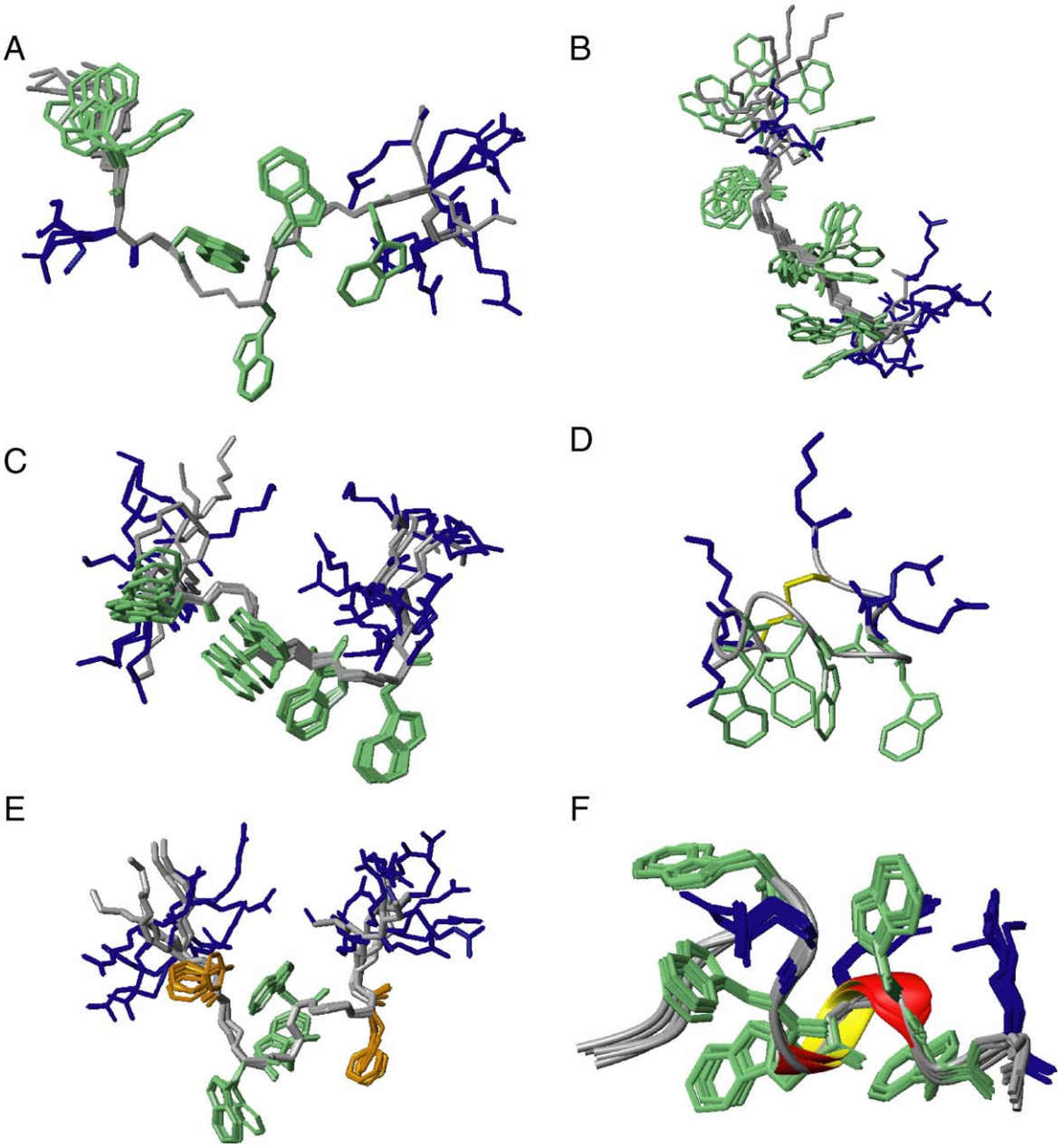

Fig. 3. (A and B) Structures of indolicidin determined in DPC and SDS micelles, respectively. (C) Linear CP-11 in the presence of DPC micelles. (D) Structure of the disulfide bonded, cyclic CP-11 as solved in DPC micelles, highlighting the clear segregation of Trp and Arg residues from each other. (E) Tritrpticin as determined by NMR in SDS micelles showing the wedge shape consisting of hydrophobic amino acids flanked by the hydrophilic residues. (F) PuroA structure in the presence of SDS micelles. Notice the cation–π arrangement between Trps 5 and 7 and Arg 6 on the left side of the peptide. Trp residues are highlighted in pale-green, Phe is shown in orange, and Lys and Arg residues are presented in blue color. The disulfide bridges are shown in yellow stick representation. Additionally, in Figs. 4 and 5, His residues are shown in cyan and Gln in pink.

either terminal followed by Phe and two Pro residues that flank the three central Trp residues (Table 1). The peptide was found to be structurally poorly defined in Tris buffer, but it adopted a better defined conformation in SDS micelles [54]. This structure consists of a turn–turn segment that revolves around the two proline residues (Fig. 3). This way, the peptide adopts an amphipathic conformation in SDS micelles, resembling a wedge shape with the Trp residues in the narrow part of the structure, mediated by two turns and leading to the hydrophilic side of the peptide which is made up of the Arg residues [54]. In SDS micelles, the hydrophobic core is defined much better than the cationic part of the peptide, which shows a lot of variability (Fig. 3). The hydrophobic residues were deduced to rest in the interfacial region of the bilayer based on Trp fluorescence quenching experiments, while the cationic Arg residues interact with the anionic surface charges of the bilayer [54]. To adopt an amphipathic structure, tritrpticin sacrifices many intra-molecular hydrogen bonds that would be created if it formed a regular secondary structure such as a helix or a β-sheet. Modeling tritrpticin as a sheet or helix based on its primary structure, it becomes obvious that the peptide cannot form an amphipathic structure in those conformations [54]. The amphipathic

arrangement must therefore provide an energetic advantage, considering the peptide prefers this conformation over regular secondary structure and its attendant stabilizing forces. The driving forces behind the wedge-shaped, amphiphilic structure are thought to be the Trp residues and the two Phes that all lie in the same vicinity such that they can rest in the interfacial region of the membrane (Fig. 3).

