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1 *Type of the Paper (Review)*

2 **pH dependent antimicrobial peptides and proteins,**  
3 **their mechanisms of action and potential as**  
4 **therapeutic agents**

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12

13 **Abstract:** Antimicrobial peptides (AMPs) are potent antibiotics of the innate immune system that  
14 have been extensively investigated as a potential solution to the global problem of infectious  
15 diseases caused by pathogenic microbes. A group of AMPs that are increasingly being reported are  
16 those that utilise pH dependent antimicrobial mechanisms and here, we review research into this  
17 area. This review shows that these antimicrobial molecules are produced by a diverse spectrum of  
18 creatures, including vertebrates and invertebrates, and are primarily cationic although a number of  
19 anionic examples are known. Some of these molecules exhibit high pH optima for their  
20 antimicrobial activity but in most cases, these AMPs show activity against microbes that present  
21 low pH optima, which reflects the acidic pH generally found at their sites of action, particularly the  
22 skin. The modes of action used by these molecules are based on a number of major structure /  
23 function relationships, which include metal ion binding, changes to net charge and conformational  
24 plasticity, and primarily involve the protonation of histidine, aspartic acid and glutamic acid  
25 residues at low pH. The pH dependent activity of pore forming antimicrobial proteins involves  
26 mechanisms that generally differ fundamentally to those used by pH dependent AMPs, which can  
27 be described by the carpet, toroidal pore and barrel-stave pore models of membrane interaction. A  
28 number of pH dependent AMPs and antimicrobial proteins have been developed for medical  
29 purposes and have successfully completed clinical trials, including kappacins, LL-37, histatins and  
30 lactoferrin, along with a number of their derivatives. Major examples of the therapeutic application  
31 of these antimicrobial molecules include wound healing as well as the treatment of multiple cancers  
32 and infections due to viruses, bacteria and fungi. In general, these applications involve topical  
33 administration, such as the use of mouth washes, cream formulations and hydrogel delivery  
34 systems. Nonetheless, many pH dependent AMPs and antimicrobial proteins have yet to be fully  
35 characterized and these molecules, as a whole, represent an untapped source of novel biologically  
36 active agents that could aid fulfillment of the urgent need for alternatives to conventional antibiotics,  
37 helping to avert a return to the pre-antibiotic era.  
38

39 **Keywords:** antimicrobial peptides and proteins; pH dependent antimicrobial activity; invertebrates;  
40 vertebrates;

41 **PACS:** J0101  
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43 **1. Introduction**

44 A multiplicity of synergistic factors, including diminished pharmaceutical investment, clinical  
 45 over-prescription and misuse by the food industry has led to the increasing occurrence of microbial  
 46 pathogens with multiple drug resistance (MDR) and rendered infectious diseases the leading cause  
 47 of global mortality [1]. This bleak situation led the World Health Organization (WHO) to recently  
 48 predict that the uncurbed rise of MDR pathogens could see conditions in the 21st Century return to  
 49 those of the pre-antibiotic era when no antimicrobials were available for the treatment of many  
 50 common diseases [2]. In response, a major analysis by the WHO and a report from the O'Neill review,  
 51 sponsored by the UK Government, have concluded that the problem of antimicrobial drug resistance  
 52 can only be fully addressed by a coordinated global approach that operates through a number of  
 53 major interventions (Table 1) [3]. In particular, intervention six (Table 1) proposed the urgent  
 54 development of novel products and strategies that could provide alternatives to conventional  
 55 antibiotics, which has generated intensive research into antimicrobial design [4]. Examples of this  
 56 research range from revisiting old anti-infective strategies, such as phage therapy, which was popular  
 57 in Eastern European countries in the early 20th Century [5], to recently reported antimicrobial  
 58 strategies, such as the development of compounds whose antibiotic activity can be regulated by light  
 59 and sound [4,6-8]. One particularly promising approach proposed by O'Neill [3] was the therapeutic  
 60 development of antimicrobial peptides (AMPs), which are potent antibiotics of the innate immune  
 61 system [9,10]. The activity of these peptides against microbes involves relatively non-specific modes  
 62 of action at multiple sites with the result that microbial resistance to AMPs has a low incidence and  
 63 is generally due to inherent rather than adaptive mechanisms [11]. Based on these observations, the  
 64 generally held view is that microbial resistance to AMPs is unlikely to approach those of conventional  
 65 antibiotics, endowing these peptides with a major medical advantage [10], and currently, a number  
 66 of AMPs are in clinical trials (Table 2).  
 67

68 **Table 1:** Major areas of intervention to combat antimicrobial drug resistance

1.	A global public awareness campaign.
2.	Improve sanitation and hygiene to prevent the spread of infection.
3.	Reduce the unnecessary use of antimicrobials in agriculture and their dissemination in the environment.
4.	Improve the global surveillance of drug resistance and antimicrobial consumption in humans and animals.
5.	Promote new rapid diagnostics to reduce use of unnecessary antimicrobials.
6.	Promote the development and use of vaccines and alternatives
7.	Improve the number, pay and recognition of people working in infectious disease.
8.	A global innovation fund for early stage and non-commercial research and development.
9.	Better incentives to promote investment for new drugs.

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Table 1 was derived from [3]

**Table 2:** Major examples of AMPs in clinical trials or in development

Antimicrobial peptides	Indication	Phase	Company
Pexiganan (MSI-78), an analogue of magainin.	Topical cream for the treatment of diabetic foot infections and ulcers.	3	Dipexium Pharma /MacroChem / Genaera
Isegran (IB-367), a derivative of protegrin 1.	Mouthwash for the treatment of chemotherapy induced oral mucositis.	3	Ardea Biosciences / national Cancer Institute.
	Mouthwash for the treatment of ventilator-associated pneumonia.	3	IntraBiotics Pharmaceuticals.
PAC-113 (P-113) a synthetic derivative of histatin 3 and histatin 5.	Oral gel for the treatment of candidiasis		Pacgen Biopharmaceuticals
Omiganan (MBI 226, MX-226, CSL-001), an analogue of indolicidin.	Topical cream for the treatment of skin antisepsis, prevention of catheter infections / Rosacea.	3	Mallinckrodt / Cutanea Life Sciences, Inc.
	Topical cream for the treatment of usual type vulvar intraepithelial neoplasia / moderate to severe inflammatory acne vulgaris / mild to moderate atopic dermatitis.	3	Cutanea Life Sciences, Inc.
OP-145, a derivative of LL-37.	Ear drops for treatment of chronic bacterial middle-ear infection.	2	OctoPlus
hLF1-11, a derivative of lactoferrin.	Intravenous administration for treatment of neutropenic stem cell transplantation patients. Prevention of bacteraemia and fungal infections.	1/2	AM Pharma.
Brilacidin, (PMX-30063), a defensin mimetic.	Intravenous administration for treatment of acute bacterial skin and skin structure Infection caused by Gram-positive bacteria, including methicillin-resistant <i>Staphylococcus aureus</i> (MRSA).	3	Cellceutix.
	Oral rinse for the treatment of ulcerative mucositis associated with chemo / radiation therapy of cancer.	2	Cellceutix.
Arenicins, naturally occurring AMPs.	For the treatment of infections due to MDR Gram-positive bacteria.	Preclinical	Adenium Biotech
Novexatin (NP213), a synthetic AMP.	Brush on treatment for fungal infections of the toenail.	1/2	NovaBiotics
C16G2, a synthetic specifically targeted AMP.	Mouthwash for the treatment of tooth decay caused by <i>Streptococcus mutans</i>	2	C3 Jian, Inc.
Lytixar (LTX-109), a peptidomimetic.	Topical antibiotic for the treatment of nasal carriers of MRSA.	1/2	Lytix Biopharma.
	Topical cream for the treatment of infections due to Gram-positive bacteria.	2	Lytix Biopharma.

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Table 2 was derived from [12-15]

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In order to develop AMPs as medically relevant anti-infective agents, there have been numerous investigations into their antimicrobial mechanisms, which to date has shown that membrane interaction is a requirement for virtually all of these mechanisms [9,10,16,17]. These investigations have also shown that there are a number of major drivers in the membrane interactions of AMPs of which the most important are charge, hydrophobicity and amphiphilicity [9,18,19]. The vast majority of AMPs are cationic to help facilitate the targeting of microbes through direct electrostatic interaction with anionic components of their membranes [19,20]. Nearly all AMPs are also amphiphilic, which generates hydrophobic surfaces that are able to drive the partitioning of these peptides into microbial membranes and hydrophilic surfaces that are able to stabilize these hydrophobic interactions via electrostatic associations with the head group regions of these membranes [21,22]. Based on these investigations, a variety of models have been proposed to describe the antimicrobial action of AMPs with those most frequently reported appearing to be variants of the barrel stave pore and carpet type mechanisms, which involve membrane disruption via discrete channel formation and non-specific solubilisation respectively [23].

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There have been many advances in understanding the mode of action used by AMPs but although a number of earlier studies showed that pH can modulate the antimicrobial activity of these peptides, no major review of this area of research appears to have been presented in the literature [24-28]. However, it is now becoming increasingly clear that pH is a major driver in the membrane interactions and biological activity of not only many AMPs but also a number of antimicrobial proteins produced by eukaryotes (Table 3). To update on these antimicrobial molecules, here, we present an overview of recent progress in the understanding of their modes of action along with the development of their therapeutic and biotechnological potential.

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## 2. An overview of pH dependent peptides and proteins with antimicrobial activity

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In the 1980s and 1990s, a series of seminal studies, including work on the African clawed frog, *Xenopus laevis*, and a number of mammals, led to what many take to be the first major description of eukaryotic AMPs such as magainins, defensins and SAAPs [29]. However, in 1956, phagocytin from humans, rabbits, horses and guinea pigs was reported to exhibit non-membranolytic activity against a range of Gram-positive and Gram-negative bacteria that was enhanced by low pH [24,25]. The peptide was not characterised or further investigated and today, it is not even known as to whether phagocytin was rediscovered later and given an alternative name [30]. However, it would appear to be a matter of historical fact that what was most likely the first AMP to be reported from eukaryotes showed a pH dependent mode of action [24,25]. Since these earlier studies, it is now known that these peptides are produced by virtually all multicellular organisms [31,32] and that an increasing number of these molecules possess pH-dependent activity (Table 3).

