Structure-Activity Relationships of Multifunctional Host Defence Peptides

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Abstract: Host defence peptides (HDPs) are multi-functional inducers and effectors of host immunity. Through their direct antimicrobial activity HDPs have for been successfully utilized for many years as topical antibiotics and food preservatives. The more recent appreciation of HDP immunomodulatory activities offers additional opportunities for application as systemic antimicrobials, anti-inflammatory agents and vaccine adjuvants. HDPs have demonstrated proof-of-principle success in each of these applications. Optimization of HDPs for these objectives will benefit from a greater comprehension of the structural basis of their various activities. Such an understanding will facilitate rational design and/or selection of peptides with enhanced properties. This is complicated, however, by the diversity of HDP sequences, structures and mechanisms of action. Furthermore, while the ability of HDPs to undergo template-driven formation of bioactive structures enables these small peptides to perform a diverse range of actions it also complicates efforts to understand contributions of particular structural features to specific activities. With recognition of these limitations, but consideration of the emerging importance of this exciting class of molecules, we review the current understanding of the structural basis of select HDP activities as well as present strategies for HDP selection and optimization.

Keywords: Host defence peptides, structure-activity relationship, peptide design, immunomodulatory, antimicrobial, vaccine adjuvant, anti-inflammatory.

1. INTRODUCTION

1.a. General

Host defence peptides (HDPs) are an evolutionarily conserved mechanism of innate immune defence found in all complex life forms [1]. Approximately one thousand such peptides have been identified (www.bbcm.units.it/~tossi/pag1.htm) from bacteria, plants, insects, birds, fish and mammals. Family members are highly diverse in terms of their sequence but are generally united by their small size (typically 12 to 50 amino acids), overall cationic charge (+2 to +9) and amphipathicity (up to 50% hydrophobic residues) [2, 3]. Natural variants are typically synthesized as inactive precursors with generation of mature, active species through proteolytic processing [4].

1.b. Overview of Nomenclature, Biological Roles and Therapeutic Applications

Based on their potent *in vitro* antimicrobial activity it was initially postulated that these peptides contributed to immune defence through direct attack on the microbe. This presumed mechanism of action dictated the strategies and criteria employed for selection of peptides of perceived therapeutic value. It is now apparent, however, that this activity does not encapsulate the full spectrum of their biological activities nor does it reflect the sophistication of their contributions to immune defence. These peptides are now recognized to modulate a variety of host immune functions including non-membranous host targets for modulation of host immunity. This has prompted re-evaluation of HDP: 1) biological roles/mechanisms, 2) therapeutic potentials, 3) criteria for selection/optimization and 4) nomenclature. This broader perspective is most easily reflected in the increasingly accepted description of these peptides as host defence peptides rather than antimicrobial peptides. In the interests of clarity we will adopt and maintain the HDP nomenclature independent of the function under consideration. The greater challenge in considering HDPs as immunomodulatory molecules is in re-evaluating the considerable literature amassed from the limited perspective of direct antimicrobial activity. Information obtained from these initial studies on the membrane permeating properties of these peptides, as well as their ability to undergo template driven formation of bioactive structures, remains of considerable value to immunotherapeutic applications. In particular, as toxicity is a primary obstacle limiting application of HDPs as systemic therapies it is imperative to understand how various peptide biophysical parameters and modifications influence the tendency to disrupt host membranes. Furthermore, if a similar trend of template driven formation of bioactive structure is observed for immunomodulatory functions this will impact strategies for design and select of peptide immunotherapeutics. Accordingly, we believe that considerations of the future immunotherapeutic potentials of HDPs must evolve

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from their origins as direct antimicrobials with appropriate consideration of the associated literature and findings.

1.c. Significance to Immune Defence

The contribution of HDPs to host immunity is highlighted by disorders relating to HDP deficiency. The resulting diseases are complex but the shared phenotype of increased susceptibility to infection underscores a conserved role for HDPs in immune defence. For example, individuals suffering from specific granule deficiency syndrome experience increased frequency and severity of bacterial infections as a presumed consequence of the absence of α-defensins [5]. In morbus Kostmann syndrome the absence of LL-37 and α-defensins [human neutrophil peptides (HNP) 1-3], is associated with oral bacterial infection and severe periodontal inflammatory disease even after neutrophil growth factor therapy [6]. Individuals with low copy numbers of the human β defensin (hBD)-2 gene have increased likelihood for development of Crohn's disease [7]. Furthermore, low levels of hBD-2 and -3expression in lesions caused by atopic dermatitis correlate with increased susceptibility to skin infections [8]. Genetic investigations in mice further support the importance of HDPs in immune defences. Knock-outs of β-defensin-1 [9, 10], cathelicidin-related antimicrobial peptide (CRAMP) [11], and matrix metalloproteinase (MMP)-7 [12], which is required for proteolytic activation of the pro-forms of enteric α-defensins, all result in increased susceptibility to infection. While HDP deficiency is associated with increased susceptibility to infection, it is of greater interest from a therapeutic perspective that elevated levels of HDPs, through engineered expression or therapeutic administration, enhance the ability to withstand microbial challenge. For example, increased endogenous expression of hBD-2 and -3 and LL-37 in psoriasis [13] and bronchoalveolar inflammation [14] are associated with resistance to infection, while engineered expression [15-18], or administration [19, 20], of various HDPs has been shown to increase the ability to prevent or clear a spectrum of infections.