The mechanism of tritrpticin involves interactions with the bacterial membrane, but what exactly occurs is difficult to resolve. Tritrpticin induces membrane leakage to various degrees in different types of model membranes [55]. The peptide interacts more strongly with LUVs made up of partly negatively charged phospholipids, such as DOPE:DOPG or DOPC:DOPG mixtures [55]. In contrast, it only causes very poor leakage of LUVs consisting of DOPC:DOPE. This is in agreement with a red-edge effect that is observed in fluorescence experiments, indicating that tritrpticin inserts into negatively charged membranes more strongly and therefore has a greater lytic effect [55]. Such membrane insertion by tritrpticin was postulated to cause positive curvature strain, which may eventually lead to toroidal pore formation at high enough peptide concentrations in accordance with the SMH model [56–60]. This mechanism has been well documented for the antimicrobial peptide magainin 2 [57]. This theory is consistent with another study that examined tritrpticin's ability to form ion channels [61]. It showed that tritrpticin readily forms pores and induced current jumps in azolectin black lipid membranes (BLM), exhibiting strong single channel conductance values, ranging from hundreds to thousands of pS [61]. Early results show that when other BLMs were used without an overall negative charge, the peptide induced less ion channel activity [61]. The ion selectivity of channels formed by tritrpticin was tested as well, revealing that the pores select for cationic species [61]. Although this is counter-intuitive considering tritrpticin's high concentration of positively charged amino acids, it agrees with other research on antimicrobial peptides. Analogous to magainin 2, when tritrpticin inserts into the membrane, the three consecutive Trp residues form a large hydrophobic centre, which causes lateral pressure that leads to positive curvature strain [55]. During subsequent pore formation, the interactions of the Arg residues with the anionic lipid headgroups will pull the lipids along into the pore, forming a composite pore not only lined with cationic charges from the peptide's Arg sidechains but also from the anionic headgroups of the membrane lipids (Fig. 1) [61]. Naturally, it is much more favorable for cationic species to traverse a pore lined with both anionic and cationic charges rather than attempting to overcome repulsive electrostatic forces stemming from cation–cation interactions.

4. Puroindoline

The puroindoline proteins are small, cationic proteins isolated from wheat seeds [62]. Two isoforms are known to exist, puroindoline A (PIN-a) and puroindoline B (PIN-b). The proteins are about 13 kDa in size, contain five disulfide bonds, and an unusually Trp rich region. They serve two roles in wheat seeds. Firstly, they are responsible for wheat grain hardness [63] and secondly, they possess antifungal and antibacterial activity to protect the seeds (Table 2). For example, it has been demonstrated that transgenic rice that originally did not express the puroindolines, shows greatly increased resistance to diseases when expressing the proteins [64]. Puroindolines have been found to co-localize in the aleurone and the endosperm regions of seeds [65]. The aleurone surrounds the starchy endosperm and presents the initial point of invasion for potential pathogens. The puroindolines are therefore located ideally to protect the seed from an attack. In nature, the proteins can act cooperatively with each other and with other peptides such as α-purothionins to combat pathogens [65,66]. These properties of puroindolines have made them very interesting for study, especially in regards to the food industry, as these proteins could potentially be used as natural food preservatives [65]. This seems to be a viable idea, considering that puroindolines are still found intact in foods after both heat treatments and long-term storage, both of which occur during food production [65].

The Trp-rich regions of the puroindoline proteins have been isolated as the potential active centres for antimicrobial/antifungal activity (Table 1) [39]. While the Trp rich region of PIN-b (puroB) does not possess any antimicrobial activity, the short 13 residue peptide of the Trp rich region of PIN-a (puroA) showed antimicrobial activity against both Gram-positive and Gram-negative bacteria (Table 2) [39]. The structure of this 13 residue peptide was determined in anionic SDS micelles by homonuclear NMR methods [39]. Although unstructured in aqueous solution, puroA adopted a well-defined structure in SDS micelles. The peptide has a helical segment spanning from Trp8 to Trp10, with the remainder of the peptide forming a coil, but not adopting any distinct helical conformation (Fig. 3) [39]. Overall, this forms an amphipathic structure with the cationic sidechains resting on the hydrophilic side, as seen so often in antimicrobial peptides [39]. An interesting facet of this structure is that the sidechain of Arg6 rests directly between the two indole rings of Trps 5 and 7, which is energetically favorable due to previously described cation–π effects (Fig. 3) [39].

Puroindolines likely interact with phospholipid membranes, as they show both strong adsorption to phospholipid monolayers and are purified with a Triton X-114 extraction, highlighting their interactions with hydrophobic species [62,67]. Studies with puroA suggest similar interactions with membranes since the peptide adopts a well defined conformation in SDS micelles and also because the peptide is capable of inducing calcein leakage from vesicles [39]. In addition, research on both the full length protein and on the puroA peptide reveal that puroindoline prefers negatively charged phospholipids rather than zwitterionic headgroups, suggesting that electrostatic interactions play an important role in determining its target [39,68]. Although it seems apparent that the two species both interact with bacterial or fungal membranes, it is still uncertain whether the puroA peptide and the full-length protein act in the same fashion. The puroA peptide is only 13 residues long and fluorescence quenching studies reveal that the peptide resides in the interfacial region of the membrane [39]. This suggests that the peptide would act in a fashion similar to magainin 2, which promotes

positive curvature in the bilayer and at high enough concentrations, causes toroidal pore formation [57]. For the intact puroindoline proteins as well as other thionins, channel formation has been reported that leads to membrane permeabilization in *Xenopus laevis* oocytes and is facilitated by a negative membrane potential [69,70]. Interestingly, the membranes disintegrate at elevated protein concentrations as the channels begin to aggregate, similarly to what has been observed in PIN-a microscopy studies [68]. Thus, it seems that the puroA peptide has a different mechanism of action than the intact PIN-a protein.

The full-length proteins have been shown to act synergistically not only with purothionins, but also with each other, enhancing each other's potency to varying degrees [65]. Small amounts of PIN-a were found to have large effects on bacteria after previous exposure to sub-lethal concentrations of PIN-b and vice versa [65]. It was also pointed out that PIN-a may act in a different manner from PIN-b; for example, the PIN-b protein may have an intracellular target [65]. This theory goes well with the observed synergy between the two proteins. PIN-a is inactive at 1/3 its MIC, but upon addition of only 5 μg/mL of PIN-b, almost 90% inhibition is achieved [65]. Thus, PIN-a can be envisioned as a 'gate-opener' for its counterpart, enabling PIN-b to enter into the cell at lower concentrations than when the latter is present alone.