**Table 3.** AMPs with pH dependent activity.

Vertebrates	AMPs	Host organism	Key references
Fish	Gaduscidin-1 and gaduscidin-2	<i>Gadus morhua</i>	[33,34]
Amphibians	Chensinin-1	<i>Rana chensinensis</i>	[35,36]
	Esculentin-2EM	<i>Glandirana emeljanovi</i>	This work
	Dermaseptin PD-3-7	<i>Pachymedusa dactylos</i>	[37]
Humans	Phagocytin		[24,25]
	Psoriasin		[38-40]
	$\beta$ -microseminoprotein		[41].
	LL-37		[42]
	Hep-25 and hep-20		[43-48]
	Histatins		[49,50]
	Lactoferrin		[51]
	DCD-1(L)		[52-54]
	Kappacin A and kappacin B		[55,56]
Rabbits	Phagocytin		[24,25]
	Platelet microbiocidal proteins		[57]
	NP1 and NP2		[58,59]
Horses	Phagocytin		[24,25]
Guinea pigs	Phagocytin		[24,25]
Mice	CRAMP		[42]
Cattle	Lactoferricin B		[60,61].
Invertebrates	AMPs	Host organism	Key references
Marine	Myticin C	<i>Mytilus galloprovincialis</i>	[62,63]
	KPS-1	<i>Atrina pectinate</i>	[64]
	Ci-PAP-A22 and Ci-MAM-A24	<i>Ciona intestinalis</i>	[65-68]
	Clavaspilin and clavansins	<i>Styela clava</i>	[69-80]
	Styelins	<i>Styela clava</i>	[72,73,81]
Terrestrial	Hebraein	<i>Amblyomma hebraeum</i>	[82]
	Amoebapores	<i>Entamoeba histolytica</i>	[83-87]
	Acanthaporin	<i>Acanthamoeba culbertsoni</i>	[88]
	Caenopores	<i>Caenorhabditis elegans</i>	[89-93]

## 111 2.1. Fish

112 Gaduscidin-1 (gad-1) and gaduscidin-2 (gad-2) were AMPs identified in the Atlantic cod, *Gadus*  
113 *morhua* and shown to be highly, constitutively expressed in immune-relevant tissues [94,95]. Low pH  
114 was found to enhance the activity of both peptides against *Escherichia coli*, which appeared to involve  
115 membrane interaction, but interestingly, although gad-1 and gad-2 were predominantly  $\alpha$ -helical at  
116 neutral pH, acid conditions led to a large decrease in the levels of  $\alpha$ -helicity possessed by these  
117 peptides [33]. These results contrast with most  $\alpha$ -helical AMPs where an enhanced capacity for  
118 membranolytic and antimicrobial activity is generally associated with increased levels of this  
119 secondary structure [96,97]. It was proposed that gad-1 and gad-2 each possessed a structural  
120 plasticity, which facilitated an appropriate balance between amphiphilic and mixed hydrophobic /  
121 hydrophilic structural features that promoted maximal levels of membrane interaction and  
122 antibacterial activity [33]. Gad-1 and gad-2 were found to be histidine rich AMPs and the enhanced  
123 capacity of these peptides for membranolytic and antimicrobial activity at low pH appeared to

124 involve these residues [33]. It is well established that histidine (pKa 6.5) is uncharged at physiological  
125 pH but fully positively charged at low pH, thereby enhancing the potential of AMPs for interaction  
126 with anionic membranes under acid conditions [50,98]. However, there also appeared to be a complex  
127 relationship between the level of histidine residues possessed by these AMPs and their pH dependent  
128 capacity for membrane interaction [33,34]. In response, molecular dynamic simulation studies were  
129 undertaken and predicted that the number of sequential histidine pairs contained by gad-1 and gad-  
130 2 were important to their ability for membrane disruption [34]. These AMPs possess one and two of  
131 these histidine pairs respectively [94] and the N-terminal regions of both peptides, which included  
132 this motif, were preferentially located proximal to membrane channels with which gad-1 and gad-2  
133 were associated [34]. Based on the topology of the peptide-lipid interactions mediating the formation  
134 of these channels, it was suggested that the antimicrobial action of gad-1 and gad-2 may involve the  
135 use of a disordered toroidal pore type mechanism of membrane disruption [34,99,100].

## 136 2.2. Amphibians

137 Chensinin-1 is a histidine rich peptide produced by the frog, *Rana chensinensis* and recent  
138 studies showed that low pH enhanced the positive charge of the peptide [35] and thereby, its ability  
139 to kill Gram-positive bacteria, such as *Bacillus cereus* [36]. This antibacterial activity appeared to  
140 involve the adoption of an extended structure, similar to that of other AMPs that are rich in specific  
141 residues [101,102], which induced lysis of the *B. cereus* membrane [36]. Interestingly, the peptide  
142 showed no activity against Gram-negative bacteria [36,103], which appeared to involve high affinity  
143 binding between chensinin-1 and lipopolysaccharide (LPS) in the outer membrane of these bacteria  
144 [103]. Maximin H5 from the toad, *Bombina maxima*, was also recently found to be ineffective against  
145 Gram-negative bacteria due to high affinity binding to phosphatidylethanolamine (PE) in the  
146 cytoplasmic membrane (CM) of these organisms [104,105]. Similar PE mediated mechanisms have  
147 been proposed to mediate the resistance of microbes to other AMPs [104,105], supporting the growing  
148 view that receptors could play a variety of roles in the biological activities of these peptides [23,98,106-  
149 108]. Esculentin-2EM (E2EM, previously gaegurin 4) is an  $\alpha$ -helical peptide isolated from the frog,  
150 *Glandirana emeljanovi* (formerly *Rana rugosa*) KIM [109,110], that is able to kill protozoa, fungi, Gram-  
151 positive bacteria and Gram-negative bacteria [110-112]. E2EM possesses a C-terminal cyclic region  
152 stabilized by a disulphide bond (Rana box) that is conserved across many ranid AMPs and helps  
153 stabilise pore formation by the peptide thereby promoting its antimicrobial action (Figure  
154 1)[109,110,112-115]. Several models have been proposed to represent pore formation by E2EM and  
155 the best supported by experimental evidence appear to be the toroidal pore and barrel stave  
156 mechanisms (Figure 1). Here we present data showing that the linear reduced form of E2EM (E2EM-  
157 lin) possesses antimicrobial activity consistent with recent studies showing that the reduction of  
158 cysteine-stabilized AMPs to generate peptides with novel mechanisms of antimicrobial activity may  
159 form part of some innate immune systems [116,117]. Our results showed that E2EM-lin was active  
160 against both Gram-positive and Gram-negative bacteria and appeared to exhibit pH dependent  
161 antimicrobial activity, which parallels the molluscan cysteine stabilized AMPs, myticins, whose  
162 reduced forms were described above to show pH dependent antibacterial and antiviral action [62,63].  
163 It was found that under the low pH conditions associated with the skin of frogs [118], E2EM-lin had  
164 a general ability to induce the lysis of both anionic and zwitterionic membranes, which was enhanced  
165 at higher pH (Table 4). These data clearly suggest that the C-terminal disulphide bond in the E2EM  
166 Rana box region does not play a major role in its ability for membrane pore formation (Figure 1),  
167 which supports earlier work [114,115]. In the case of DMPG, which is a key component of membranes  
168 within Gram-positive bacteria [119], E2EM-lin induced relatively low levels of membrane lysis at acid  
169 pH (< 25%). However, a shift to alkaline pH led to a large increase in the lytic activity of the peptide  
170 to circa 95%, which was accompanied by a correspondingly large increase in its  $\alpha$ -helical content of  
171 circa 25% to give levels approaching 75% (Table 4). Previous studies have shown that E2EM-lin and  
172 E2EM adopt highly similar  $\alpha$ -helical structures in membranes and undergo oligomerization to form

173 pores [115,120]. Based on these data, we speculate that high pH may enhance the ability of E2EM-lin  
 174 to lyse DMPG membranes by increasing the potential of the peptide for pore formation through an  
 175 increased capacity for self-association. The segments of E2EM-lin involved in pore formation are  
 176 strongly amphiphilic  $\alpha$ -helices with wide hydrophobic faces that would be maximized by alkaline  
 177 pH, promoting the potential for the mutual interactions involved in the formation of multimeric  
 178 species (Figure 1). In the case of DMPE, which is often taken to represent the membranes of Gram-  
 179 negative bacteria [119], E2EM-lin induced high levels of membrane lysis at acid pH (60%). A move to  
 180 alkaline pH led to a relatively low increase in the lytic activity of the peptide, which was around 25%  
 181 and was accompanied by a decrease in its  $\alpha$ -helical content of 20% to give levels of circa 30% (Table  
 182 4). These data would seem to indicate that E2EM-lin uses a different mechanism of lysis in the case  
 183 of DMPE membranes and it has previously been suggested that the peptide may adopt a number of  
 184 lipid interactive forms [115]. The functional significance of our data is not fully clear but given the  
 185 very high levels of membrane lysis induced by E2EM-lin at higher pH for each lipid investigated,  
 186 biological relevance is suggested, which forms the basis of ongoing investigations.  
 187

188 **Table 4.** The  $\alpha$ -helical content and lysis levels of E2EM-lin

Lipid	pH	Lysis (%)	$\alpha$ -helicity (%)
DMPS	6	17	30
	8	63	49
DMPG	6	23	51
	8	94	73
DMPC	6	52	45
	8	73	15
DMPE	6	60	49
	8	83	31

189 The levels of lysis exhibited by E2EM-lin were determined using a calcein release assay and the levels of  $\alpha$ -  
 190 helicity shown by the peptide were measured using CD spectroscopy, all as previously described [105].

