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DEFENSINS AND DEFENSIN-LIKE MOLECULES: ANTIBACTERIAL MODE OF ACTION

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SUMMARY

Antimicrobial peptides are important in the innate immunity and defence mechanisms of all organisms. Several models have been proposed in order to explain their antibacterial mode of action. Most antimicrobial peptides are amphipatic and cationic, and thus an effect on the cytoplasmic membrane of susceptible bacteria has been postulated as the main mode of action. The peptides may either form a channel, hereby inducing leakage of cytoplasmic content, or the peptide may induce permeability changes in a detergent-like manner. Both modes of action may lead to the death of the bacterial cell. Intracellular targets have also been identified for some antimicrobial peptides, and include binding to macromolecules, inhibition of macromolecular biosynthesis, and inhibition of bacterial enzymes. Some peptides have also been shown to have more than one target. This review addresses the models describing the antibacterial mode of action of human defensins present in the gut. In addition, the antibacterial mode of action of related antimicrobial peptides is discussed.

INTRODUCTION

The microbial load in the intestines of mammals is enormous (*Moore* and *Holdeman*, 1974). Some of these microbes are involved in the digestion and uptake of nutrients, and hence benefits the host. The presence of pathogenic bacteria may however not benefit the host, and several mechanisms are involved in the protection of the intestine from these bacteria. The mechanisms include the presence of a normal bacterial flora, volatile fatty acids, peristaltic movements, mucus, shedding of intestinal cells, and the presence of secretory IgA antibodies (*Mahida* et al., 1997; *Israel* and *Walker*, 1988). In addition, a rapid, non-oxidative, no-memory first-line defence system, the innate immunity system, involving peptides and proteins with antimicrobial activity protect the host against possible pathogenic bacteria. Such antimicrobial peptides are present in the gastrointestinal tract across phyla. Magainins are found in the stomach and intestine of the African frog *Xenopus laevis* (*Zasloff*, 1992; *Reilly* et al., 1994), and the midgut of some insects contains cells that produce antimicrobial peptides (*Nicolas* et al., 1996). Cecropin P1, an antimicrobial peptide related to the cecropins found in insects, has been isolated from the por-

cine proximal small intestine (*Lee* et al., 1989).

In the human digestive tract, many proteins and peptides with antimicrobial activity are present (summarised by *Lehrer*, 2001). Some peptides are confined to the epithelial cells and protect them from invasion by microbes (e.g. β-defensins, hCAP18/LL-37), thus creating a barrier against microbes. Others enter the digestive system through salivary glands (e.g. histatins), from the Paneth cells in the small intestine (e.g. α-defensins), and from pancreas (e.g. β-defensins). Further, some antimicrobial proteins and peptides, i.e. lactoferrin and lactoferricin, can enter the GI-tract either through food (*Kuwata* et al., 1998, 2001) or from endogenous sources (*Kayazawa* et al., 2002). Antimicrobial peptides are also found in the gut due to their presence in migrating polymorphonuclear cells (*Handy* et al., 1995).

The defensins

The defensins comprise the largest group of mammalian peptides (*Risso*, 2000), and are present throughout the digestive tract in all mammals, including humans (Table 1). There are two subfamilies of human defensins: (i) α-defensins and (ii) β-defensins, differing from each other in the position of the cysteine residues and in the bridge formation. The mature α-defensins comprises 29-35 amino acids (*Lehrer* et al., 1993), and the β-defensins 34-42 residues (*Selsted* et al., 1993). In their mature form, all defensins share a similar structural conformation; they are all β-sheets, cycled and stabilised by three disulphide-bridges (*Risso*, 2000).

Antimicrobial spectrum and activity

Although the antimicrobial peptides in the GI-tract possess several similarities, their antimicrobial properties are distinct (Tables 2, 3 and 4). Most peptides are active against both Gram-negative and Gram-positive bacteria, some also against fungi and protozoa, while others are also active against viruses and mycobacterium. The minimal inhibitory concentrations of the peptides are in the range of 0.1-100 μg/ml. They show synergistic activity between themselves and with other host defence molecules such as lactoferrin and lysozyme (*Bals* et al., 1998a, 1998b; *Nagaoka* et al, 2000; *Singh* et al., 2000; *Garcia* et al., 2001a).