5. Combinatorial peptides

The peptides discussed so far have all been discovered and derived from an organism that naturally produces either the peptide itself or a protein that gives rise to that particular peptide. The following two Trp-rich peptides were both derived in an entirely different fashion, taking advantage of a synthetic combinatorial library made to potentially screen millions of soluble hexapeptides in a library [71]. Not only can these libraries be used to discover novel antimicrobial peptides, they can also be used to improve the activity of already known peptides [72]. The two peptides presented here were discovered as novel antimicrobial peptides and both contain two Trp and at least two Arg residues, as well as acetylated N-termini and amidated C-termini (Table 1) [71,73]. The two peptides, named combi-1 and combi-2, have antibacterial activities similar to naturally occurring peptides, with IC_{50}'s ranging from 5 to 39 μg/mL against most organisms tested [30]. Intriguingly, the combi-1 sequence is highly reminiscent of the LfcinB active centre amino acid sequence (see below and Table 1). Considering that many of the peptides derived from the screening of synthetic combinatorial libraries resemble those found in nature, it appears that this is indeed a mechanism of performing natural selection in vitro.

The structures of combi-1 and combi-2 show pronounced dependencies on their immediate surroundings. The peptides are unstructured in aqueous solution, but when introduced to either zwitterionic DPC or anionic SDS micelles, the peptides adopt an ordered structure [74,75]. Not only are the combis sensitive to the presence or absence of micelles, they also respond to the particular type of micelle present. Combi-1 is more ordered in the presence of SDS micelles than in DPC, while combi-2 shows no such difference (Fig. 4). Combi-1 only adopts an amphipathic structure in SDS micelles with its three Arg residues oriented towards one side and the remaining three hydrophobic residues on the opposite side, unlike combi-2, which adopts a coiled and amphipathic structure in both micelle types (Fig. 4) [74,75]. The interaction between the peptide and the surrounding medium seems to be governed by the sidechains and their properties, since no hydrogen bonding of the peptide's backbone was observed [74]. The amphipathic structure of combi-1 maximizes the number of favorable interactions with the micelle. The positively charged Arg residues likely attach the peptide via electrostatic interactions with the SDS headgroups while the hydrophobic aromatic residues interact with the interfacial region of the micelle [74]. Although combi-2 adopts an amphipathic structure in both environments, the structure still displays some subtle, yet significant, differences. In DPC micelles, the His residue is part of the hydrophobic portion of the peptide, while in anionic SDS micelles, the His forms part of the hydrophilic region and interacts with the negatively charged micelle (Fig. 4) [75]. This indicates that combi-2, through its His residue, possesses some structural plasticity that allows it to adapt to different environments. Combi-1 displays a clear preference for anionic surfaces not only in NMR studies, but in a number of other experiments as well. DSC data show that through its pronounced interactions with anionic lipid headgroups, the peptide changes the thermotropic phase behaviour of DPPG membranes, while it is unable to do so with DPPC membranes [74]. DSC experiments also indicate that both combi peptides form areas of higher peptide concentration only in the presence of negatively charged lipids, such as PG and not with zwitterionic lipids like PC [75]. Fluorescence quenching data further substantiate this observation since a larger blue-shift is seen in PG vesicles than PC vesicles, indicating the Trp residues are buried deeper in the anionic lipids. Clearly, the peptides have a highly specific and sensitive preference for anionic membrane surfaces resembling bacterial membranes. Other experiments provide additional evidence on how these peptides perform their bactericidal action. Calcein leakage studies indicate that both combi peptides are very poor at permeabilizing vesicles [75]. Further, the peptides are too short to span the membrane, indicating that they are unlikely to perform their lethal action through stable pore formation. Consistent with this finding, confocal microscopy studies of fluorescein-labeled peptides show that the combi peptides rapidly penetrate bacterial cells, in contrast to other peptides that remain on the surface of bacteria, where they form a peptide layer [75,76]. Taken together, these data strongly suggest that the peptides utilize their amphipathic structure to first interact with the membranes and subsequently aggregate there. How exactly they pass through the membranes is not known, but one could imagine that they may form transient toroidal pores that rapidly disintegrate and thereby move the peptides inside the cell. Spontaneous uptake into the cell has also been proposed for a number of other short, Arg-rich cationic peptides (see below) [24,77]. Once inside the cell, the peptides could interact with an intracellular target similar to the lysozyme peptides discussed below.