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Figure 1. Models for the membrane pore formation by E2EM

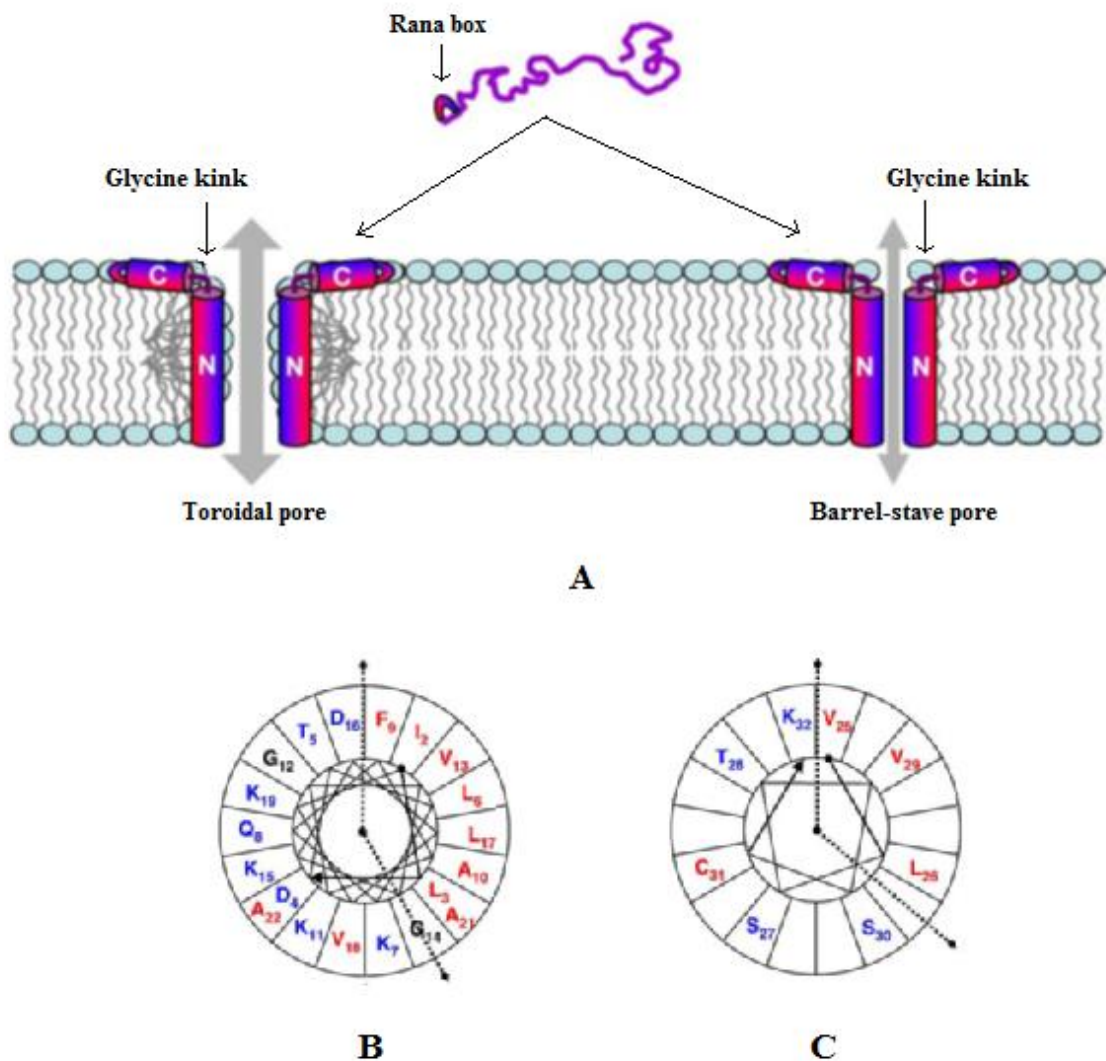


Figure 1 was revised from [115] and Figure 1A shows models for pore formation by E2EM, which are the toroidal pore and barrel stave mechanisms (Table 1) and are the best supported experimentally. In both models, the N-terminal 23 residues of the peptide spans the bilayer and a glycine kink orientates the 7 residue, C-terminal Rana box region of E2EM to lie parallel to the membrane surface. In this orientation, the Rana box region of the peptide, which is a cysteine stabilized macrocyclic structure, interacts with the lipid head-group region of the membrane and stabilises pore formation by E2EM [115]. The major difference between these models is that in the toroidal pore mechanism, the membrane leaflets deform to allow the lipid head-group region to remain in contact with the hydrophilic face of the E2EM membrane spanning region, which is not observed in the barrel stave mechanism [23]. For clarity, two monomers of E2EM are shown in the schematic pore above but oligomers formed by between five and ten peptide molecules have been proposed [115,120]. Similar models of membrane interaction appear to apply to the linear reduced form of the peptide [115], which is represented in our studies as E2EM-lin. Figures 1B and 1C show two-dimensional axial projections [121] for the membrane spanning region and Rana box domain of E2EM, respectively, that are involved in pore formation by the peptide. In both cases, these segments for amphipilic  $\alpha$ -helices with wide hydrophobic faces that our data suggest would be maximised by alkaline pH, thereby promoting the potential for the mutual interaction of E2EM monomers and the formation of multimeric species involved in pore formation.

218 In addition to cationic AMPs, anionic AMPs with pH dependent activity have also been reported  
219 for amphibians, such as dermaseptin PD-3-7, which was isolated from the frog, *Pachymedusa*  
220 *dacnicolor* [122]. In aqueous solution, the peptide showed an inherent propensity to adopt an extended  
221 conformation and self-assemble into amyloid fibrils in a reversible pH-controlled manner [37]. At  
222 low pH, dermaseptin PD-3-7 existed as amyloid-like  $\beta$ -sheet aggregates but at higher pH underwent  
223 morphological changes, which led to the formation of metastable amorphous aggregates in a manner  
224 that appeared to be mediated by deprotonation of the aspartic acid residues (pKa 3.9) and C-terminal  
225 carboxyl groups possessed by the peptide. These amorphous aggregates induced damage to cells of  
226 the insect, *Spodoptera frugiperda*, by an unidentified mechanism but showed no activity against *E. coli*  
227 and *Bacillus subtilis* [37]. Based on these observations, it was suggested that amyloid formation by  
228 dermaseptin PD-3-7 may act as a storage facility for the peptide similar to the depository function  
229 proposed for the amyloidogenesis of pituitary peptide hormones [123]. Triggered by an increase in  
230 pH, this storage facility would release a pre-formed, cytotoxic agent that contributed to the natural  
231 defence strategy of the host amphibian [37]. However, it is worthy of note that the peptide was only  
232 tested for activity against a small number of bacteria [37] and it is generally accepted that AMPs are  
233 promiscuous in their antimicrobial mechanisms [29,124] with amyloid-mediated antibacterial  
234 mechanisms increasingly being reported [23,125]. Interestingly, more recent studies on dermaseptin  
235 PD-3-7 have shown that stereochemical modification of the peptide's second residue to form the  
236 diastereomer [d-Leu2] strongly influenced the pH-triggered, morphological changes involved in  
237 amyloid formation by dermaseptin PD-3-7, inducing a fundamental change in its superstructural  
238 organization that was related to differences between the conformational propensities of these epimers  
239 [126]. It was proposed by these latter authors that epimers of PD-3-7 may play a role as anionic AMPs,  
240 or defence molecules, in the innate immune system of *P. dacnicolor* [126] and a similar proposal has  
241 been made for the production of epimeric AMPs by other frogs and toads [9].  
242

### 243 2.3. Humans and other mammals

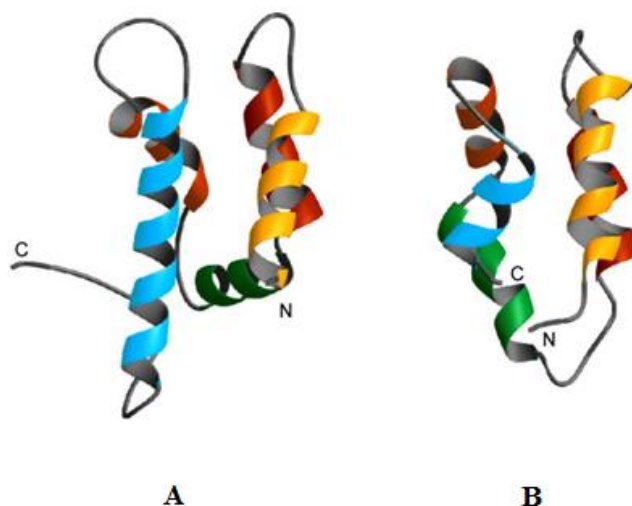
244 Psoriasin (S100A7) is a human, cysteine stabilized  $\alpha$ -helical protein [127] of the S100 family of  
245 signalling proteins [128-130] that is known to have a role in the antimicrobial defence of the skin,  
246 including serving as a multifunctional modulator of neutrophil activation [131,132]. The protein was  
247 investigated for antimicrobial activity and shown to kill *E. coli* using pH independent mechanisms  
248 that were primarily due to the depletion of  $Zn^{2+}$  [38,39] and more recently, the protein was identified  
249 in vaginal fluid, appearing to help protect the female genital tract from infection [40]. Psoriasin was  
250 also found to kill *Bacillus megaterium* but via the use of two different modes of action, which involved  
251  $Zn^{2+}$  depletion at neutral pH but membrane pore formation and oligomerisation of the protein at  
252 low pH. This pore forming mechanism was not further investigated but evidence suggested that it  
253 was likely to show some similarities to a barrel-stave pore type mode of action [38,39]. It is interesting  
254 to note that psoriasin exhibits a number of structural and functional similarities to amoebapore A  
255 (Figure 2) [39,133], which is a pH dependent antimicrobial protein from the protozoa, *Entamoeba*  
256 *histolytica* that is discussed below [85]. In particular, psoriasin possesses a histidine residue in its C-  
257 terminal region [127] similar to amoebapore A [86] and based on these similarities, it can be  
258 speculated that the enhanced action of psoriasin against *B. megaterium* at low pH may involve a  
259 histidine mediated increased ability for pore formation and oligomerisation.  $\beta$ -microseminoprotein  
260 (MSP), also named as PSP-94, is a human protein that is believed to have a protective role in prostate  
261 carcinogenesis due to its ability to suppress the growth of tumours although more recent studies have  
262 suggested that MSP may protect against prostate cancer by inhibiting fungal infection in this genital  
263 region [134,135]. This suggestion was primarily based on recent work, which showed that the acid  
264 conditions of the vagina promoted the ability of MSP in post coital seminal plasma to kill *Candidia*  
265 *albicans*. This antifungal activity appeared to involve lysis of the organism's membranes and to be  
266 mediated by a C-terminal fragment of MSP, which included a glutamic acid residue involved in the