1.d. Patterns of Expression

HDPs are present in many cell types but predominant expression is at critical interfaces of host/pathogen interaction such as phagocytic granules of immune cells, mucosal surfaces, skin,and body fluids. Depending on host species, peptide and cell type, HDP expression can either be constitutive or induced. Induced expression is normally in response to infection, tissue damage or inflammation. Specific triggers for induced expression include microbial molecular signatures, such as lipopolysaccharide (LPS) [21] or other toll-like receptor (TLR) agonists [22-24], a variety of host molecules or both. For example, in keratinocytes, human β-defensin (hBD)-2-4 is induced by tumour necrosis factor (TNF)-α, interleukin (IL)-1β, Il-2β as well as bacteria [25, 26]; LL-37 expression is responsive to both infection and injury [27]. Conversely, in intestinal epithelial cells hBD-1-3 is induced in response to enteric pathogens [24] while LL-37 appears upregulated by endogenous inflammatory molecules [28].

1.e. Immunomodulatory Activities

While many HDPs possess potent *in vitro* antimicrobial activity it is unlikely that this activity represents the primary contribution of HDPs to immune defence, primarily because many HDPs are expressed at levels below those required for antimicrobial activity. For example, levels of LL-37 at mucosal sites(2-5 μg/ml) are more than an order of magnitude lower than the minimal inhibitory concentrations (MIC) of this peptide for most bacteria in the presence of physiological salt concentrations [29]. There are, however, specific instances where HDP direct antimicrobial activity may aid in defence against microbial challenge. For example, α-defensins are found at sufficiently high concentrations (mg/mL) within intestinal crypts and granules of neutrophils to mediate direct antimicrobial activity [29]. Additionally, the antimicrobial activities of HDPs are quite sensitive to experimental conditions and reported MICs, especially when performed in dilute media, likely overestimate antimicrobial potency. While virtually all amphipathic, cationic peptides possess some degree of antimicrobial activity in dilute media, this activity is often suppressed by physiological concentrations of monovalent cations such as Na^+ or K^+ (100 mM), divalent cations such as Mg^{2+} or Ca^{2+} (1-2 mM) and polyanionic complex carbohydrates such as glycosaminoglycans, heparin sulphate, and mucins [30, 31]. Thus, the reported antimicrobial activities of many HDPs are likely artefacts of the experimental conditions. There are notable exceptions, such as protegrins, hBD-3 and θ-defensins, which possess antimicrobial activity which is largely independent of salt concentrations [32]. It is also possible to design antimicrobial peptides that are fairly insensitive to monovalent cations [33]. The physiological significance of direct antimicrobial activity is further challenged by the protection afforded by peptides with no detectable direct antimicrobial activity. For example, an immunomodulatory peptide, innate defence regulatory peptide (IDR)-1, was built to from a bovine cationic antimicrobial peptide template but designed to completely lack direct antimicrobial activity, is able to protect animals from bacterial challenges [20]. Thus, it seems likely that HDPs assist in treatment and prevention of infections by influencing host cell processes.

As an alternative mechanisms of protective effect many natural HDPs have been shown to activate a broad range of protective aspects of innate immunity including modulation of cytokines/chemokines and their receptors, recruitment of leukocytes to sites of infection, stimulation of histamine release from mast cells, angiogenesis, dendritic cell maturation and wound healing [31, 34-37]. Furthermore, although HDPs can counter infections through pro-inflammatory mechanisms, they are not per se pro-inflammatory since they also modulate the potentially deleterious consequences of inflammation suppressing certain TLR signalling responses (e.g. that stimulated by LPS) reducing LPS-induced production of proinflammatory cytokines such as TNFα [38, 39]. A representation of the various mechanism of HDP action on both bacterial and host cells is presented in (Fig. **1**).

The involvement of HDPs in the activation of immune responses has been extensively reviewed elsewhere [28, 40, 41] and will not be further elaborated on here other than to suggest that the activation of host immunity may represent a more desirable, and elegant, mechanism of peptide action than direct attack on the microbe. In particular, indirect antimicrobial activity of HDPs through activation of a broad