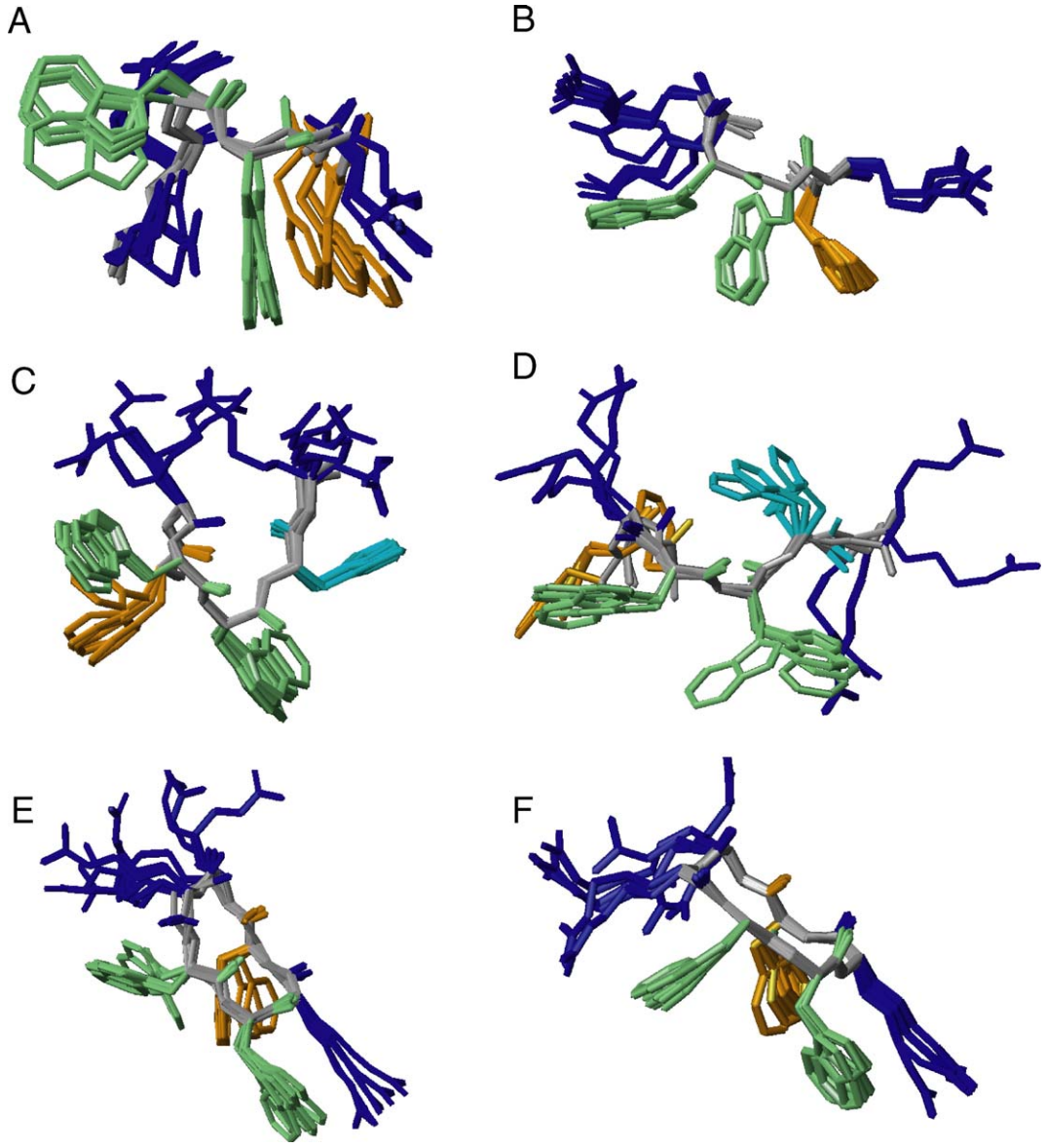

Fig. 4. (A and B) The linear combi-1 peptide in the presence of DPC and SDS, respectively. (C and D) Combi-2 peptide first in DPC and then SDS micelles. In SDS the His residue orients itself to the hydrophilic face of the peptide, whereas it remains with the aromatic residues in DPC. (E and F) Cyclo-combi in the presence of DPC and SDS, respectively. Notice the stacking arrangement between the Arg and Trp residues pointing to the bottom right. See Fig. 3 for the amino acid coloring scheme.

In an attempt to increase the potency of combi-1, various substitutions and modifications have been made to the peptide [78]. An end-to-end cyclized version of combi-1 (cyclo-combi) was found to reduce the MIC tenfold (Table 1) [79]. The structure of the cyclo-peptide indicates that cyclization enhances the peptide's amphipathicity, largely segregating the hydrophobic and hydrophilic portions of the peptide above and below the plane of the ring structure (Fig. 4) [80]. The peptide adopts a β-sheet structure in micelles, made up of two short β-turns [80]. The sidechains are well defined for the hydrophobic residues, while on the opposite side, Arg1 and Arg2 show great flexibility in both SDS and DPC micelles [80]. As expected, cyclo-combi orients its hydrophobic residues towards the micelle core, while both the backbone and Arg sidechains reside more towards the surface of the micelle [80]. Interestingly, the third Arg is in close proximity to the indole group of Trp4 in a stacked conformation, which is ideal for cation–π interactions (Fig 4). The behaviour of the Arg residues in a lipid bilayer was further investigated by MD simulations where hydrogen bonding with phospholipid headgroups and snorkeling effects of the Arg residues could be observed [81]. Formation of peptide–lipid aggregates of about one peptide to eight lipid molecules was also reported, and this requires further experimental verification [81].

6. Antimicrobial peptides from food proteins

6.1. Lactoferricins

The glycoprotein lactoferrin (LF) which is found in mammalian milk and other bodily secretions as well as neutrophils, is a large 80 kDa iron binding protein of the

transferrin protein family [82]. It has been known for a long time that LF possesses antimicrobial activity, but originally it was thought that the activity was dependent on the protein's ability to scavenge iron away from invading bacteria. Even though its iron binding properties play a role in preventing biofilm formation [83], it is now known that this is not its only mechanism of action. When the protein is digested at acidic pH by proteases such as pepsin that are found in the stomach, the products yield highly active antimicrobial peptides [84–87]. In this manner, newborns are protected from pathogenic infections, since they consume milk from their mother that not only provides nutrients, but at the same time enhances the offspring's still under developed defensive machinery. One of these antimicrobial peptides is lactoferricin (Lfcin), which is cleaved from the basic N-terminal region of LF. Bovine Lfcin (LfcinB) has been investigated most extensively since it is the most active Lfcin found across various species and also the most readily available (for a comprehensive review, see [9]). The Lfcin peptides from different species display drastic sequence differences but still share some similarities. LfcinB encompasses 25 residues, including two Trp residues at positions 6 and 8 and a disulfide bond between its two cysteine residues (Table 1). Its activity covers a remarkable range, from antibacterial activity against both Gram-positive and Gram-negative bacteria to anti-fungal activity, as well as anti-viral, and antitumor activities (Table 2) [88–90]. In addition, Lfcin can bind to endotoxin and unmethylated nucleotides and therefore has been proposed to be capable of inhibiting septic shock [88]. The 25 amino acid residue peptide exhibits similar antimicrobial activity to the parent protein and furthermore, the antimicrobial active center of LF can be reduced to an even smaller peptide. A 15 residue peptide has been constructed that only shows minimal loss of activity [91] and an 11 residue peptide showed decreased hemolytic activity compared to full length LfcinB, while retaining similar antimicrobial activity [92]. Finally, what is considered to be the active center of LfcinB was identified to be a hexapeptide spanning from residues 4 to 9 and when the C-terminal's negative charge is neutralized by amidation, this peptide indeed has an MIC value that is comparable to that of the full-length 25 residue peptide (Table 1) [93].