267 ability of the protein to form coordinate bonds with Ca<sup>2+</sup>. It appeared that MSP coordination of Ca<sup>2+</sup>  
268 at neutral pH inhibited the antifungal activity of the protein but at low pH, electrostatic interaction  
269 between the ion and the C-terminal glutamic acid of MSP (pKa 4.1) decreased, facilitating the ability  
270 of the protein to kill *C. albicans*. Porcine MSP appeared to use a similar pH dependent antifungal  
271 mechanism, suggesting that it may be a widespread innate immune factor active against *C. albicans*  
272 and possibly helping to explain the low sexual transmission rate of vulvovaginal candidiasis in  
273 humans [41]. *C. albicans* was also reported to be susceptible to the pH dependent activity of LL-37  
274 and its derivatives, KS-30, and RK-31, along with CRAMP [42], which is a murine homologue of LL-  
275 37 [136]. It appeared that KS-30, and RK-31 were produced by the proteolytic cleavage of LL-37 in the  
276 low pH environment of human sweat and that these pH conditions enhanced the ability all the  
277 peptides tested to kill *C. albicans* via permeabilisation of the fungal membrane. The use of animal  
278 models showed that although LL-37 and its derivatives were induced in murine skin in response to  
279 *C. albicans* infection, this induction did not confer subcutaneous resistance to the organism [42]. Based  
280 on these results, it was suggested that these peptides may be of primary importance in forming a  
281 barrier against fungal infections on the skin surface [42], given that the dysregulated production of  
282 LL-37 and its derivatives has been strongly associated with skin disease due to fungi and other  
283 microbes [137-139]. Hepcidin, (hep-25) is a human  $\beta$ -sheet hormone that has a well-established role  
284 in iron homeostasis [140] and has been shown to exhibit pH dependent antimicrobial activity [43]. In  
285 particular, acid conditions been shown to enhance the activity of hep-25 and several of its isoforms,  
286 such as hep-20, against the fungal pathogen, *Candida glabrata* [44], along with a range of Gram-  
287 negative bacteria, such as *Pseudomonas aeruginosa*, and Gram-positive bacteria, including  
288 *Enterococcus faecium* [45,46]. Studies on *E. coli* suggested that the antibacterial action of these  
289 peptides involved membranolytic mechanisms which were enhanced under acid conditions due to  
290 the presence of histidine residues within their primary structure [47]. Based on these observations, it  
291 was suggested that with acidic pH, the increase in positive charge of histidine residues in hep-25 and  
292 hep-20 would promote their ability to target and lyse bacterial membranes, resulting in the death of  
293 the host organism [43]. Hep-20 has also been shown to be effective against drug resistant *C. glabrata*  
294 under the low pH and physiological conditions associated with the vagina, which led to the  
295 suggestion that the peptide may form the basis of novel therapeutics for the control of vaginal  
296 infections due to the organism [48]. Another group of human AMPs rich in histidine residues are the  
297 histatins (hst), which are salivary peptides with antiviral, antibacterial and antifungal activity [141-  
298 146] and protecting the oral cavity from fungal pathogens appears to be to the primary role of these  
299 peptides [147,148]. The pH of the oral cavity is mainly governed by that of saliva, which has a range  
300 that generally varies between pH 5 and pH 8 [149,150] although significantly lower pH values can  
301 occur on the surface of teeth due to the metabolic activities of cariogenic microorganisms [151], which  
302 is able to promote the growth of fungi and form mixed species biofilms [152,153]. Low pH appears  
303 able to enhance the antifungal activity of hst-1, hst-3 [154] and hst-5 [49], which, for example,  
304 enhances the positive charge carried by hst-5 and facilitates its translocation into fungal cells to attack  
305 intracellular targets [49]. However, a detailed description of the antifungal activity of these peptides  
306 is lacking and a number of mechanisms have been proposed [141,143-146], such as complex formation  
307 with iron to interfere with the cellular metabolism of the metal in fungi, such as *C. albicans* [155].  
308 Studies on human lactoferrin, which is a multifunctional iron-binding protein [156] found that low  
309 pH enhanced the ability of sub-lethal levels of the protein to kill *C. albicans*, through multiple  
310 mechanisms, including dissipation of the proton motive force (PMF) across the CM of the organism  
311 [51]. This pH effect was attributed to increased electrostatic interactions between the peptide and  
312 anionic components of the *C. albicans* membrane, thereby enhancing the ability of lactoferrin to  
313 partition into fungal CM and generate lesions associated with PMF dissipation and the peptide's  
314 antifungal action [51]. Sub lethal levels of lactoferrin have also been shown to be effective against  
315 biofilms of *P. aeruginosa* [157] and the protein was found able to synergise the activity of other  
316 antimicrobials against biofilms formed from *P. aeruginosa* and methicillin resistant *Staphylococcus*  
317 *aeus* (MRSA) [158,159]. It was proposed that this anti-biofilm activity was due to the iron-binding

318 properties of the protein [157] but this does not appear to be the only mechanism involved in this  
319 activity [160] and acidic pH is strongly associated with these sessile microbial communities [161].  
320 Lactoferricin B, which is a potent AMP derived from bovine lactoferrin [162], has also been shown to  
321 possess pH dependent antimicrobial activity, killing bacteria and *C. albicans* at low pH via  
322 mechanisms that appeared to involve membranolysis [60,61]. Low pH was also found to enhance the  
323 activity of platelet microbiocidal proteins, which were isolated from leporine platelets and showed  
324 activity against *Staphylococcus aureus*, *E. coli*, and *C. albicans* [57]. These results strongly supported the  
325 mounting evidence that platelets serve important multiple roles in host defence against infection,  
326 including the localized release of AMPs and other antimicrobial factors in response to microbial  
327 colonization and other stimuli [163]. The pH dependent antimicrobial mechanisms of platelet  
328 microbiocidal proteins were not further characterised but other studies showed that the antifungal  
329 and antibacterial activity of a number of these AMPs involved dissipation of the PMF across the  
330 cytoplasmic membrane of target organisms, which was able to synergise the activity of conventional  
331 antibiotics [164]. These results parallel those described above for human lactoferrin, allowing the  
332 speculation that low pH enhances the ability of some leporine platelet microbiocidal proteins to  
333 interact with membranes of *C. albicans* and generate lesions associated with PMF dissipation and the  
334 peptide's antifungal action. Other leporine AMPs shown to possess pH dependent activity are the  
335 defensins, NP1 and NP2, which were found to permeabilise the outer membrane of *P. aeruginosa* most  
336 efficiently at low pH although these peptides were ineffective against the organism under these pH  
337 conditions. These AMPs are present in leporine macrophages and it was suggested that this pH  
338 dependent membranolytic activity may synergise the antibacterial action of other defence molecules  
339 under the acid conditions associated with phagocytosis [58,59].

340

341 **Figure 2.** Similarities between the structures of psoriasin and amoebapore A



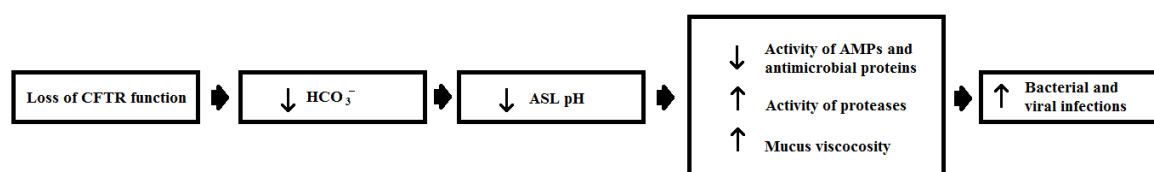
342

343 Figure 2 was revised from [39] and shows human psoriasin (A) and amoebapore A from the protozoa,  
344 *Entamoeba histolytica* (B). It can be clearly seen that these peptides show structural similarities and  
345 both have been shown to possess pH dependent mechanisms of antimicrobial activity that is  
346 enhanced by acid conditions [38-40,83-85,87]. In particular, psoriasin possesses a histidine residue in  
347 its C-terminal region [127] similarly to amoebapore A [86] and based on these similarities, it can be  
348 speculated that the enhanced antibacterial action of psoriasin at low pH may involve a histidine  
349 mediated increased ability for pore formation and oligomerisation.

350 A particularly important case of human AMPs with activity that can be influenced by pH is that  
351 found in the airway surface liquid (ASL) of individuals with cystic fibrosis (CF) [165,166], which is a  
352 lethal genetic disorder characterized by viscous mucus and bacterial colonization of the airways  
353 [167]. In the mammalian respiratory system, the ASL represents a first line of pulmonary defence by  
354 forming the interface between the environment and the host organism and helping to protect against  
355 the action of inhaled and aspirated bacteria by producing a variety of antimicrobial molecules  
356 [168,169]. These ASL molecules include AMPs, such as LL-37, HNP-1, HBD-1 and lactoferrin, along  
357 with antimicrobial proteins, such as lysozyme, surfactant protein A and surfactant protein D  
358 [165,166]. Many of these antimicrobial molecules also contribute to the pulmonary innate immune  
359 system by adorning lattices of extracellular DNA, chromatin, enzymes and other proteins to form  
360 neutrophil extracellular traps (NETs). These DNA complexes are released in response to the presence  
361 of microbial pathogens and provide a mechanism for the localised concentration of effector  
362 molecules. NETs have been reported able to eradicate microbial pathogens using a variety of  
363 mechanisms, including the action of antimicrobial attachments and proteolytic degradation, as well  
364 as neutralizing their activity by forming a physical barrier that prevents the dissemination of these  
365 pathogens [166,170-172]. More recent studies have shown that the Human Short Palate Lung Nasal  
366 Epithelial Clone 1 (SPLUNC1), which is a protein expressed in the upper airways of the lung, plays  
367 multiple roles in pulmonary innate immunity. These roles include: the direct inhibition of bacterial  
368 growth, the prevention of microbial biofilm formation and the regulation of other AMPs and  
369 antimicrobial proteins, such as LL-37, HBD-2 and lysozyme [173-176]. However, in CF, mutations in  
370 the cystic fibrosis transmembrane conductance regulator (CFTR) gene leads to reduced  $\text{HCO}_3^-$   
371 secretion and produces an abnormally acidic pH in ASL [177,178], which studies on humans and  
372 animal models have suggested can negatively affect the efficacy of ASL antimicrobial molecules and  
373 predispose CF airways to microbial infection (Figure 3) [165,179]. For example, recent studies on a  
374 porcine CF model showed that the low pH of the ASL inhibited the activity of LL-37, lactoferrin and  
375 other AMPs when directed against *S. aureus* and *P. aeruginosa* [180,181], which are known, major CF  
376 pathogens [182,183]. These acid conditions also reduced the ability of ASL AMPs to synergize their  
377 activities when in combination with each other and with antimicrobial proteins, such as lysozyme  
378 [180,181]. It has been further proposed that conditions of low pH in CF airways could reduce the  
379 efficacy of AMPs and antimicrobial proteins that adorn NETs [170] along with the antimicrobial and  
380 other biological activities of SPLUNC1 [179,184]. The mechanisms by which the low pH impairs the  
381 activity of AMPs and antimicrobial proteins in CF airways are currently unclear but it has been  
382 suggested that these mechanisms include a variety of contributions. For example, it has been  
383 proposed that low pH in CF airways may mediate the degradation of AMPs *via* the activation of host  
384 proteases, such as cysteine cathepsins, and microbial enzymes, such as aureolysin of *S. aureus* and  
385 elastase of *P. aeruginosa*, and the immobilization of AMPs through binding to mucins, which are large,  
386 anionic glycoproteins and the primary component of mucus. It has been further proposed that low  
387 pH in CF airways may induce conformational changes in AMPs that reduce the ability of these  
388 peptides to bind microbial membranes and cell wall components, such as lipid II  
389 [165,166,170,180,181,185]. In addition to reducing the activity of AMPs, low pH appears able to  
390 negatively impact on other defence factors of the ASL; for example, by increasing the rheological  
391 properties of secreted mucins, decreasing ciliary beat frequency, impairing phagocyte function and  
392 depleting ASL volume [165,166,179,185]. Based on these observations, it has been proposed that  
393 connections between the loss of CFTR, reduced ASL pH, and impaired CF host defense function  
394 could provide a paradigm for the identification of new therapeutic targets and strategies to reduce  
395 the morbidity associated with CF lung disease [165].