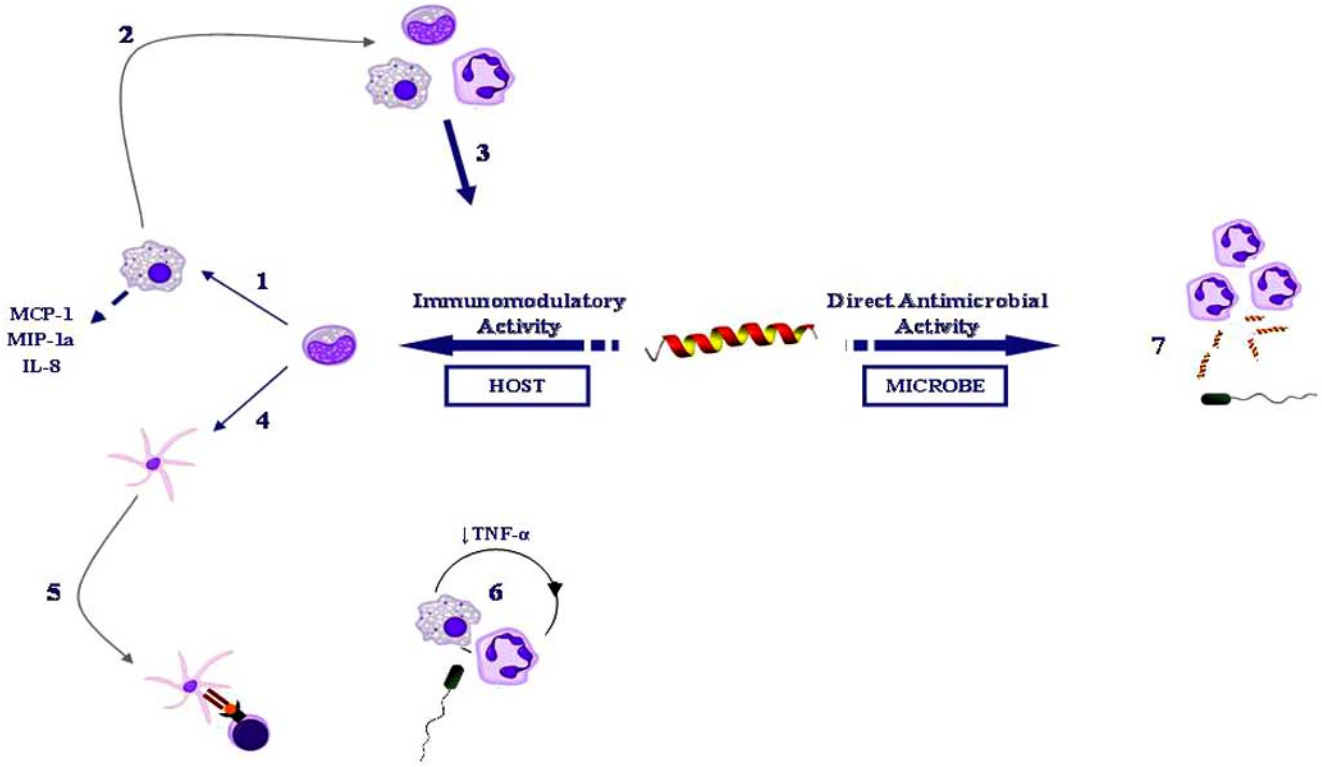

Fig. (1). Immunomodulatory and Direct Antimicrobial Activities of Host Defence Peptides. HDPs exert their anti-infective activities through either direct antimicrobial activity or through modulation of the host immune response. HDPs target monocyte maturation and differentiation to macrophages resulting in the release of effector molecules such as cytokines and chemokines (1). In turn, these chemokines, as well as the HDPs themselves, promote the leukocyte chemotaxis (2-3). HDPs also promote monocyte differentiation to DCs (4). HDPs have been demonstrated to promote the expression of co-stimulatory molecules on DCs and the expression of IL-12 (5). HDPs may also suppress pro-inflammatory responses through the suppression of pro-inflammatory molecule expression (6). HDP-mediated direct antimicrobial activity has been demonstrated as a physiological anti-infective activity for those HDPs that are present at physiological concentrations that match their respective MIC values and are not inhibited by high salt or divalent cation concentrations.The direct antimicrobial mechanisms of HDPs may involve interactions of HDPs with specific membrane and cytoplasmic targets or membrane lytic activities (7).

range of host defence mechanisms is less likely to induce resistance in microbes than direct microbial killing. Secondly, since structural features that bestow direct antimicrobial activity closely overlap with those determining toxicity, selection of peptides which operate through activation of host immunity may partially address limitations imposed by peptide toxicity. Collectively, activation of innate immune responses HDPs appear to represent a new paradigm for antimicrobial therapy.

2. HDP PHYSICAL CHARACTERISTICS

Investigations of HDP structure-activity relationships have historically focused on direct antimicrobial activity. This has included considerations of how different structural classes, as well as biophysical parameters independent of structural class, correlate with the ability to associate with and/or disrupt membranes. Properties of interest include net charge, hydrophobicity, amphipathicity, flexibility and propensity for self-assembly. While these investigations have given priority towards direct antimicrobial activity, it is anticipated that many of these biophysical parameters are also imperative for HDP immunomodulatory activities. In addition, recent, more detailed, structure-activity relationship studies involving thousands of peptides and neural network modelling have indicated tremendous complexity of such relationships with a dependency on multiple parameters, including inductive parameters that measure the variation of properties over the sequence of the peptide [42].

2.a. HDP Structural Classes

Given their small size and conservation of gross biophysical properties, it might be anticipated that all HDPs would adopt similar three dimensional structures. This is not the case, however, as a number of diverse, and often dynamic, structural classes are observed. HDPs typically group into four structural classes; extended, α-helical, loop and β sheet. Diagrams of representative members of each class are presented (Fig. **2**).