Several inhibitors of antimicrobial activity have been described and inhibition of activity by NaCl has been implicated in cystic fibrosis (*Smith* et al., 1996; *Goldman* et al., 1997). HBD-3 is the only β-defensin that is salt-insensitive (*Harder* et al., 2001). The inhibition by NaCl is also dependent on the microbe, as high NaCl concentrations inhibits the activity of HNP-1 against Gram-positive and Gram-negative bacteria, but have no effect on the activity of HNP-1 against mycobacterium or Herpes simplex virus-1 (*Daher* et al., 1986; *Miyasaki* et al., 1990; *Ogata* et al., 1992; *Miyakawa* et al., 1996). For LL-37, NaCl inhibits the activity against methicillin resistant *S. aureus*, but does not influence the activity against vancomycin resistant *Enterococcus faecium* (*Turner* et al., 1998). Divalent cations such as Ca^{2+} and Mg^{2+}, serum and albumin have also been reported to inhibit the activity of some peptides (see Tables 2, 3 and 4).

The other GI-tract peptides covered in this review, lactoferricin B, magainin 2, cecropin P1 and LL-37, also show a broad spectrum of activity covering Gram-positive and Gram-negative bacteria, fungi, viruses, and protozoa (Table 2). Among these peptides, lactoferricin B is the only peptide with antiviral activity (*Andersen* et al., 2001).

Table 1: The human defensins categorised by family and localisation in the gut

Family	Peptide	Localisation	References
α-defensin	HNP 1- 4	neutrophil cells	Ganz et al., 1985; Wilde et al., 1989
	HD-5 and -6	Paneth cells	Jones and Bevins, 1992, 1993; Zhao et al., 1996
β-defensin	HBD-1	small intestine, colon, pancreas	Zhao et al., 1996; Gropp et al., 1999; O'Neil et al., 1999)
	HBD-2	gastric mucosa, colon	O'Neil et al., 1999; Hamanaka et al., 2001
	HBD-3	colon	Hase et al., 2002

Table 2: Antibacterial peptides located in the GI-tract of their respective host, the antimicrobial spectrum and identified inhibitors of the antimicrobial activity

Peptide	Origin	Spectrum	Inhibitors	References
Magainin 2	African frog	G+, G-, Fungi, Protozoa	none identified	Zasloff, 1987; Zasloff et al., 1988; Bessalle et al., 1990; Cirioni et al., 1998; Giacometti et al.,1998, 1999, 2000; Andra J. et al., 2001
Cecropin P1	Porcine	G+, G-, Fungi, Protozoa	none identified	Lee et al., 1989; Moore et al., 1996; Cirioni et al., 1998 Giacometti et al., 1998, 1999 and 2000; Smeianov et al., 2000; Andra et al., 2001
Lfcin B (17-41)	Bovine	G+, G-, Fungi, Protozoa Envelope virus	KCl, NaCl, $MgCl_2$, $CaCl_2$,$Fe_2(SO_4)_3$, $FeCl_3$, pH < 6.5, anions	Bellamy et al., 1992; Turchany et al., 1995; Ulvatne and Vorland, 2001; Andersen et al., 2001; Ulvatne et al., submitted
LL-37/hCAP-18	Human	G+, G-, Protozoa	Serum, NaCl, Ca^{2+}	Agerberth et al., 1995, Larrick et al., 1995; Bals et al., 1998a, Johansson et al., 1998; Turner et al., 1998, Nagaoka et al., 2000; Singh et al., 2000, Travis et al., 2000

Table 3: The antimicrobial spectrum and identified inhibitors of the antimicrobial activity of human β-defensins

Peptide	Spectrum	Inhibitors	References
HBD-1	G+, G-, Adenovirus	NaCl	Goldman et al., 1997; Singh et al., 1998, 2000; Valore et al., 1998; Gropp et al., 1999
HBD-2	G+, G-, Fungi	NaCl	Harder et al., 1997; Bals et al., 1998b; Singh et al., 1998, 2000; Hamanaka et al., 2001
HBD-3	G+, G-, Fungi	none identified	Harder et al, 2001; Garcia et al., 2001b
HBD-4	G+, G-, Fungi	NaCl	Garcia et al., 2001a