396

397

398 **Figure 3.** The pH of airway surface liquid and the pathogenesis of cystic fibrosis

399

400 Figure 3 was revised from [165] and shows a scheme for how changes in ASL pH may influence the  
 401 pathogenesis of CF. In CF, the loss of CFTR function results in decreased  $\text{HCO}_3^-$  conductance across airway  
 402 epithelial cells and leads to low pH in the ASL. Under these pH conditions, ASL AMPs, such as LL-37,  
 403 HNP-1, HBD-1 and lactoferrin, and antimicrobial proteins, such as lysozyme, surfactant protein A and  
 404 surfactant protein D, have reduced activity. Lower pH also leads to the increased viscosity of mucins,  
 405 decreased ciliary beat frequency, impaired phagocyte function and depleted ASL volume. These effects  
 406 lead to a decrease in the antimicrobial efficacy of the ASL and subsequently contribute to increased  
 407 respiratory infections in the CF airway, caused by both viral and bacterial pathogens [165,166,179,185].

408

409 A number of anionic AMPs with pH dependent activity have been identified in humans,  
 410 including DCD-1(L), which is proteolytically cleaved from dermcidin, which is also an anionic  
 411 peptide and found in human sweat [9,52,186,187]. DCD-1(L) is characterised by its broad range  
 412 antimicrobial activity, killing fungi, such as *C. albicans*, as well as Gram-positive bacteria, including  
 413 MRSA, Gram-negative bacteria, such as *Salmonella typhimurium*, and acid fast bacteria including  
 414 rifampin- and isoniazid-resistant *Mycobacterium tuberculosis* [188-193]. The peptide appears to exhibit  
 415 pH dependent antibacterial action [188] whereby low pH induces the peptide to adopt  $\alpha$ -helical  
 416 structure on the bacterial membrane surface, leading to ion channel formation *via*  $\text{Zn}^{2+}$  stabilized  
 417 DCD-1(L) oligomers and death of the host organism through membrane disruption [52,53]. A more  
 418 recent study gives general support to this model and suggested that under acid conditions, the  
 419 negative charge on DCD-1(L) becomes neutral, which facilitates membrane partitioning and  $\text{Zn}^{2+}$   
 420 dependent membrane channel formation *via* either a barrel-stave pore or a toroidal pore type  
 421 mechanism [54]. Another example of AAMPs with pH-dependent activity are kappacin A and  
 422 kappacin B, which are classed as food peptides and appear to be cleaved from  $\kappa$ -casein in bovine milk  
 423 by digestion with chymosin in the human stomach [194]. These peptides exhibit potent activity  
 424 against a range of Gram-positive and Gram-negative bacteria, including *Streptococcus mutans*,  
 425 *Porphyromonas gingivalis* and *Actinomyces lundii* [55,56], which make a major contribution to  
 426 supragingival dental plaques [195,196]. Characterisation studies showed that the antibacterial  
 427 mechanisms used by kappacins involved the pH-dependent lysis of microbial membranes, which  
 428 was enhanced by acid conditions [56]. The active region of kappacins included a phosphorylated  
 429 serine residue that was essential for antibacterial activity [56,197] and interestingly, these peptides  
 430 showed significantly different pH optima for this activity that resulted from a single residue  
 431 difference in the sequence of their active regions [55,197]. Kappacin A showed the highest  
 432 antibacterial activity of the two peptides and possessed an aspartic acid residue in its active region,  
 433 which was replaced by an alanine residue in the corresponding location of kappacin B [55,197,198].  
 434 The functional significance of this difference in sequences is not known although it has been proposed  
 435 that the additional negative charge possessed by kappacin A may enhance its ability to bind metal  
 436 ions [9]. It had been demonstrated that the presence of  $\text{Ca}^{2+}$  and  $\text{Zn}^{2+}$  ions enhanced the antibacterial  
 437 activity of kappacins and it was suggested that these ions may form a cationic salt bridge between  
 438 kappacins and anionic components of the bacterial membrane, thereby facilitating membrane  
 439 binding and antibacterial action [197]. It has been proposed that the membrane interactive  
 440 conformation of these peptides may be a proline-kinked amphiphilic  $\alpha$ -helix but conformational



441 changes observed in the peptide in the presence of membrane mimic could not be clearly assigned to  
442 any particular secondary structures and the structure of kappacins remains unclear [9,56,197,198].  
443

#### 444 2.4. Marine invertebrates

445 Myticin C (myt C) exists as a number of isoforms in the bivalve mollusc, *Mytilus galloprovincialis*  
446 [199-201] and has been shown to possess activity against fish viruses, including Hemorrhagic  
447 Septicaemia virus and Infectious Pancreatic Necrosis virus [202]. Several studies were conducted on  
448 the antimicrobial activity of a reduced form of myt C, (myt Cc, [62,63]) based on the proposal that the  
449 endogenous reduction of cysteine-stabilized AMPs to produce peptides with higher levels of  
450 antimicrobial activity may form part of some innate immune systems [116,117]. It was found that the  
451 antiviral activity of myt C, myt Cc and derivatives of both these peptides was enhanced by low pH.  
452 These studies also showed that only under acid conditions did these various peptides possess activity  
453 against Gram-positive bacteria and Gram-negative bacteria. Structure function studies on *E. coli*  
454 suggested that the antimicrobial action of myt C and its variants was membranolytic and involved  
455 low pH mediated increases in their levels of  $\alpha$ -helicity and  $\beta$ -hairpin elements within their molecular  
456 architecture [62,63]. In addition to their pH dependent antimicrobial activity, both myt C and myt Cc  
457 possessed chemotactic activity and appeared to be the first chemokine/cytokine-like molecules  
458 identified in bivalves [63,202]. These results added to the increasing evidence that AMPs can serve as  
459 cytokines [29,203] and interestingly, it is also becoming clear that these latter peptides are able to  
460 exhibit antimicrobial activity [204,205] that is enhanced by low pH [206,207]. Molluscs are also a  
461 source of histidine containing AMPs with pH dependent activity, such as the peptide, KPS-1, which  
462 was isolated from, *Atrina pectinata*, and under acid conditions, inhibited the growth of a range of  
463 Gram-negative bacteria, including *P. Aeruginosa*, *S. typhimurium* and *Enterobacter sakazakii* [64]. Ci-  
464 PAP-A22 and Ci-MAM-A24 are representative peptides of the Ci-PAP and Ci-MAM families of  
465 AMPs from the solitary tunicate (Sea squirt), *Ciona intestinalis* [65,66], which appear to be produced  
466 in haemocytes and granulocytes of the organism. [208,209]. Both peptides were found to be  
467 predominantly  $\alpha$ -helical and to possess antimicrobial activity [68,209,210] that appeared to have a  
468 pH dependency with optima that varied according to the target microbes [65-68]. In the case of Ci-  
469 PAP-A22, the activity of the peptide against fungi, Gram-negative and Gram-positive bacteria was  
470 enhanced by neutral pH except for *B. megaterium* which was more efficiently killed by Ci-PAP-A22  
471 at acid pH [65]. In contrast, Ci-MAM-A24 showed enhanced activity against *B. megaterium*, *B. subtilis*,  
472 *E. coli* and *P. aeruginosa* at low pH but neutral pH optima for action towards *S. aureus*, *Staphylococcus*  
473 *epidermis*, *Serratia marsecens* and *Klebsiella pneumoniae*. The peptide was also found to exhibit pH  
474 independent antimicrobial activity, killing comparable levels of *Yersinia enterocolitica* and fungi under  
475 low and neutral conditions of pH [66]. The antimicrobial activity of both Ci-PAP-A22 and Ci-MAM-  
476 A24 appeared to involve a membranolytic mechanism that had characteristics consistent with a  
477 'carpet' or 'toroidal pore, type model (Table 1). To help explain the differences in pH dependent  
478 antimicrobial activity shown by these peptides it was suggested that histidine mediated variation in  
479 their positive charge may facilitate optimal membrane interaction on a species- specific basis [65,66]  
480 and of course varying lipid composition will mean differing bacterial systems exhibit different  
481 changes to key parameters such as lipid packing at varying pH. Interestingly, Ci-MAM-A24 was  
482 found to be more potent than Ci-PAP-A22 and appears to be the first AMP reported to kill an intra-  
483 amoebic pathogen [67]. It was demonstrate that Ci-MAM-A24 was able to kill *Legionella pneumophila*,  
484 which is a Gram-negative parasite responsible for Legionnaire's disease [211], whilst the organism  
485 was replicating intracellularly in *Acanthamoeba castellanii* [67]. It is well established that *A. castellanii*  
486 acts as a vector for this bacterium [212,213], which efficiently replicates in the acidic environment of  
487 host amoebal phagosomes [214,215]. Ci-MAM-A24 was also able to kill Mycobacteria, in murine  
488 macrophages [210] and these acid fast bacteria are known to replicate in the acidic compartments of  
489 these host cells [216]. Given the pH dependent antimicrobial activity of the peptide, it is tempting to

490 speculate that the ability of Ci-MAM-A24 to kill these various bacterial parasites was potentiated by  
491 the low pH of their host cell environments. Clavaspirin, clavansins and styelins were isolated from  
492 another solitary tunicate, *Styela clava* and these pH dependent AMPs were found to be rich in both  
493 histidine and phenylalanine residues [72,73]. In general, it was found that clavaspirin and clavansins  
494 possessed pH dependent antibacterial and antifungal activity [69-71,80] with low pH enhancing the  
495 ability of these AMPs to adopt  $\alpha$ -helical structure and permeabilize the membranes of these  
496 organisms [72,73]. It appeared that the protonation of histidine residues under low pH conditions  
497 promoted the ability of these AMPs to target microbial membranes whilst the presence of their  
498 glycine and phenylalanine residues provided them with the conformational flexibility and structural  
499 hydrophobicity to facilitate bilayer partitioning [74-79]. Styelins, which are rich in phenylalanine  
500 residues, were found to show activity against both human bacterial pathogens and marine bacteria,  
501 such as *Psychrobacter immobilis* and *Planococcus citreus*, [72,217]. The best characterised of these AMPs  
502 is styelin D, which possesses  $\alpha$ -helical structure and is highly unusual in that it contains twelve post-  
503 translationally modified residues [81]. For example, the peptide contained multiple  
504 bromotryptophan residues, which are found in the AMPs of other marine organisms [218-222] and  
505 play an important role in the life of sea sponges and lower marine invertebrates [223]. Styelin D's  
506 post-translationally modified residues enhanced the peptide's membranolytic action at low pH but  
507 only against Gram-positive bacteria. It was suggested that a role for these extensive modifications  
508 may be in preserving activity against certain organisms under the acid conditions found in  
509 haemocytes of *S. clava* where the styelins are active [81].