The α-helical and extended structure classes are defined in part by their structural flexibility and absence of disulfide bonds. HDPs of these classes often have random, dynamic structures in solution but adopt defined, amphipathic conformations when in contact with hydrophobic environments such as membranes. The amphipathic α-helical class of HDPs is the most abundant and well characterized class of

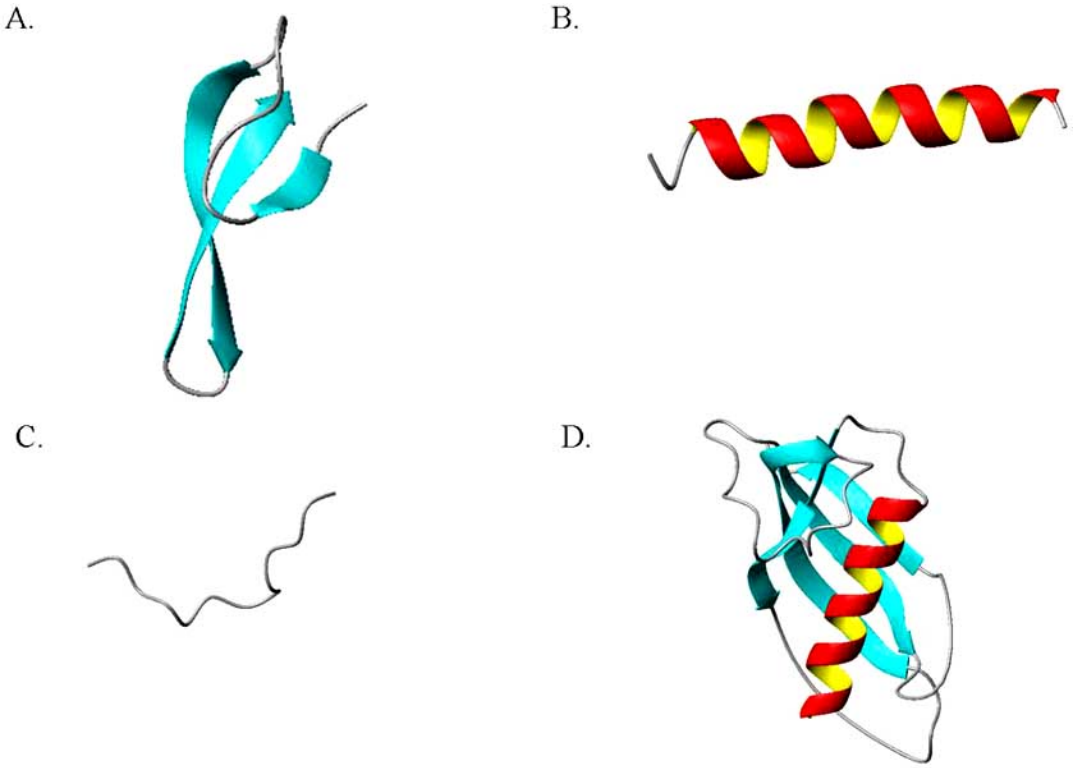

Fig. (2). Structures of host defence peptides representing the major structural classes. A. β-sheet class (HNP-3; PDB 1DFN). **B**. linear α-helical class (magainin; PDB 2MAG). **C**. extended class (indolicidin; 1G89). **D**. cysteine-stabilized α-β (protegrin-3; 1PFP).All structures were made with MOLMol and the color schema are as follows: red/yellow,α-helical propensity; aqua, β-sheet propensity; gray, extended or coil.

HDPs. HDPs of this class are usually short (<30 amino acids), devoid of cysteine residues, and unstructured or linear in non-hydrophobic environments. Examples of this class include alamethicin [43], bee venom melittin [44], the frog magainins [45], and human LL-37 [46]. The less common extended class of HDPs are often enriched in particular types of amino acids such as Gly, Pro, Trp and Arg residues and include indolicidin, a bovine neutrophil peptide, and the porcine peptide fragment tritpticin [47]. In hydrophobic environments the extended class of HDPs are stabilized by hydrogen bonding and van der Waals forces in contrast to the intra-molecular stabilization forces found within the α-helical HDPs.

The other two HDP classes, the loop and β-sheet peptides, are respectively defined by the presence of one or more disulfide bonds. The bovine neutrophil HDP bactenecin (also termed dodecapeptide) is an example of a loop peptide. The more common β-sheet HDPs are stabilized by two to four disulfide bridges, which usually represent the most conserved and defining structural element. For example, β-defensins have three disulfide bonds in the arrangement Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6 while α-defensins have alternative patterns of disulphides Cys1-Cys6, Cys2-Cys5 and Cys3-Cys4. In addition to disulfide patterns, a structural motif termed the γ-core has been identified and appears to be conserved throughout the defensin branch of β-sheet HDPs [48, 49]. This motif is also observed amongst many chemokines and, given the demonstrated direct chemotactic activity of many HDPs, indicates a potential evolutionary precursor of both molecules. In addition to defensin-like peptides there are a number of peptides with two disulphides that form β-hairpins such as the porcine HDP protegrin and horseshoe crab polyphemusins.