Table 4: The antimicrobial spectrum and identified inhibitors of the antimicrobial activity of human α-defensins

Peptide	Spectrum	Inhibitors	References
HNP1	G+, G-, Fungi, Protozoa, Envelope virus Mycobacterium, Chlamydia	NaCl , Mg^{2+}, Ca^{2+}, pH, serum, albumin, NaBr, KCl	Ganz et al., 1985, Daher et al., 1986, Lehrer et al., 1988, Shafer et al., 1988, Miyasaki et al., 1990, Ogata et al., 1992, Aley et al., 1994, Turchany et al., 1995, Miyakawa et al., 1996, Yasin et al., 1996, Turner et al., 1998, Nagaoka et al., 2000, Newman et al., 2000, Bastian and Shafer, 2001
HNP2	G+, G-, Fungi, Envelope virus, Mycobacterium, Chlamydia	pH < 4,0 Serum	Ganz et al., 1985; Daher et al., 1986; Lehrer et al., 1988; Miyasaki et al., 1990; Ogata et al., 1992; Miyakawa et al, 1996; Yasin et al., 1996, 2000; Newman et al., 2000
HNP3	G-, Fungi, Envelope virus, Mycobacterium	pH < 4,0	Ganz et al., 1985; Daher et al., 1986; Miyasaki et al., 1990; Ogata et al., 1992; Miyakawa et al., 1996; Newman et al., 2000
HNP4	G+, G-, Fungi	none identified	Wilde et al., 1989; Gabay et al., 1989
HD5	G+, G-, Fungi, Adenovirus	NaCl, pH	Porter et al., 1997; Gropp et al., 1999
HD6	No studies performed		

GI-tract peptides are thus active against a wide range of Gram-positive and Gram-negative bacteria, as well as fungi, protozoa, viruses, and mycobacterium.

DISCUSSION

Mode of action of antimicrobial peptides

Due to the amphipatic, cationic structure of most antimicrobial peptides, an effect on the cytoplasmic membrane of susceptible bacteria has been postulated as the main mode of action (*Ganz* and *Lehrer*, 1998). After an initial interaction between peptide and bacterial cell surface, the peptide will traverse to the outer leaflet of the cytoplasmic membrane and cause an increased permeability, which eventually leads to cell death.

In Gram-negative bacteria, the antibacterial peptides are thought to cross the outer membrane through a mechanism called "self-promoted-uptake" (*Hancock* and *Bell*, 1988). Divalent cations in the LPS are replaced by the peptide, causing an increased permeability of the outer membrane, which allows more peptide molecules to cross the outer barrier. For Gram-positive bacteria, the initial interaction is shown to be with the (L)TA (*Vorland* et al., 1999), yet there are no good explanation for the subsequent crossing of the thick peptidoglycan layer present in Gram-positive organisms.

Several models have been proposed in order to explain the effect antimicrobial peptides have on the cytoplasmic membrane (Table 5). In general, the peptides may act by destabilising and hereby permeabilising the membrane, or by forming distinct pores/channels in the membrane. For the former effect, the most known models include the formation of a peptide carpet (*Gazit* et al., 1995) and thinning of the membrane (*Ludtke* et al., 1995; *Berneche* et al., 1998; *Heller* et al., 2000). For pore-forming peptides, the models include the barrel-stave model (*Shai*, 1999; *Bechinger*, 1999), the wormhole model (*Matsuzaki* et al., 1996; *Ludtke* et al., 1996), and the two-state model (*Huang*, 2000). Dependent upon the character of the pore, the formation of pores may lead to leakage of ions and cytoplasmic content, influx of water, or both.

Despite the focus on bacterial membranes as targets for antimicrobial peptides, several antimicrobial peptides have been shown to have intracellular targets. These include binding to DNA, RNA and/or proteins (*Park* et al., 1998; *Otvos* et al., 2000; *Kragol* et al., 2001), inhibition of macromolecular biosynthesis (*Boman* et al., 1993; *Subbalakshmi* and *Sitaram*, 1998; *Castle* et al., 1999; *Patrzykat* et al., 2002) and inhibition of bacterial enzymes (*Nishikata* et al., 1991; *Couto* et al., 1993; *Andreu* and *Rivas*, 1998).