510  
511

## 512 2.5. Terrestrial invertebrates

513 Hebraein is produced by the tick, *Amblyomma hebraeum* [224] and showed acid pH optima for its  
514 activity against *E. coli*, *S. aureus* and the fungus, *C. glabrata* [82], a major cause of vulvovaginal  
515 candidiasis in diabetics [225]. The peptide possessed an  $\alpha$ -helical structure except for a short C-  
516 terminal extension containing multiple histidine residues, which appeared to be required for activity  
517 against these organisms [82]. Based on these observations, it was suggested that the acidic pH  
518 induced in the physiological environment when a tick blood-feeds would increase the cationicity of  
519 hebraein and thereby its membrane interactivity and antimicrobial potency [82]. However, the  
520 activity of hebraein against *S. aureus* appeared to be independent of this histidine cluster and *C.*  
521 *albicans* was not susceptible to the action of the peptide suggesting that it possessed a variety of  
522 antimicrobial mechanisms, which were influenced by the target organism [82]. Interestingly, these  
523 latter studies showed hebraein to possess homology and structural similarities to microplusin, which  
524 is a  $\text{Cu}^{2+}$  chelating peptide isolated from another arachnid, the cattle tick, *Rhipicephalus microplus*, with  
525 broad range antimicrobial activity [226-228]. Studies on the Gram-positive bacterium, *Micrococcus*  
526 *luteus*, and the fungus, *Cryptococcus neoformans*, suggested that  $\text{Cu}^{2+}$  chelation involving histidine  
527 residues promotes the antimicrobial activity of microplusin by depriving vital cellular processes of  
528 the ion, such as haeme-copper terminal oxidases that contribute to cell respiration [228-230].  
529 Amoebapores are a family of cystein stabilized antimicrobial proteins with  $\alpha$ -helical structures that  
530 are found in the cytoplasmic granules of the protozoan parasite of primates, *Entamoeba histolytica*  
531 [83,85,231], and interestingly there is evidence to suggest that amoeba-like peptides may have been  
532 amongst the first eukaryotic AMPs to emerge [232]. Amoebapore A is the best characterized of this  
533 family of proteins and has been shown to exhibit pH dependent activity against Gram-positive  
534 organisms, such as *M. luteus*, and Gram-negative bacteria, including *E. coli* [83,84,87], which appears  
535 to involve pore formation in the membranes of target organisms [84]. Both pore formation and the  
536 antibacterial activity of the protein were enhanced by low pH, which appears to derive from an  
537 increased ability of amoebapore A to self-associate and form oligomers with some similarities to a  
538 barrel-stave pore type mode of action [83-85]. Elucidation of the structure of amoebapore A indicated



539 that a C-terminal histidine residue acted as a molecular switch that triggers the formation of active  
540 dimers from inactive monomers, which leads to the construction oligomeric pores in target cell  
541 membranes, [86]. In addition to amoebapore A, two isoforms of the protein, amoebapores, B and C,  
542 are included in the amoebapore family and all three isoforms differ markedly in their primary  
543 structure and spectra of antibacterial activity, synergising their combined efficacy. Amoebapores, B  
544 and C are believed to have similar antibacterial mechanisms to amoebapore A and the acidic pH  
545 optima of these proteins is consistent with the low pH conditions encountered in the  
546 amoebic intracellular vesicles, which form their site of action [83,85,231]. More recently,  
547 acanthaporin, which is another protozoan protein with pH dependent antimicrobial activity was  
548 described in the *Acanthamoeba culbertsoni*. At neutral pH, acanthaporin appears to exist as an inactive  
549 dimer but low pH triggers the histidine mediated production of monomers and the formation of  
550 membrane pores, which promoted the activity of the peptide against a variety of bacteria [88].  
551 Caenopores, also known as saposin-like proteins (SPP), are cystein stabilized helical proteins that are  
552 found in the nematode, *Caenorhabditis elegans* [93,232-235] and are distantly related to amoebapores  
553 with which they share structural and functional features [89,92,236,237]. Many of the genes encoding  
554 SPP proteins in *C. elegans* are induced in response to microbial challenge [232] and several of their  
555 gene products have been reported to exhibit pH dependent antimicrobial activity, including SSP-1  
556 [89,90], SPP-3 [91], SPP-5 [92] and SPP-12 [90]. These studies showed that that low pH enhanced the  
557 ability of caenopores to kill a wide range of microbes, including Gram-negative bacteria, such as *E.*  
558 *coli*; Gram-positive bacteria, including *Bacillus thuringiensis*; yeasts, such as *Saccharomyces cerevisiae*;  
559 and amoebae, including *Dictyostelium discoideum*. For each of these proteins, antimicrobial activity  
560 appeared to be based on an ability to form pores in membranes of target organisms under acid pH  
561 conditions and it was suggested that this ability was mediated by the multiple internal  
562 histidine residues possessed by caenopores. Due to these residues, the positive charge of these  
563 proteins is enhanced under acid conditions, increasing the potential for interaction with anionic  
564 components of microbial membranes and possibly mediating pore formation, as described for  
565 amoebapores [90-93,236,237]. It was observed that the pH dependent activity of these proteins would  
566 appear to reflect the pH conditions at the site of their functional action, such as SPP-1 and SPP-5,  
567 which are active in the acidic environment of the *C. elegans* intestine [92,93,236,237].  
568

### 569 3. Potential applications of pH dependent antimicrobial peptides and proteins

570 In response to the growing demand for new antibiotics with novel mechanisms of action, the  
571 number of AMPs and antimicrobial proteins entering clinical trials is accelerating [12] and included  
572 within these antimicrobial molecules are a number that have been reviewed here (Table 2). Currently,  
573 the only pH dependent anionic AMPs that appear to have been commercially developed are  
574 kappacins. Based on their activity against oral pathogens [55,56], preparations including these  
575 peptides and zinc have been patented [238] and are available as a dental care products [194,198]. It  
576 has also been shown that these peptides exhibit increased antimicrobial activity in foods with high  
577 calcium contents [194], which, taken with the history of the safe use of  $\kappa$ -casein, led to the proposal  
578 that kappacins may be used as a preservative [239]. In the case PD-3-7, epimers of this peptide appear  
579 to be the only amyloid forming amphibian anionic AMPs so far reported and have the potential to  
580 progress understanding of the role of residue chirality in the formation of disease-related amyloid  
581 and aid the design of amyloid-based nanomaterials [126]. The development of functional amyloids  
582 as novel nanostructure materials for multiple purposes, such as drug delivery and tissue repair /  
583 engineering, is a growing area of technology [240,241] and recently, techniques have been developed  
584 to detect epimeric AMPs in the complex skin secretions of frogs and toads [242].

585 The most researched of the cationic AMPs reviewed here for potential medical development is  
586 LL-37, which is a prospective broad range antimicrobial agent that is also able to induce wound  
587 healing and angiogenesis as well as modulate apoptosis [243]. This potential is though limited in

588 some cases by the pleiotropic effects of the peptide [244]. For example, the peptide shows a variation  
589 in its sensitivity to cancer types, promoting proliferation, migration, and tumorigenesis in breast,  
590 lung, and prostate cancers through receptor signaling but suppresses proliferation and induces  
591 apoptotic and autophagic cell death in gastric cancer, colon cancer, and T-cell leukemia [107].  
592 However, wound treatment is a globally prevalent and economic burden, which makes the  
593 pleiotropic ability of LL-37 to exert healing properties and combat multiple microbial pathogens an  
594 attractive platform that has been used to develop potential therapeutic strategies for wound  
595 treatment [245]. For example, a clinical phase I/II study conducted by Pergamum on LL-37 led to a  
596 patent [246] and showed that topical application of the peptide was safe and enhanced wound  
597 healing in patients with chronic venous leg ulcers and diabetic patients suffering from infected  
598 wounds [245,247]. Wound infection is a major complication in diabetic patients and in particular,  
599 infected foot ulcers is one of the most serious and frequent of these complications, which accounts  
600 for over 50% of all lower limb amputations performed on these patients [248]. More recently, several  
601 studies have developed biodegradable drug delivery system that facilitated the controlled sustained  
602 release of LL-37 and other wound healing agents, such as lactate and serpin A1, from nanoparticles.  
603 LL-37 and these agents acted synergistically in the treatment of full thickness excisional wounds,  
604 significantly promoting wound closure, reducing bacterial contamination and enhancing anti-  
605 inflammatory activity. These systems offered several advantages over therapies commonly used to  
606 treat chronic wound infections, which are often limited due to factors, such as the lack of controlled  
607 delivery and the depth of skin infections [249,250]. A number of LL-37 related peptides have also  
608 shown the potential for therapeutic development [243,251], such as OP-145, which was developed by  
609 OctoPlus, and when the peptide was included in cream formulations for nasal application, these  
610 preparations were found to be efficacious in the eradication of MRSA carriage [252]. The anterior  
611 nares are the main reservoir for colonization by *S. aureus* and the nasal carriage of MRSA is an  
612 important risk factor for subsequent infection and transmission of this pathogen, which has led to  
613 intensive efforts to identify agents able to efficiently reduce MRSA colonization [253]. The completion  
614 of phase I/II clinical trials by OP-145 also showed that the peptide was safe and efficacious as a  
615 treatment for chronic otitis media, or chronic bacterial middle-ear infection (Table 2) [254]. This  
616 disease afflicts millions of people worldwide and is highly recalcitrant to treatment by conventional  
617 antibiotics, which is now known to be primarily due to bacterial biofilms [255]. Another derivative  
618 of LL-37, 60.4Ac, has also proven to be beneficial in the treatment of patients with otitis media [245]  
619 and more recently the peptide showed the potential for development as a novel local therapy to treat  
620 patients with burn wounds infected with multidrug-resistant bacteria, including MRSA [256]. Burn  
621 wounds are one of the most common and devastating forms of trauma and the infection of these  
622 wound by drug resistant bacterial pathogens is rapidly becoming a serious therapeutic challenge in  
623 the care of burn patients [257].