The structure of LfcinB in aqueous solution was solved by homonuclear NMR and shows that the peptide adopts a short, twisted, antiparallel β-sheet conformation (Fig. 5) [94]. The β-sheet gives the peptide an amphipathic structure, with most of the hydrophobic residues residing on one side of the β-sheet plane, while the majority of the hydrophilic residues rest on the other side. The hydrophobic residues are nested together tightly, revealed by a dense cluster of NOEs observed during structure calculations [94]. The hydrophilic residues, although resting on one side of the peptide are pointing away from each other, probably to minimize unfavorable electrostatic repulsions. The LfcinB structure in solution at low salt concentration contrasts the conformation of the peptide in the full-length LF protein (Fig. 5). In the crystal structure, an α-helix stretches over half the peptide, whereas a β-sheet is present in solution [95]. The peptide therefore undergoes a major conformational change when it is cleaved from LF (Fig. 5). The transition from helix to sheet has recently been modeled by MD simulations and highlight two major forces that are responsible for the interconversion of secondary structure [96]. Firstly, in the helical conformation the positive charges all lie on the same side, which makes the peptide quite unstable unless salt is present to minimize repulsive electrostatic interactions. Secondly, the conversion appears to also be driven by a hydrophobic collapse, which favors the β-sheet conformation when the peptide is excised from LF [96]. As part of the intact LF protein, the peptide forms a number of stabilizing, long-range hydrophobic contacts with other parts of the LF protein [94]. In the short LcinB peptide many hydrophobic sidechains become exposed to the solvent, which is unfavorable and therefore leads to a hydrophobic collapse. The structure of the active center hexapeptide of LfcinB and the 11 residue peptide ($LfcinB_{4-14}$) were solved when bound to SDS micelles (Fig. 5) [97,98]. Neither of the peptides adopts any canonical secondary structure, but they are both well defined and, not surprisingly, adopt amphipathic conformations [97,98]. 5-doxyl stearic acid spin label experiments conducted with the hexapeptide indicate that the Trp residues are anchored in the interfacial region of the micelle while the three Arg residues protrude to interact with the negative charges of the SDS micelles [97]. In addition to these linear peptides, a cyclized analogue of $LfcinB_{4-14}$ was made by disulfide bonding two cysteines that were added at both termini (Table 1) [98]. This peptide displays a similar structure as the linear version, but had a more clearly defined segregation of hydrophilic and hydrophobic residues, which has been credited for the cyclic peptide's increased antimicrobial activity [98]. Mutational studies on a 15 residue LfcinB peptide provide interesting insights as well. The replacement of either Trps 6 or 8 by Ala deletes its entire antibacterial activity [99]. Replacement by unnatural amino acids such as β-diphenylalanine that possess larger hydrophobic groups or increasing the number of Trp residues decreases the MICs against *E. coli* and *S. aureus* in most cases [100,101]. This trend is not followed indefinitely, as increasing the hydrophobic content too much eventually leads to increased hemolytic activity. These studies demonstrate the importance of Trp's anchoring ability and sidechain size and shape, rather than its hydrogen bonding ability and amphipathic character [25].

Human Lfcin (LfcinH) is significantly larger than the bovine peptide, consisting of 49 amino acids and possessing a similar loop to LfcinB that is constrained by a disulfide bond (Table 1). Its peptide sequence was recently determined by NMR, Edman degradation and mass spectrometry analysis, following pepsin digestion of human LF from milk [102]. Originally, another preparation yielded a slightly different product, that contained two separate fragments held together by an additional disulfide bond [87]. The structure of LfcinH is poorly defined in SDS and DPC micelles, but exhibits a partially folded structure in water and is well defined in a more hydrophobic solvent consisting of 4:4:1 methanol–chloroform–water [102]. This solvent has been used in other studies to mimic a membrane environment [103,104]. In this solvent, LfcinH adopts an amphipathic, partially helical structure (Fig. 5) [102]. This, unlike LfcinB, is