2.b. Peptide Charge

Nearly all HDPs are cationic with net charges ranging from +2 to +9 [50]. As a notable exception, anionic HDPs have been observed in the lung surfactant of humans and ruminants [51-53]. Anionic antimicrobial peptides are bactericidal for both Gram-negative and Gram-positive bacteria through an unknown, zinc-dependent, mechanism [51-55]. For the most part, however, cationicity is highly conserved amongst HDPs. Cationicity has been postulated as a critical feature to drive the specificity of interaction between HDPs and bacteria through electrostatic interaction with highly electronegative bacterial cell membranes. To date there have few reports indicating bacterial receptors for HDPs (PhoQ and Lipid II in the case of the lantibiotic nisin) and most initial interactions between cationic peptides and bacteria are mediated by polyanionic cell envelope molecules. These include negatively charged lipids of the bacterial cytoplasmic membrane, such as phosphatidylglycerol, phosphatidylserine and cardiolipin, LPS of the outer membrane of Gram-negative bacteria and teichoic and techuronic acids associated with cell walls of Gram-positive bacteria. In contrast, zwitterionic and neutral membrane lipids of the eukaryotic cell membrane represent a poor target for HDPs. Furthermore, differences in the magnitude of membrane potential

gradients across cytoplasmic membranes of bacterial (~ -140 mV) and host (~ 15 mV) cells may also guide HDP specificity. The large electrochemical gradients in prokaryotic cells have been postulated to drive the influx of peptides into the cell [56] and it has been demonstrated that the binding of HDPs to membranes is sensitive to membrane potential, as even minor reductions in membrane potential (-20 mV) increased binding constants of selected HDPs by 200-fold [57]. Thus electrostatic attraction, and hence peptide cationicity, represents a critical parameter to drive the action, and ensure the selectivity, of microbial membrane interaction and in some instances disruption. Interestingly, cationic charge is also characteristic of nuclear localization signal peptides, a class of cationic peptides that readily translocate into eukaryotic cells, and several studies have demonstrated that these peptides also have direct antimicrobial activity e.g. HIV peptide TAT [58].

HDP derivatives of varying net charges, or with different sequence positioning of the cationic residues, demonstrate that antimicrobial activity correlates with overall cationicity. This correlation is limited, however, as increases in cationicity above a particular threshold usually do not afford further enhancement of antimicrobial activity but instead increase toxicity and reduce spectrum of activity [55]. It has been suggested that an absolute measure of charge, rather than the specific positioning of charged residues, is the determining factor for antimicrobial activity and toxicity. However recent, more detailed, structure-activity relationship studies suggest that this may be somewhat simplified and these observations may overemphasize the relation between HDP charge and cell selectivity [42].

2.c. Hydrophobicity

In addition to positively charged residues, HDPs, and in particular those with strong antimicrobial activity, also contain high proportions of hydrophobic amino acids. Hydrophobicity is essential for antimicrobial activity as it dictates peptide insertion into bacterial membranes. HDP hydrophobicity also contributes to toxicity. Investigations of magainin analogues have demonstrated that increases in mean peptide hydrophobicity, while maintaining constant charge, helicity and hydrophobic moment, promotepermeabilization of neutral, but not anionic, membranes [59] suggesting a correlation between peptide hydrophobicity and toxicity. Similarly, toxicity of the bovine cathelicidins bovine myeloid antimicrobial peptide (BMAP)-27 and BMAP-28 mapped to a highly hydrophobic C-terminal region. In these examples peptide toxicity was attenuated through either C-terminal truncation,or substitution of hydrophilic residues into this region with retention of antimicrobial activity [60]. Hodges *et al*. demonstrated a similar correlation between peptide hydrophobicity and hemolytic activities of gramicidin analogs (e.g. GS_{14}) [61]. Thus HDPs may have evolved to be moderately hydrophobic with a preference for microbial membrane interaction [62].

2.d. Amphipathicity

Hydrophobicity provides a measure of the relative content of hydrophobic residues but does not consider the influence of distribution of these groups on higher order peptide properties. Amphipathicity elaborates on hydrophobicity by characterizing the relative placement of hydrophobic and hydrophilic residues. As such, amphipathicity provides a more descriptive measure of how hydrophobic residues contribute to antimicrobial behaviour and toxicity. For HDPs it has been demonstrated that peptide amphipathicity contributes to both antimicrobial activity and toxicity [62]. Decreased amphipathicity with maintenance of sequence, charge, and hydrophobicityin gramicidin S, a cyclic β-sheet HDP with high permeabilization activity towards neutral membranes, results in increased antimicrobial activity and decreased hemolytic activity [61]. Thus, similar to hydrophobicity, increasing amphipathicity above a critical threshold can result in non-specific interactions between HDPs and host cell membranes leading to toxicity [61, 63].

2.e. Phase Transition

That amphipathicity is a critical feature for HDP antimicrobial activity raises questions of peptide solubility. It is likely that multiple peptide conformations are adopted in solution with exclusive formation of amphipathic species following contact with target cell membranes through segregation of amino acid side chains (termed here phase transition by analogy to membrane structural transitions). This would serve multiple roles in improving solubility, minimizing toxicity, and limiting formation of membrane-permeating or -disrupting species until bacterial contact. This phase transition process has also been termed context-specific activation or functional amplification [62]. Two mechanisms of phase transition have been proposed involving dynamic formation of conformations or complexes that increase solubility and decrease membrane disruption capability. The first mechanism involves sequestering hydrophobic regions through intramolecular conformational changes which minimize the density of hydrophobic residues in particular regions. Alternatively, formation of homomeric complexes may permit mutual sequestering of hydrophobic patches. This would likely apply to HDPs whose limited flexibility (such as e.g. defensins) prevents shielding of hydrophobic regions through intramolecular conformational rearrangements.