Mode of action of defensins

Several lines of evidence argue for a hypothesis involving the cytoplasmic membrane as the bactericidal target for defensins:

(i) Defensins (HNP-1) sequentially permeabilise the outer and inner membrane of *E. coli* (*Lehrer* et al., 1989),
(ii) Defensins (HNP-1) form voltage-dependent channels in artificial membranes (*Kagan* et al., 1990),
(iii) Defensins induce leakage of cytoplasmic content (*Lehrer* et al., 1989; *Cociancich* et al., 1993),
(iv) Defensins induce leakage of vesicle content from negatively charged liposomes (*Wimley* et al., 1994),

Table 5: The main models describing the mode of action of antibacterial peptides

Cytoplasmic membrane					Cellular functions			
Permeabilising		Poreforming			DNA, RNA, proteins		Bacterial enzymes	
carpet	thinning	barrel stave	worm-hole	two-state	binding	synthesis	pseudosubstrate	binding
cecropins LL-37	magainin 2 protegrin 1 Melittin	alamethicin "defensins"	magainins	magainins protegrin 1	buforin II pyrrhocoricin	PR-39 indolicidin apidaecin	histatin	NAP-2
1,2	3,4,5,6	7,8,9,10,11	12,13,14	15,16	17,18	19,20,21,22	23,24	25,26

1: Gazit et al., 1995
2: Shai, 1999
3: Oren et al., 1999
4: Ludtke et al., 1995
5: Berneche et al., 1998
6: Heller et al., 2000
7: Wimley et al., 1994
8: Ludtke et al., 1996
9: Bechin-ger, 1999
10: Oren et al., 1999
11: Shai, 1999
12: Ludtke et al., 1995, 1996
13: Matsuzaki et al., 1996
14: Matsuzaki, 1998
15: Ludtke et al., 1995
16: Huang, 2000
17: Park et al., 1996, 1998
18: Kragol et al., 2001
19: Agerberth et al., 1991
20: Boman et al., 1993
21: Subbalakshmi and Sitaram, 1998
22: Castle et al., 1999
23: Nishikata et al., 1991
24: Andreu and Rimas, 1998
25: Couto et al., 1993
26: Andreu and Rimas, 1998

(v) Defensins (HNP 1-3) are active against enveloped viruses, but not against non-enveloped viruses (*Daher* et al., 1986),
(vi) The effect of defensins is abolished by membrane-depolarising agents (*Lehrer* et al., 1988), and
(vii) Metabolically active microbes are more susceptible to human α-defensins than resting microbes (*Lehrer* et al., 1989).

However, NMR studies have shown that it is not possible to use the same model to describe the mode of action for all defensins (*Hoover* et al., 2001). HNP-3 dimers cannot be modelled using HBD-2 monomers, and HBD-1 monomers cannot be arranged into HBD-2 or HNP-3 type dimers. Hence, the exact mechanism of all defensins is not known, and there are currently two models describing the mode of action of defensins. One model describes the formation of multimeric pores in the cytoplasmic membrane (*Wimley* et al., 1994), and the other involves non-specific electrostatic interactions between negatively charged moieties in the membranes and the positive charges of the side chains of defensin molecules (*Hill* et al., 1991). Both mechanisms may lead to permeability changes, cell rupture/lysis and death.

The multimeric pore

Wimley et al. (1994) have published the results of an extensive study performed on HNP-2. HNP-2 binds to negatively charged vesicles through electrostatic interactions, induce fusion of the outer monolayer of vesicles, and cause leakage of vesicle content through pores with a maximum diameter of approximately 25 Å. The authors further present a multimeric model of such a pore made from HNP-2 molecules, based upon the crystal structure of defensins showing dimers with the form of a basket (*Hill* et al., 1991). This basket has a hydrophobic bottom and a polar top. The model pore is composed of 6 defensin dimers arranged with the polar basket tops lining a ~20 Å pore. The hydrophobic basket bottom face outwards towards the bilayer of the membrane. This channel allows the leakage of rather large molecules (up to ~4,400 Da).