624 Histatins and their derivatives show the potential for a wide range of therapeutic and  
625 biotechnical application [141], particularly in the field of dentistry and bio-dental research [258]. For  
626 example, the hst-5 derivative, JH8194, is a promising candidate to act as a surface substrate in dental  
627 implants to prevent peri-implantitis and peri-implant mucositis whilst decreasing infections  
628 [259,260]. A major focus in the medical development of histatins has been in the preparation of  
629 formulations to treat oral diseases and infections [141]. For example, highly effective hydrogel  
630 delivery systems for the topical and oral application of hst-5 have been developed for the treatment  
631 of oral candidiasis [261], which is the most common opportunistic fungal infection in  
632 immunocompromised populations [262]. High potential for the topical treatment of this fungal  
633 condition was also demonstrated when derivatives of hst-5 were conjugated to spermidine and tested  
634 on immunocompromised murine models [263]. Compared to hst-5, these conjugates exhibited a  
635 higher clinical half-life, enhanced uptake into *Candida* cells, and greater candidacidal efficacies, and  
636 were proposed to be viable alternatives to azole antifungals [263], which are commonly used to treat  
637 oral candidiasis [262]. A compound derived from hst-5 and hs1-3, P-113 (PAC-113), developed by  
638 Pacgen, was evaluated in Phase I/ II clinical studies for the treatment of both oral candidiasis and

639 gingivitis, and was found to be safe and effective in the treatment of both conditions (Table 2)[12].  
640 Gingivitis is the most common form of periodontal disease, affecting up to 15% of the adult  
641 populations worldwide and primarily due to *Porphyromonas gingivalis*. Untreated the condition can  
642 lead to periodontitis, the chronic destruction of connective tissues, and ultimately result in loss of  
643 teeth [264]. P-113 has been patented [265] and most recently, it has been shown that the candidal  
644 efficacy of the peptide was greatly enhanced when it was modified by coupling to other AMPs and  
645 their derivatives [144] Another histatin, hist-1, was conjugated to a silver metallopharmaceutical and  
646 the conjugate was found to have wound healing properties coupled to potent activity against  
647 bacteria, which included MRSA, indicating the potential for development of novel multifunctional  
648 therapeutics [266]. Clavanins are attractive candidates for development as drugs against bacteria  
649 associated with sepsis, which is rapidly becoming a problematic nosocomial infection [267], and  
650 recently developed nanoparticle formulations of these peptides, showed high promise as a drugs  
651 against polymicrobial sepsis with morphological characteristics suitable for administration via  
652 injection [80]. Derivatives of clavanins have also been developed to combat biofilms formed by, *S.*  
653 *mutans*, which is a major contributor to dental plaque and one of the major etiological factors involved  
654 in causing caries [268]. Dental caries is one of the most prevalent, preventable infectious diseases  
655 affecting humans and is recognized as the primary cause of oral pain and tooth loss [269].

656 The major medical development of the antimicrobial proteins reviewed here appears to be  
657 lactoferrin, which as described above is an iron binding protein but, like LL-37, is pleiotropic and also  
658 displays broad range antimicrobial activity using a number of mechanisms, which includes the  
659 release of derivative AMPs *via* hydrolysis by proteases [162,270,271]. Lactoferrin and its related  
660 peptides shows the potential for a number of clinical uses, ranging from wound healing and the  
661 detection of bacteria to the treatment of microbial infections both alone and in combination with other  
662 clinically relevant agents [272]. A full description of these medical uses is beyond the scope of this  
663 review but lactoferrin and its derivatives have featured in multiple clinical trials [160,272,273] and  
664 have numerous entries in a recently constructed database of bioactive peptides derived from milk  
665 proteins [274]. As major examples, lactoferrin and its derivatives have been extensively investigated  
666 as potential drugs for the treatment of common viral infections including the common cold, influenza,  
667 viral gastroenteritis and herpes [275] whilst the inhibitory effects of these proteins and peptides  
668 against the proliferation of multiple cancers, has suggested a potential role in cancer prevention [276].  
669 It is well established that many AMPs and antimicrobial proteins have anticancer activity that  
670 generally appears to involve mechanisms of membranolysis that are similar to those used by these  
671 molecules in their action against microbes [277,278], which in some cases shows pH dependence [98],  
672 as recently described [279,280]. Advanced clinical trials have shown that the administration of  
673 lactoferrin has no significant side effects and that the protein has efficacy in treating iron deficiency  
674 anemia in pregnant women [281], sepsis in premature neonates, which is a common and severe  
675 complication in new-born infants [282] and infections due to *Helicobacter pylori*, which is causally  
676 associated with gastritis and peptic ulcer diseases [283]. A major example of the medical potential of  
677 lactoferrin is the development of ALX-109 by Alaxia, which is a combination of the protein and  
678 hypothiocyanite for the treatment of CF [160]. This drug combination has been granted orphan drug  
679 status by American and European licensing agencies and has been shown to enhance the ability of  
680 conventional antibiotics to eliminate biofilms of *P. aeruginosa* growing on CF airway epithelial cells  
681 [284]. Derivatives of lactoferrin, have also shown the potential for therapeutic development such as  
682 hLF(1-11), which was developed by AM Pharma, and in clinical trials the peptide was safely injected  
683 into neutropenic stem cell transplantation patients [160]. Neutropenia is defined as a reduction in the  
684 absolute number of neutrophils in the blood circulation, predisposing individuals to severe or fatal  
685 infections [285], and currently, hLF(1-11), awaits development for the prevention of bacteremia and  
686 fungal infections in immunocompromised individuals (Table 2)[273]. Lactoferricin B is cleaved from  
687 the N-terminal region of bovine lactoferrin under acid pH conditions and has an extremely wide  
688 spectrum of antimicrobial activity against bacterial, fungal and parasite species as well as showing  
689 anti-catabolic and anti-inflammatory effects [162,270,271]. Based on these abilities, this peptide has

690 featured in numerous preclinical trials and shows the potential for a variety of therapeutic purposes,  
691 including the treatment of ocular infections, osteo-articular gastro-Intestinal and dermatological  
692 diseases, along with applications in veterinary practice and the food industry [272]. The commercial  
693 importance of lactoferrin and its derivatives is perhaps underlined by the fact that the recombinant  
694 human protein has been expressed in transgenic cattle to provide the large-scale production of  
695 lactoferrin for pharmaceutical use [286]. The recombinant protein has also been expressed in microbes  
696 and higher plants in the search for bioreactors with the capacity for large-scale production, which,  
697 led to lactoferrin expression also being used as a tool for the enhancement of plant resistance to  
698 pathogens [286].

699

#### 700 4. Discussion

701 AMPs and antimicrobial proteins with pH dependent action against microbes appear to receive  
702 relatively little attention in the literature but, as this review has shown, these molecules are produced  
703 by a diverse spectrum of eukaryotes, including: vertebrates, such as fish, humans, horses, cattle,  
704 rabbits, guinea pigs, mice, frogs and toads, as well as invertebrates, such as ticks, parasites, worms  
705 and mollusks (Table 3). Around two thirds of the molecules reviewed here are cationic AMPs and  
706 antimicrobial proteins with most of those that remain possessing net negative charges [287]. It is  
707 generally recognized that the incidence of anionic antimicrobial molecules is low and that, in general,  
708 their occurrence appears to be a strategy to synergize the antimicrobial activity of their cationic  
709 counterparts [9,288]. For example, the proteolytic processing of the sweat borne peptide, dermcidin,  
710 to yield DCD-1(L), described above, also produces a number of other anionic AMPs, such as SSL-46  
711 (net charge -2) and LEK-45 (net charge -2) [289]. These sweat-derived anionic AMPs are continually  
712 secreted and are believed to synergize the activity of cationic AMPs in the constitutive innate defense  
713 of human skin by modulating surface colonization by microbes rather than responding to injury and  
714 inflammation as observed for inducible peptides, such as LL-37 [186].

715 The pH dependence of the antimicrobial molecules reviewed here was found to vary with pH  
716 with some, such as E2EM-lin, exhibiting high pH optima (Table 4) whilst others, such as Ci-PAP-A22  
717 and Ci-MAM-A24, exhibited optima at either neutral or acid pH depending on the target organisms  
718 [65-68]. Again depending on the target microbes, several antimicrobial molecules, including the latter  
719 peptides and psoriasin, showed the ability to employ both pH dependent and pH independent  
720 activity [38-40,65-68]. However, most of the AMPs and antimicrobial proteins reviewed here  
721 exhibited low pH optima, which is consistent with the acidic pH found at their sites of action,  
722 particularly the skin [131,290]. Consistent with these observations, the major structure / function  
723 relationships that promote the pH dependent activity of the antimicrobial molecules reviewed here  
724 are those involving amino acid residues that become protonated under acid conditions, including  
725 histidine, aspartic acid and glutamic acid residues. Under these pH conditions, the protonation of  
726 these residues will have the overall effect of increasing the cationicity or decreasing the anionicity of  
727 the parent molecule thereby, enhancing its ability to target and interact with negatively charged  
728 components of microbial membranes. Typical examples include hebraein [224] and clavanins [74-79],  
729 and in the case of Ci-PAP-A22 and Ci-MAM-A24, it appears that the histidine mediated variation in  
730 the cationicity of these peptides facilitates optimal interaction with target microbial membranes on a  
731 species to specific basis [65,66]. However, given the high incidence of histidine residues in the  
732 antimicrobial molecules reviewed here, it is worthy of note that the possession of these residues is  
733 not necessarily sufficient for a pH dependent mode of antimicrobial action. This point is well  
734 illustrated by Pc-pis, from the yellow croaker, *Pseudosciaena crocea*, which includes a number of  
735 histidine residues in its primary structure and displays pH independent antimicrobial activity.  
736 However, the addition of a histidine residue to its sequence generated a peptide with antimicrobial  
737 activity optimal at low pH and a wider spectrum of antimicrobial activity [291].

738 A second major structure / function relationship for histidine, aspartic acid and glutamic acid  
739 residues in the antimicrobial action of pH dependent AMPs and proteins reviewed here is to facilitate  
740 the binding of metal ions. For example, the binding of Ca<sup>2+</sup> by MSP at low pH potentiates the activity  
741 of the peptide by alleviating inhibitory mechanisms that are mediated by the ion [41] and metal ion  
742 binding by histidine residues appears able to promote microbial death through depletion of these  
743 ions for a number of antimicrobial molecules, such as histatins [141,143,155]. In contrast, the binding  
744 of metal ions appears to potentiates the activity of some antimicrobial molecules reviewed here by  
745 promoting their capacity to form peptide-membrane or peptide-peptide salt bridges and thereby  
746 disrupt microbial membranes, as proposed for kappacins [197] and DCD-1(L) respectively [54].  
747 However, the most common structure / function relationships for histidine, aspartic acid and  
748 glutamic acid residues in the antimicrobial action of the molecules reviewed here is to directly  
749 promote the disruption of target microbial membranes. For example, in the case of several  
750 antimicrobial proteins, the protonation of histidine residues appears to be a molecular switch that  
751 initiates oligomerisation and the formation of discrete channels or pores by the protein, as in the case  
752 of acanthaporin [88]. In some cases though, histidine, aspartic acid and glutamic acid residues appear  
753 to play multiple roles in promoting the activity of their parent antimicrobial molecules. For example,  
754 the N-terminal regions of gad-1 and gad-2 include a number of sequential histidine pairs that appear  
755 to be important to their ability for lipid targeting and interaction, channel formation and thereby the  
756 disruption of microbial membranes at low pH [33,34,94,99,100].