For α-helical peptides, phase transition usually occurs through intramolecular rearrangements that minimize formation of hydrophobic domains in the absence of an amphiphilic acceptor such as bacterial membranes. Interaction with the anionic phospholipids of bacterial membranes promotes adoption of amphipathic α-helices. Induction of these structures is promoted and stabilized to achieve optimal association between cationic residues of the peptide and anionic membrane components. Many classical α-helical peptides have been shown to conform to this model. For example, PGLa, an amphibian HDP, is disordered when exposed to neutral membranes but adopts an ordered helical conformation in membranes composed of anionic membrane molecules such as phosphatidylglycerol and phosphatidylethanolamine [64]. Similarly, induction of the helical conformation for magainin peptides occurs in the presence of outer membrane LPS or lipid A [65]. Furthermore, anionic artificial membranes induce helical conformations of ovispirin and two of its derivatives, while zwitterionic environments restrain helix formation [66]. Helix stability was highest for

native ovispirin with an increased ability to retain secondary structure in the presence of neutral membranes, indicating that its increased toxicity, compared to the analogs, might correlate with helix stability.

Conformations of β-sheet HDPs tend to be less dynamic as a consequence of constraints imposed by disulfide bonds. However, within this class of peptides hinge regions have been identified which allow for the adoption of structures with increased hydrophobic surfaces [67]. Powers *et al*. have identified a hinge-like region in polyphemusin I that may aid in LPS binding, peptide translocation and antimicrobial activity [68]. In general, peptides of this class have limited flexibility and lack the ability to transition to substantially different structures. Instead they might maintain solubility through formation of higher-order complexes in which hydrophobic surfaces associate to minimize contact with water. Therefore, β-sheet peptides may undergo formation of quaternary structures prior to membrane interaction. The ability of β-sheet HDPs to form multimeric complexes is supported by the observation that several α- and β-defensins form higher-order structures during crystal packing [69].

2.f. Self-Assembly

The ability to assemble into homomeric complexes, as observed in phase transitions of β-sheet HDPs, has consequence for various peptide activities. Formelittin, a highly lytic HDP, a core leucine zipper motif is crucial for toxicity but not antimicrobial activity [70]. Substitution of Leu to Ala within this motif does not influence antimicrobial activity but impairs hemolytic activity. This indicates distinct peptide structures are necessary for membrane permeation in zwitterionic and negatively charged membranes. Interestingly, the Leu to Ala substitutions impaired self-association and localization in zwitterionic membranes but not in anionic membranes. This suggests differences in red blood cell membrane permeability might reflect loss of self-association capabilities. The investigators concluded these substitutions perturbed helix assembly, membrane permeability, membrane localization, and secondary structure characteristics within zwitterionic membranes. These characteristics were not altered, however, within negatively charged membranes and antimicrobial activity was retained as a consequence.

Similar correlations between HDP oligomerization and hemolytic activity have been demonstrated. For example, a truncated version of LL-37 compromised hemolytic, but not antimicrobial activity, which correlated with reduced ability to oligomerize [71]. Chen *et al*. demonstrated a similar relationship between peptide self-association and reduced cytotoxicity in an α-helical peptide with high antimicrobial and hemolytic activities [72]. Contributions of self-assembly to toxicity are further supported by D- amino acid substitutions that reduce the tendency to self-associate as well as hemolytic activity. The reduced potential of D-amino-acid substituted peptides to self-associate is likely a mechanistic explanation for previous reports of reduced hemolytic activity of D-amino acid containing peptides. Propensities for self-association may also explain why increased hydrophobicity has been shown to correlate with increased toxicity as peptides of low hydrophobicity generally demonstrate weaker self-association [31, 73].

2.g. Considerations of Biophysical Properties to Immunomodulatory Applications

From a broader perspective the biophysical characteristics that define HDPs, in particular their charged/amphipathic nature, and ability for template-driven formation of bioactive structures, may also be of significance from immunotherapeutic perspectives. Firstly, the charged/amphipathic nature of these peptides may make the peptides "sticky" with strong potential for complex formation with a range of host molecules. Furthermore, the structural flexibility of HDPs might suggest these peptides to belong to a subdivision of the inherently unstructured proteins which utilize conformational flexibility to achieve a broader range of physical functions through multiple effectors. These characteristics could contribute to complex formation with multiple, biologically relevant, host effectors rather than the traditional paradigm of a single ligand for a single receptor. This also raises important practical considerations with respect to discriminating true biological effectors from false positives which may arise. Such promiscuity of complex formation will also complicate efforts of peptide optimization as modifications towards a particular activity achieved through one receptor may impact or introduce other interactions with unforeseen consequences.

3. PAST AND PRESENT THERAPEUTIC APPLICATIONS

The earliest, and to date only, successful commercial utilizations of cationic peptides have been through their direct antimicrobial activities in topical applications (polymyxin B and gramicidin S) and food preservatives (nisin). These peptides are highly modified, non-ribosomal peptides or lantibiotic bacteriocins from bacterial sources where, in the absence of a specific host defence system, evolutionary selection for potent direct antimicrobial activity occurred. In general, bacterial antimicrobial peptides (bacteriocins) are often more potent direct antimicrobials than eukaryotic HDPs [74].The success of these therapeutic applications, coupled with the emerging threat of drug-resistant bacteria, prompted further investigation of HDP antimicrobial potential. Additional studies verified HDPs from prokaryotic and eukaryotic sources possess potent, fast-acting activity against a broad spectrum of Gram-negative and Gram-positive strains, including multi-drug resistant strains [75, 76]. The low frequency of resistance selection and ability to generate peptides with enhanced activities through sequence manipulation further support the consideration of HDPs as next generation antibiotics (Table **1**). Although concerns have been raised regarding potential toxicities associated with systemic administration, topical applications of HDPs appear well-tolerated. For example, a phase I study of the protegrin analog IB 367 demonstrated significant decreases in oral microflora with no clinically significant toxicity [77].