Non-specific electrostatic interactions

Aley et al. (1994) reports of cell aggregation and dramatic changes in morphology of *Giardia lamblia* trophozoites after exposure to HNP-1. The mode of action was interpreted to involve binding and lysis, an event that appeared to involve charge interactions. Further, the high-resolution crystal structure of HBD-2 show that peptide monomers are capable of forming an octameric structure with a uniform positively charged outer surface (*Hoover* et al., 2000). However, the structural and electrostatic properties of the HBD-2 octamer support an electrostatic charge-based mechanism of membrane permeabilisation by beta-defensins, rather than a mechanism based on formation of bilayer-spanning pores.

Electrostatic interactions may lead to cell death through a detergent like effect, where the formation of a carpet of peptide molecules in the membrane results in membrane disruption at a critical ratio of lipid:peptide (*Shai*, 1999). The interactions may also cause separation of the polar lipid head groups of the phospholipids in the cytoplasmic membrane, as they are pushed aside by the hydrophobic residues of the membrane associated peptide molecules (*Ludtke* et al., 1995). As a result, gaps will be formed between the head groups, inducing physical stress on the bacterial cytoplasmic membrane, and result in the collapse of the membrane.

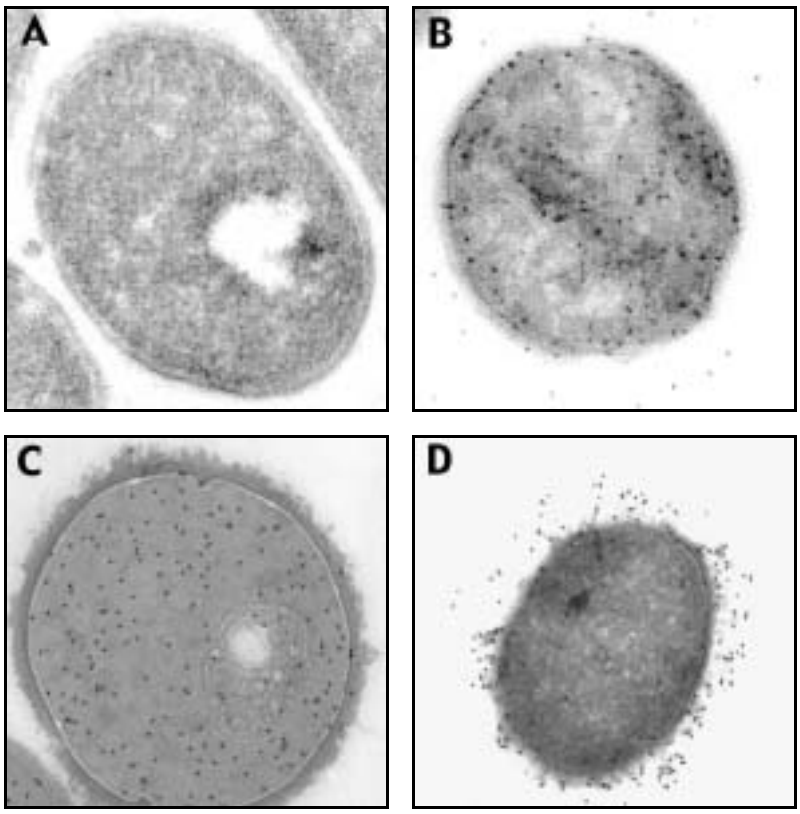

Figure 1: Electron micrographs of bacteria exposed to antimicrobial peptides, immunolabelled with polyclonal antibodies towards the respective peptide, and further visualised with gold-marked protein A. Panel A; Negative control (*E. coli* not exposed to any peptide). Panel B; *E. coli* exposed to magainin 2 for 30 minutes. Panel C; *S. aureus* exposed to lactoferricin B for one hour. Panel D; *E. coli* exposed to cecropin P1 for 30 minutes. The micrographs have previously been published by *Haukland* et al. (2001).