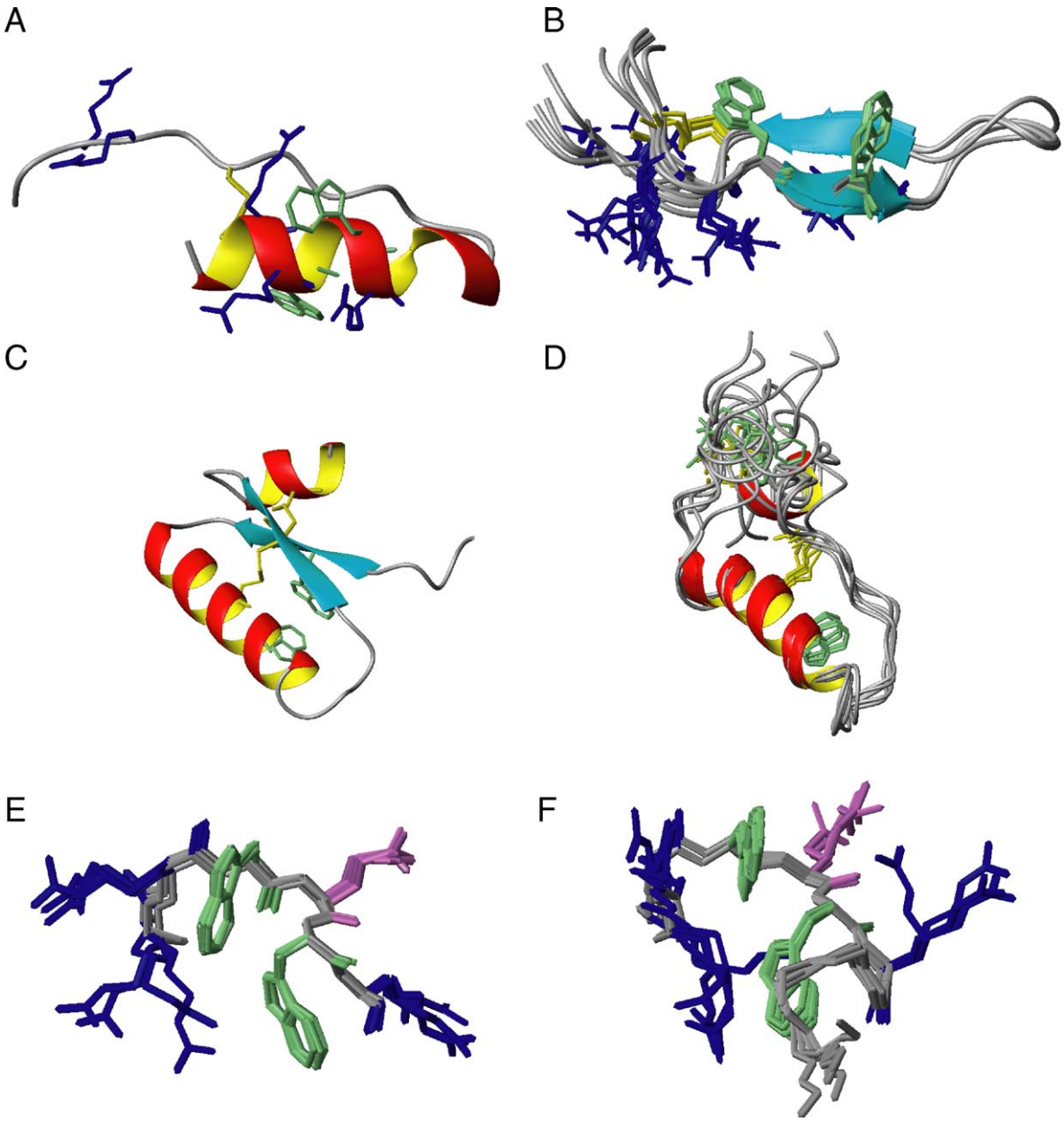

Fig. 5. (A and C) Ribbon diagrams taken from the crystal structures of intact bovine and human lactoferrin, respectively. Each fragment corresponds to the amino acid range of the matching lactoferricin peptide. (B and D) Bovine and human lactoferricin peptides, determined in water and membrane mimetic solvents, respectively. LfcinB undergoes a clear transition is secondary structure from helix to sheet, while LfcinH retains a partially helical conformation. (E) Structure of the LfcinB active centre hexapeptide as determined in SDS micelles. (F) The slightly longer $LfcinB_{4-14}$ peptide solved in SDS micelles. Notice the similarity in conformations between the two. For an explanation of the color scheme, see Fig. 3.

similar to the conformation seen in the crystal structure of human LF [105]. In intact human LF, the segment corresponding to LfcinH forms an α-helix and the first strand of a larger β-sheet (Fig. 5). The peptide does not form a β-strand in any solvents tested and does not adopt any helical structure in an aqueous environment to prevent unfavorable contacts by hydrophobic residues, such as Phe21 or Val35, with the hydrophilic solvent [102]. Rather, LfcinH undergoes a hydrophobic collapse, which is reflected by a significant number of NOEs observed in water. The highest number of long range interactions in this partially folded state are observed for Trps 9 and 23, which reinforces the important role Trp residues play along the protein folding pathway (Fig. 6) [102]. In a more lipophilic solvent, such as the one mentioned above, and in the intact human LF protein, the peptide forms stabilizing hydrophobic contacts with the solvent or the rest of the protein, respectively, that favor helix formation. Another study involving the N-terminal part of the LfcinH peptide bound to endotoxin reveals that the peptide may after all be able to undergo a similar transition as LfcinB, since the LPS bound peptide displays CD spectra reminiscent of a β-sheet peptide [106]. It has yet to be determined what exact effect LPS binding has on LfcinB and what implications any potentially induced structural changes have on antimicrobial activity.

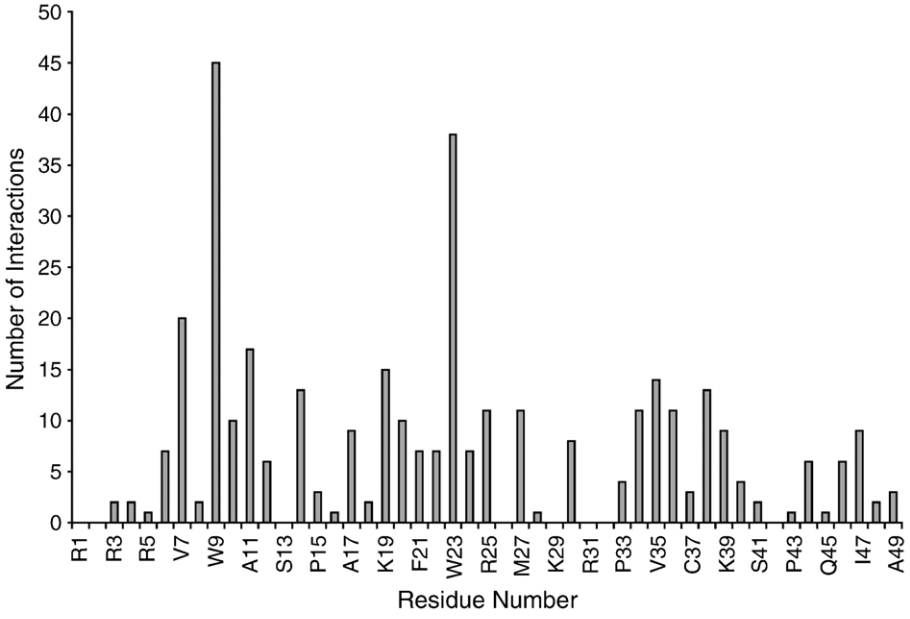

Fig. 6. Number of non-sequential, interresidue NOE interactions of LfcinH observed in water. Trps 9 and 23 display the highest number of interactions in the only partially folded peptide. (Reproduced with permission from [102], © 2006, the American Society for Microbiology. All rights reserved).