To date three non-bacterially produced cationic peptides have advanced into Phase III clinical trials (Pexiganan, Iseganan, and Omiganan). Two of these (Omiganan and Pexiganan) have demonstrated efficacy in Phase III clinical trials [35]. Pexiganan (MSI-78; Genaera, PA, USA), a C-terminally modified magainin 2 derivative, was investigated for use as a topical antimicrobial in treatment of mild to

Table 1. Therapeutic Applications of Host Defence Peptides

Peptide	Company	Target	Clinical Stage
MX-226	Migenix (Vancouver, BC, Canada)	Catheter-associated infections	Phase IIIb
MX-594AN	Migenix (Vancouver, BC, Canada)	Acne Vulgaris	Phase II
CLS001	Migenix (Vancouver, BC, Canada)	Rosacea	Phase II
PG-1	IntraBiotics (Mountain View, CA, USA)	Peritoneal infections	*Phase III*
IB 367	IntraBiotics (Mountain View, CA, USA)	Chronic respiratory infections	Phase II
Plectasin (fungal defensin)	Novozymes A/S (Bagsvaerd, Denmark)	Systemic (anti-Gram positive) infections	Preclinical
IMX942	Inimex (Vancouver, BC, Canada)	Selective stimulation of innate immunity	Preclinical
hLF1-11	AM-Pharma	Direct antimicrobial activity to address allogenic bone marrow stem cell transplantation-associated infections	Phase II
Neuprex (rBPI21)	XOMA (Berkley, CA, USA)	Meningococcaemia and Crohn's Disease; stem cell transplants	Phase II/III; Phase I/II

moderate diabetic foot ulcer infections [47]. Although Pexiganan was demonstrated to have low-associated toxicities and equivalent clinical outcomes to ofloxacin, a fluoroquinolone antibiotic, it did not, however, demonstrate additional benefit to currently available therapies and was accordingly rejectedby the FDA [78]. Omiganan/MX-226, developed by Migenix (Vancouver, Canada), for prevention of catheter colonization and associated infections, has demonstratedstatistically significant efficacy and recently completed confirmatory Phase IIIb clinical trials [35, 79]. It is currently being prepared for a new drug approval (NDA) application [47, 78, 80, 81].

Recently, investigations of non-antimicrobial HDP activities have demonstrated therapeutic efficacy in the modulation of host immune responses highlighting the potential for alternative HDP applications. For example MX-594AN (Migenix; an alternative formulation of Omiganan) completed phase II clinical trials as a topical therapeutic for mild to moderate acne vulgaris and demonstrated significant efficacy against a range of acne lesions, although the primary activity was anti-inflammatory [80]. MX-594AN (under the name CLS001) demonstrated significant therapeutic effects in Phase II clinical trails for treatment of the non-infectious inflammatory skin disease Rosacea. Recently Inimex introduced IMX-942, a non-antimicrobial, 5-amino acid IDR peptide, into Phase I clinical trials for treatment of fevers secondary to chemotherapy of bone marrow transplant patients. Similarly hLF1-11, an N-terminal derivative of human lactoferrin, has successfully navigated Phase 1 trials and while originally described as an antimicrobial peptide appears to now be described as a modulator of immunity.

4. FUTURE THERAPEUTIC APPLICATIONS

The ability of HDPs to modulate various aspects of host immunity offers exciting potential for future application as immunotherapeutics. Such utilization could bein multiple contexts. **(a) Immune Stimulating Antimicrobial Agents:** The involvement of HDPs in the activation of host immunity may represent a more desirable, and elegant, mechanism of antimicrobial action than direct attack on the microbe. In particular, indirect antimicrobial activity resulting from the activation of a broad range of host defence responses is less likely to induce resistance responses than direct targeting of microbes by HDPs. Secondly, there is greater potential for safety as structural features that bestow direct antimicrobial activity closely overlap with those determining toxicity. Thus activation of innate immune responses by HDPs appears to represent a new paradigm for antimicrobial therapy and may be utilized as stand-alone treatments or in combination with conventional antibiotics [82]. The broad spectrum anti-infective activities engendered might be especially useful where the etiological agent of infection is unknown; **(b) Anti-Inflammatory Agents in the Presence or Absence of Infection:** Suppression of pro-inflammatory cytokine responses by HDPs, coupled with the chemoattraction of immune cells, may represent a therapeutic mechanism for suppressing inflammation. The anti-inflammatory action of HDPs could be utilized in treatment of chronic and acute inflammatory conditionsor in combination therapies with conventional antimicrobials against the inflammatory sequellae of certain infections; and **(c) Vaccine Adjuvants:** the ability for HDPs to initiate and polarize adaptive immune responses has potential applications as vaccine adjuvants to induce more potent responses against a range of antigens.

This could reduce immunization doses and frequencies as well as to enhancing responses to weakly immunogenic antigens.