757 A further major structure / function relationship involved in the mechanisms of the antimicrobial  
758 molecules reviewed here is pH related conformational change in  $\alpha$ -helical architecture, which is by  
759 far the most common secondary structural element identified in these AMPs and antimicrobial  
760 proteins. Indeed, it well established that histidine, glutamic acid and aspartic acid residues have a  
761 strong potential for  $\alpha$ -helical formation that is enhanced by low pH [98,292]. The pore forming  
762 antimicrobial proteins reviewed here are strongly  $\alpha$ -helical (Figure 2) and it is known that changes to  
763 the levels of  $\alpha$ -helical architecture possessed by these proteins are enhanced by low pH, which  
764 promotes their pore forming mechanisms and are key to their ability to kill microbes [39,86,88,93]. A  
765 full description of these conformational changes is beyond the scope of this review but as an example,  
766 the protonation of C-terminal histidine residues by low pH promotes conformational changes that  
767 lead to the construction of hexameric membrane pores via the formation of active dimers from  
768 inactive monomers in the case of amoebapores [83,85,231] caenopores [89-93] and psoriasisin [38-40].  
769 The pore forming mechanism of acanthaporin, shows similarities to those of these latter proteins and  
770 also results in the formation of hexameric membrane pores. However, in the case of acanthaporin,  
771 the low pH mediated protonation of C-terminal histidine residues promotes conformational changes  
772 that induce pore formation via the formation of active dimers from inactive monomers [88]. Strictly,  
773 based on its size, lactoferrin is an antimicrobial protein, but it is often classified with AMPs due to its  
774 ubiquity in body fluids and its ability to kill bacteria using membrane interactive mechanisms with  
775 similarities to those of these latter peptides [29]. However, lactoferrin was first characterized as an  
776 iron binding protein and sequestration of the metal was initially believed to form the basis of its  
777 antibacterial mechanism although the protein is now known to use multiple iron-independent  
778 mechanisms in its activity against microbes [162,293].

779 In relation to the AMPs reviewed here low pH generally increased their levels of  $\alpha$ -helical  
780 secondary structure and thereby enhanced their capacity for membrane interaction and antimicrobial  
781 activity. However, alkaline conditions promoted maximal levels of  $\alpha$ -helical structure in E2EM-lin,  
782 which appeared to promote monomer association, pore formation and membrane interaction at the  
783 peptide's high pH optimum (Table 4, Figure 1). In contrast, to these latter AMPs, gad-1 and gad-2  
784 were found to possess minimal levels of  $\alpha$ -helical structure under the low pH conditions that were  
785 optimal for their membrane interactions and antimicrobial activity [33]. These observations would  
786 seem to clearly indicate that pH dependent structural plasticity is an important factor in the  
787 antimicrobial mechanisms of many of the AMPs reviewed here. This form of structural plasticity  
788 would appear to be key to facilitating the appropriate balance between the amphiphilicity and

789 hydrophobicity of these peptides that is required for their membranolytic action at optimal pH, as  
790 proposed for gad-1 and gad-2 [33]. Reinforcing this proposal, other amino acid residues have been  
791 reported to contribute to the structural plasticity of the  $\alpha$ -helical AMPs reviewed here including  
792 glycine, phenylalanine and post-translationally modified residues. These residues appear to enhance  
793 the conformational flexibility and structural hydrophobicity of tunicate clavaspilin, clavanins and  
794 styelins for bilayer partitioning and antimicrobial action at their low pH optimum [72,73].

795 The antimicrobial mechanisms of several AMPs reviewed here appear to be described by models  
796 of membrane interaction, including variants of the carpet, toroidal pore and barrel-stave pore  
797 mechanisms. These models of membrane interaction differ fundamentally to the pore forming  
798 mechanisms of the antimicrobial proteins described above and were primarily proposed to describe  
799 the membrane spanning abilities of AMPs, which are generally up to 50 residues in length [23]. A  
800 number of novel antimicrobial mechanisms for AMPs have also been revealed by this review, such  
801 as that described for human lactoferrin, which at sub lethal levels appears to kill microbes via the pH  
802 dependent dissipation of microbial PMF [51]. The microbial PMF is an emerging potential target for  
803 the development of novel AMPs and antimicrobial proteins based on the fact that the temporary  
804 membrane perturbations caused by their action can have a large negative impact on bacterial  
805 metabolism, affecting a diverse array of cellular processes that depend upon the PMF [294-299]. This  
806 review has also described novel examples of pH dependent AMPs produced by the reduction of  
807 cysteine stabilized parent AMPs including myt Cc [63,202] and E2EM-lin (Table 4, Figure 1, [114,115])  
808 and it has been recently shown that the free cysteines of reduced AMPs play an important role in  
809 their antimicrobial activity [116,117]. Moreover, it was speculated above that the antimicrobial  
810 activity of E2EM-lin may involve pore formation via self-association and interestingly, recent work  
811 has suggested that free cysteine residues may play a role in the antimicrobial activity of AMPs by  
812 facilitating the oligomerisation of these peptides [300]. Taken together, these reports show that AMPs  
813 with pH dependent antimicrobial activity contribute to the accumulating evidence that the  
814 endogenous reduction of cysteine-stabilized AMPs is a strategy used by hosts to generate novel  
815 peptides that enhance the efficacy of their antimicrobial capacity [116,117].

816 A number of the AMPs and antimicrobial proteins reviewed here, along with their derivatives,  
817 have been developed for multiple medical purposes, which in some cases has led to patents and the  
818 successful completion of clinical trials, and include kappacins, LL-37, histatins, lactoferrin and  
819 clavanins. Major examples of the application of these AMPs and proteins include the treatment of  
820 multiple cancers along with viral infections, such as the common cold; bacterial infections, including  
821 those associated with implants, otitis media, neutropenia and CF; and fungal infections, particularly,  
822 those detrimental to oral health. These AMPs and proteins also show the potential to induce wound  
823 healing, such as for diabetic patients and burn victims, and interestingly, a recent report has indicated  
824 that wound healing is accelerated by an acidic environment, which promotes a range of beneficial  
825 effects including increases in antimicrobial activity and the enhancement of epithelization and  
826 angiogenesis [301]. In general, the therapeutic administration of the AMPs and proteins involve  
827 topical application, such as the use of mouth washes, cream formulations and hydrogel delivery  
828 systems. These observations raise an interesting point in that most clinical trials to date involve the  
829 treatment of skin infections or the prevention of surface colonization by microbes, particularly sessile  
830 forms of these organisms, which potentially, can indicate a wide variation in local pH conditions. A  
831 comprehensive understanding of the effect of pH on the antimicrobial activity of the molecule under  
832 development would therefore seem necessary. Nonetheless, this is not generally the case and data  
833 cited in the literature in relation to the antimicrobial activity of AMPs and proteins is usually that  
834 determined under neutral pH conditions [133]. These observations clearly suggest that when  
835 characterizing the antimicrobial action of AMPs, the optimal pH for their action against individual  
836 microbes should be determined. This point is well illustrated by recent studies, which investigated  
837 the antimicrobial action of a range of synthetic AMPs and found that high pH inhibited the action of  
838 these peptides against fungi and Gram-negative bacteria but the opposite pH trend was observed  
839 for Gram-positive bacteria [302].

840 **5. Conclusions**

841 This review has shown that AMPs and proteins with pH dependent antimicrobial activity are  
842 increasingly being reported and that progress has been made in understanding the structure /  
843 function relationships and mechanisms underpinning this activity. This review has also shown that  
844 there has been considerable therapeutic development of pH dependent antimicrobial molecules to  
845 treat a variety of infections and other conditions. However, one of the biggest therapeutic and  
846 biotechnical developments of these antimicrobial molecules has been to provide guidance to the  
847 design of novel compounds with pH dependent activity against: bacteria [303,304], fungi [50,305,306]  
848 and cancer cells [307,308] as well as applications involving drug [309,310] and gene delivery [311,312].  
849 As a specific example, most AMPs designed to target the low pH of tumor tissue are cationic and  
850 cytotoxicity to healthy tissue at physiological pH has often been an issue for these peptides  
851 [98,279,280]. To address this issue, a peptide based on magainin 2 from *X. laevis* was designed to  
852 possess a negative charge at neutral pH that switched to a strong positive charge at low pH for cancer  
853 targeting. Designated, HE, this novel peptide, killed human renal adenocarcinoma at low pH *via*  
854 membranolytic mechanisms and was nontoxic towards healthy human cells across low and neutral  
855 pH conditions, making it a promising lead compound for cancer therapy [313]. As a further example,  
856 chronic infections due to *P. aeruginosa* are responsible for the majority of the morbidity and mortality  
857 in patients with CF and the persistence of these infections is largely due to the organism adopting a  
858 biofilm mode of growth, thereby acquiring high resistance to most antibiotics [314,315]. In response,  
859 the peptide, WLBU2, was designed and found able to prevent biofilm formation by *P. aeruginosa*  
860 under the low pH and high salt conditions characteristic of the CF airway without negative effects  
861 on human airway epithelial cells. WLBU2 was also found able to synergize the action of commonly  
862 used antibiotics, such as tobramycin and meropenem, making the peptide an attractive proposition  
863 to help address the critical need for novel therapeutics able to suppress chronic CF lung infections  
864 [316]. Using another approach, it has also been proposed that increasing the airway pH in CF  
865 individuals by activating CFTR independent HCO<sub>3</sub><sup>-</sup> transport pathways or by inhibiting proton  
866 pumps could help prevent or reduce bacterial and viral infections associated with the disease  
867 [165,180,181]. Nonetheless, this review has shown that many pH dependent AMPs and antimicrobial  
868 proteins have yet to be fully characterized and it is proposed that these antimicrobial molecules merit  
869 far more research attention than they currently receive. Indeed, pH dependent AMPs and  
870 antimicrobial proteins appear to represent an untapped source of novel biologically active agents that  
871 is awaiting full exploitation and could aid fulfillment of the urgent need for alternatives to  
872 conventional antibiotics, helping to avert a return to the pre-antibiotic era.  
873

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877

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879 **References**

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