The Lfcin peptides display a variety of functions as described earlier. LfcinB has a broad range of action against many microbes, while LfcinH is bacteriostatic against a number of Gram-positive and Gram-negative species (see [9]). A large determinant for target selectivity are the electrostatic interactions between target and peptide. The cationic character of the peptides and the negatively charged membranes of bacteria (i.e. through PG, LPS, teichoic acid) provide an ideal interaction to attract the peptides to these cells. Although electrostatic forces are important for selectivity and the interactions of peptides and membranes, it does not appear that the Lfcins actually perform their activity by disrupting the membrane. Vesicle leakage studies conducted on the LfcinB peptides show that they only poorly induce leakage, especially when compared to other antimicrobial peptides that are known to kill bacteria through membrane disruption, such as tritrpticin [55,98]. Lfcins do, however, interact with negatively charged membranes. DSC results show that negatively charged DPPG liposomes display subtle changes upon LfcinB binding, while DPPE and DPPC liposomes remain unaffected even at high peptide to lipid ratios [24]. Both the human and bovine Lfcins are capable of binding LPS, indicating that they do interact with the outer membrane at least in Gram-negative bacteria. In fact, an 11 residue peptide of LfcinH has been shown to form ordered aggregates when LPS is present, which could create a large amphipathic structure that has an increased ability to disrupt the outer membrane and through this membrane damaging effect, permits individual peptides to move across [106]. This theory is supported by a lag time in bacterial action that is observed and corresponds to the time needed for peptide aggregation, as well as by blister-like formations in the outer membrane of Gram-negative bacteria, known as blebs [88,106,107]. Therefore, it is likely that Lfcins are able to interact with the membranes, but it is unlikely that their major function is to cause pores in the cytoplasmic membrane. Rather, it seems the Lfcins act on intracellular targets, which interferes with the normal functioning of microorganisms. LfcinB transmission electron microscopy data shows that the peptide indeed moves into the cytoplasm of both *S. aureus* as well as *E. coli* and that the cytoplasmic components are only affected after bleb formation [107,108]. This is supported by DSC heating scans on DiPoPE bilayers, which undergo a phase transition from a bilayer to a hexagonal phase at about 43.5 °C under normal conditions. Upon addition of LfcinB or its active center, the temperature of this phase transition increases, similar to what is observed with magainin 2 [24,57]. This pattern is consistent with transient toroidal pore formation, resulting in the peptide being moved inside the cell. The two Trp residues are particularly important in LfcinB, mutating either of them results in a complete loss of antimicrobial activity [99]. Trp anchors the peptide in the membrane and presents a vital interaction with the membrane to facilitate the peptide's entry into the cell. It has actually been proposed that the Lfcin peptide region acts as a "tugboat" that pulls the intact LF protein into the cytoplasm, as it has been observed that even the full-length LF protein can enter cells spontaneously [109]. This pattern is also seen in a number of other peptides such as buforin II and penetratin that have short regions responsible for their spontaneous transfer across the plasma membrane [110]. These peptides are known as cell-permeable peptides (CPPs) and interact with the membrane via electrostatic and/or hydrophobic interactions after which they may form pores or inverted micelles to shuttle inside the cell [110]. Similar to antimicrobial peptides, Arg residues and cation–π interactions play a crucial role in CPP–membrane interactions. For example, MD studies of penetratin highlight important cation–π interactions of Trps 48 and 56 and Arg 52 to shield the peptide from the aqueous phase when interacting with the membrane [111]. Thus, it appears Lfcin and LF utilize a

similar mechanism to enter cells as CPPs and their proteins. Once inside the cell, LfcinB is capable of binding and affecting DNA and RNA synthesis in both Gram-negative and Gram-positive bacteria [112]. LfcinB affects *S. aureus* in a faster manner, while in *E. coli* cells the process takes a longer period of time, suggesting that the target is more accessible in the former [112]. The observation that Lfcins act intracellulary helps explain how these peptides can have such a broad range of target organisms. As long as an initial favorable electrostatic interaction takes place between the cationic peptide and anionic cells of bacteria, fungi, or even tumor cells, the peptide will be able to traverse the membrane and move inside where it performs its activity.

6.2. Lysozyme peptides

Alexander Fleming discovered lysozyme in 1922 and it has been intensely studied and served as a model protein in many cases ever since. The protein is found in a range of different locations, including nasal secretions, tears, milk, as well as inside the body, stored in neutrophil granulocytes, monocytes, and macrophages [113]. Lysozyme is best known for its muramidase action on the peptidoglycan layers of bacterial membranes. It specifically attacks this compound and breaks it down enzymatically, thereby destroying the outer membrane which also kills the bacterial cells. Despite its well documented properties, it has only recently been observed that the denatured protein, which lacks the muramidase activity, is still bactericidal (Table 2) [114]. Furthermore, when the protein is digested by various proteases, the resulting mixtures are antimicrobial, reminiscent of the Lfcin peptides. The first such discovery demonstrated that clostripain digested hen egg white lysozyme (HEWL) yields a pentadecapeptide spanning over residues 98–112 (LysC) of the full protein that has antibacterial activity, yet possesses no muramidase activity (Table 1) [86]. Further studies have confirmed this, and shown that naturally occurring human enzymes such as pepsin and trypsin are capable of breaking down lysozyme to release antimicrobial peptides [115–117]. The homologous region in human lysozyme has been found to possess antimicrobial activity as well [118]. A shorter portion of the peptide derived from HEWL, consisting of residues 106–112 retained its activity as long as the Asn residue at the N-terminal end is mutated to an Arg; incidentally, this gives that particular stretch of the peptide the same sequence as in human lysozyme (Table 1) [86]. Two more residues found to be of critical importance for activity were Trps 108 and 111, mutating only one of these residues to a tyrosine deletes most of the antimicrobial activity [86]. The structures of LysC and the corresponding C-terminal region in human lysozyme (residues 107–113, LysH) were determined recently by homonuclear NMR in SDS and DPC micelles [119]. The structure of LysC was also solved in water. The peptides show some differences from what is seen in the crystal structure of lysozyme as they do not form the same helix–loop–helix conformation (Fig. 7). The N-terminal region in LysC is disordered in all three matrices, while the C-terminal region is well defined [119]. In fact, all regions corresponding to the C-terminal helix in the full-length lysozyme were found to be well ordered in both peptides (Fig. 7). The two Trp residues in the C-terminal portion of the peptides stay together on the same side of the peptide in each case, which enables them to simultaneously interact with the membrane [119]. LysC exhibits various degrees of α-helicity in the C-terminal end, which is reminiscent of its conformation in the crystal structure. The remainder of the well-defined regions in the C-terminal form coiled structures. Other studies on slightly different HEWL peptides and the corresponding region in hLZ reveal that both have strong antimicrobial activity against Gram-positive and Gram-negative bacteria as well as fungi and that the N-terminal region is less active than its C-terminal counterpart [118]. More specifically, the N-terminal segment was mostly inactive against Gram-negative bacteria, indicating the C-terminal region is absolutely necessary for activity against these organisms [118]. Overall, it appears that combined, the N-terminal helix enters the membrane and forms a pore, while the C-terminal helix associates with the lipid headgroups, such that the two Trp residues reside in an interfacial location [118].