Targets other than the membrane

The idea of antimicrobial peptides as multi-target substances is growing. Results involving other effects than those of the cytoplasmic membrane have been published. For example, in addition to its permeabilising effects, HNP-1 also causes a reduction in bacterial macromolecular biosynthesis and a drop in the colony count (*Lehrer* et al., 1989). Further, the magainins have been extensively studied as pore-forming peptides (*Matsuzaki*, 1998), and several models have been used to explain the interaction between magainin and the cytoplasmic membrane (see Table 5). Despite these effects, *Haukland* et al. (2001) have shown that magainin 2 are capable of residing in the bacterial cytoplasm (Figure 1b). Cecropin P1 does not exhibit this feature, and are confined to the bacterial cell wall (Figure 1d), consistent with the carpet model proposed for the mode of action of this peptide (*Gazit* et al., 1995).

While proposing the model of the multimeric pore, *Wimley* et al. (1994) also points out that the actual *in vivo* mechanism for cell leakage may involve

at least three steps. These included the initial interaction of monomeric defensins and cell surface through electrostatic interactions, the oligomerisation of defensins, and at last pore formation. Translocation of the peptide via pore formation is possible as a fourth step, allowing the peptide to interfere with any intracellular process.

Evidence for the translocation of several antimicrobial peptides are accumulating, and involve magainin 2 (*Haukland* et al., 2001), lactoferricin B (*Haukland* et al., 2001), and buforin (*Park* et al., 1998). For defensins, *Sharma* and *Khuller* (2001) showed that HNP-1 is an efficient inhibitor of DNA-synthesis in *Mycobacterium tuberculosis*. They suggest that the cytoplasmic membrane is the primary target for HNP-1. Binding to this target causes permeabilising of the membrane, and thus enhanced access to the secondary, intracellular target.

Lichtenstein (1991) has also made the proposal of two targets for defensins. Working on tumour cells, they report that initial effects on the plasma membrane were not sufficient for subsequent lysis. A second phase was required which involved the continued presence of defensin. They conclude that there is two phases of interaction between defensins and tumour cells, where the initial effect is on the cell membrane, and the second phase is mediated intracellularly by defensin internalised through a permeabilised membrane. A two-phased bactericidal activity is also proposed for HBD-2 and *E. coli* (*Tomita* et al., 2000). Lactoferricin B also interacts with membranes (*Ulvatne* et al. 2001), and can be traced into the cytoplasm at sub-inhibitory concentrations (Figure 1c) (*Haukland* et al., 2001). Unpublished results show that lactoferricin B have an effect of macromolecular biosynthesis (*Ulvatne* et al., in prep.).

CONCLUSION

At this point, there is no doubt that most antimicrobial peptides, including the defensins, are membrane active molecules. Through their interaction with the cytoplasmic membrane, they may cause severe damage to the bacterial cell and cell death. It is likely that bacteria may compensate for the formation of pores/channels in the membranes, while a detergent like effect is irreversible since it involves a complete rupture of the bacterial integrity. The bacterial cell is not a closed system, and the cell is in some kind of equilibrium with its surroundings through sensing systems. Transport of nutrients, waste products, and other extracellular products are constantly crossing the cytoplasmic membrane, and some of this transport happens through pores. An efficient killing by pores must therefore be swift, rapid and sudden, to ensure that the bacteria do not initiate a defence response. Antimicrobial peptides may, by utilising another secondary target, be even more efficient in the battle against pathogenic bacteria.

Due to the fact that most antimicrobial peptides also exhibit other effects, the *in vivo* effect and exact mode of action of antimicrobial peptides are hard to elucidate (*Scott* and *Hancock*, 2000). The other effects involve interactions with host cells to stimulate gene-expression from genes encoding transcription factors, chemokines, chemokine receptors, integrins etc, products that also are part of the innate immunity (*Hancock* and *Rozek*, 2002). Antimicrobial peptides are therefore multi-functioning ef-

fector molecules involved in the delicate balance between microbes and host, and their *in vivo* role must be regarded as the whole interplay between the different functions the peptides may have. Therefore, further studies on defensins and other antimicrobial peptides must be performed in order to understand the *in vivo* antibacterial mode of action.

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