Interestingly, the segment cut-out by clostripain in HEWL corresponds to a helix–loop–helix segment in the full-length

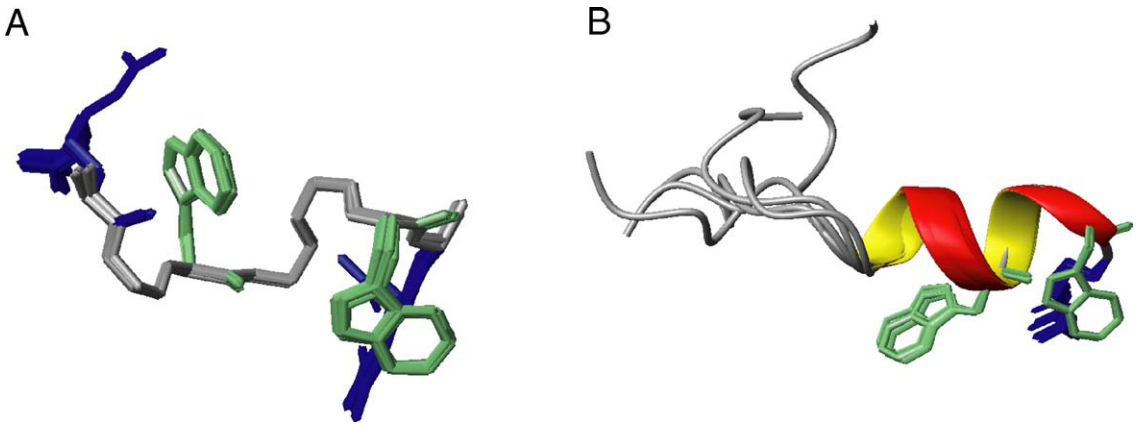

Fig. 7. (A) LysH structure in SDS micelles, spanning over residues 107–113 in the full-length human lysozyme protein. (B) LysC peptide in DPC micelles, representing residues 98–112 in HEWL. The well ordered C-terminal region is overlaid, which is almost identical to the sequence found in the LysH peptide. The color scheme is the same as the one presented in Fig. 3.

protein. This secondary structural motif has been observed in other antimicrobial peptides as well (e.g. cecropin) and is also seen in other regions of lysozyme. An investigation into any potential antimicrobial peptides derived from human c-type lysozyme (cLZ) revealed the presence of four antimicrobial peptides released through pepsin digestion of cLZ at low pH, mimicking a newborn's stomach [115]. The four peptides are all made up of a segment in the N-terminal region of the protein that constitutes another helix–loop–helix pattern in the protein [115]. They contain part of the N-terminal domain disulfide bonded to a short cationic helix in the C-terminal region of the protein.

LysH interactions with model membranes have been studied extensively. Confocal microscopy data shows that the peptide enters the cytoplasm of *E. coli* and *S. aureus* cells shortly after introduction [119]. In support of this finding it was discovered that other human lysozyme peptides can spontaneously migrate across the outer and inner membranes of Gram-negative bacteria, similar to what has been observed in other cationic, Arg rich peptides such as buforin II as described above [76,118,120]. Furthermore, the peptide shows a preference for membranes composed of negatively charged lipids, stressing the importance of the highly basic nature of antimicrobial peptides. Experiments conducted with L- and D-enantiomers of LysH provide further clues on their mechanism of action. The D-enantiomer has less activity than the L-enantiomer against a number of organisms tested, while both enantiomeric peptides show equal behaviour in regards to membrane association [86,119]. This strongly suggests that the difference in peptide activity is derived from an intracellular event, probably involving the peptide and an intracellular target molecule. In fact, it has been observed that the short lysozyme peptides inhibit DNA and RNA synthesis shortly after their introduction to bacterial cells, and only after this event are the inner and outer membranes further permeabilized [121]. In summary, it appears that the lysozyme peptides can cross both the inner and outer membranes, bringing them inside the cytoplasm of the bacterial cells, where they affect an intracellular target, likely one involved in DNA and RNA synthesis, which subsequently leads to the breakdown of the bacterial membranes and ultimately leads to cell death.

7. Conclusions

The combi peptides were discovered by scanning all possible combinations of hexapeptides. Remarkably, the hexapeptides found to possess the highest levels of activity were rich in Trp and Arg residues, emphasizing the importance of these two residues to antimicrobial peptides [73]. The peptides examined in this paper, as well as newly described peptides such as Pac-525 [122] or the de novo designed peptide WLBU2 [123,124], further illustrate this concept and show that these residues may play an important role as antimicrobial peptides are being tailored for clinical use. Additionally, many of the peptides discussed here consist of very few amino acids. Indolicidin, tritrpticin, and puroA are made up of 13 amino acids, while combi-1 and combi-2, as well as the active center of LfcinB are 6 amino acids short. This makes their synthesis cheaper compared to longer peptides, alleviating some of the concerns regarding peptide production costs. Another intriguing aspect of these peptides is the broad range of their activities. Many peptides function against both Gram-negative and Gram-positive bacteria and also possess a number of other activities. For example, LfcinB displays antitumor activity against a number of cancer cell lines, while it does not affect normal cells [125–127]. LfcinB's ability to distinguish between normal and cancerous cells is thought to involve the change in surface charge that occurs in a cancer cell, resulting in cells with a higher anionic content than normal. This represents a common theme seen in the peptides discussed, which all depend on electrostatic interactions between the cationic peptides and the anionic bacterial surface. Subsequently, the Trps play a role in membrane association because they preferably interact with the interfacial region of membranes. Trp residues also play a key role in protein folding. For example, they form native or nonnative hydrophobic contacts in unfolded proteins which serve as nucleation sites during protein folding [29]. Similarly, this can be applied to antimicrobial peptides, where in aqueous solution, the Trp residues help maintain a few hydrophobic contacts which retain the structure in a conformation favorable for subsequent membrane interactions.

Food proteins such as lysozyme and lactoferrin bear particular significance in the field of antimicrobial peptides. The fact that they represent "nutritional immunity" is exciting because it is already well known that these proteins are tolerated by the human body. In many countries today, lysozyme is used extensively as a natural preservative amounting to over 100 tons being used for this and similar purposes [116]. Therefore, these proteins provide an attractive vehicle for antimicrobial peptides, especially since it has been discovered that the inactive form can be ingested and subsequently activated by naturally occurring human or pathogenic proteases. The latter scenario would provide the advantage that the antimicrobial peptide is only activated at the actual location where it is needed. If one of these proteins could be manipulated to contain a yet to be discovered, perfect antimicrobial peptide at an enzymatic cut-site, this may just provide an ideal mechanism for antimicrobial peptide delivery to certain locations. Such a peptide may just end up being a Trp- and Arg-rich peptide that displays promiscuity against pathogenic targets due to the properties of these two residues. Overall, because of their many advantageous properties, it appears that Trp and Arg residues are exceedingly suitable to contribute to potent antimicrobial agents that eventually may be used on humans.

Acknowledgements

The research is supported by an operating grant from the Canadian Institutes for Health Research. E.J.P. and H.J.V. hold Scholarship and Scientist awards, respectively, of the Alberta

Heritage Foundation for Medical Research. The authors would also like to thank L.T. Nguyen and J.L. Gifford for helpful insights and discussions.

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