4.a. Immunomodulatory Antimicrobials

While the precise mechanisms by which HDPs modulate host immunity have not been clearly defined it appears as though these functions are receptor-mediated and involve modulation of intracellular signalling pathways. Thus far most investigations related to HDP immunomodulatory activities have focused on chemotaxis and chemokine production. From these investigations various mammalian HDPs have been shown to be chemotactic for blood neutrophils, mast cells, monocytes, T cells and immature dendritic cells [83]. Cathelicidins from many species including humans, bovine, mouse, pig and chicken have been shown to be chemotactic for a wide range of peripheral blood cells *in vitro* and *in vivo* [34]. For example, PR-39, a Pro-Arg rich porcine cathelicidin, has been implicated in neutrophil chemotaxis [84] and chicken cathelicidin-2 (CATH-2) has been demonstrated to induce the expression of monocyte-chemoattractant protein-1 (MCP-1) in human peripheral blood mononuclear cells while levels of TNF-α, IL-6, and IL-8 were unchanged [85]. Most of the cathelicidin investigations of HDP immunomodulatory activities have focused on human cathelicidin hCAP18/LL-37. LL-37 has been implicated in the chemotaxis of peripheral blood monocytes, neutrophils, and CD4+ T cells *in vitro* [83] and the retention of these activities in the presence of serum supports their physiological relevance. Physiological concentrations of LL-37 have also been demonstrated to be chemotactic for rat peritoneal mast cells and promote the induction of histamine release and intracellular Ca^{2+} mobilization within these cells [86]. As LL-37 induces the expression of a broad range of chemokines it has been debated whether these are direct chemotactic activities or indirectly related to the induction of chemokines [87]. Indeed, LL-37 might play a role as a mediator of immune responses with amplification loops as LL-37-mediated degranulation of mast cells would result in the release of inflammatory mediators and increased vascular permebilizationpotentially increasing neutrophil infiltration to sites of inflammation [88].

A variety of cellular and cytoplasmic receptors have been implicated in LL-37-mediated immunomodulatory responses. While these investigations have focused on receptors mediating chemotaxis to LL-37, receptors involved in alternative LL-37-mediated responses have also been reported. There is strong evidence to suggest that the chemotactic activities of LL-37 are mediated through formyl peptide receptor-like 1 (FPRL1) [83]. Indeed, LL-37-mediated chemotaxis was specific for cells known to express FPRL1 such as monocytes, neutrophils, and T cells. Yang *et al.* demonstrated that monocyte chemotaxis and Ca^{2+} mobilization in response to LL-37 depended on the Gi-protein coupled receptor (GPCR) and FPRL1 [83]. Mobilization of Ca^{2+} could be cross-desensitized by pertussis toxin, an antagonist for GPCRs as well as peptide antagonists of the orphan receptor FPRL1. Differentiation of monocytes into immature dendritic cells ablated the chemotactic and Ca^{2+} mobilization activities of LL-37 which was postulated to result from loss of functional FPRL1. Further supporting this receptor-ligand interaction, endothelial cell angiogenesis in response to LL-37 is also mediated by FPRL1 [89]. Alternatively, Niyonsaba *et al.* have shown that LL-37 has at least two types of receptors for chemotaxis of mast cells, namely high- and low-affinity receptors, neither of which is FPRL1 [86]. As treatment with pertussis toxin or a phospholipase C inhibitor inhibited LL-37-mediated chemotaxis, this suggests involvement of receptors coupled to the GPCR-phospholipase C signalling pathway.

Receptors involved in modulation of apoptosis by LL-37 have also been identified. Barlow *et al.* have demonstrated that LL-37 modulates apoptotic pathways in primary human innate-immune effector cells through purinergic receptor P2X, ligand-gated ion channel, 7 (P2X7), a member of the ATP-activated ionotropic P2X receptor family, and GPCR rather than FPRL-1 [90]. LL-37 also induces caspase-dependent cell death in primary airway epithelial cells and this has been suggested to involve activation of P2X7 in a mechanism analogous to apoptosis induction in human cervical epithelial cells [91]. Adding further complexity, an investigation by von Haussen *et al.* demonstrated that LL-37 acts as a growth factor for lung cancer cells through epidermal growth factor receptor (EGFR) pathway [92]. This is consistent with a report from Tjabringa *et al.* suggesting that EGFR is required for the activation of epithelial cell signalling responses to LL-37 [93]; however, P2X7 and FPRL-1 do not appear to be involved as inhibition of these receptors did not modulate LL-37tumourigenic activity. It should be noted, however, that an all D-LL-37 enantiomer retained tumourigenic activity suggesting that activation of EGFR signalling is due to membrane disruption rather than receptor interaction. Collectively, however, these investigations demonstrate the ability of LL-37 to modulate distinct biological objectives through functional interact with a number of receptors Members of the defensin family have also been demonstrated to possess potent immunomodulatory activities. Similar to the cathelicidins, defensins have broad chemotactic activities for a variety of cell types. Specifically, HBD-3 induces monocyte migration [94] and HBD-1 and HBD-2 are chemoattractants for immature dendritic cells and memory T cells [95]. HBD-1-4, similar to LL-37, induces IL-18 secretion in primary human keratinocytes [96]. Investigations of potential host cell receptors have identified chemokine receptor 6 (CCR6) as a defensin receptor. For example, the induction of HEK293 cell migration by β-defensinsis dependent on CCR6 expression and CCR6 has been implicated in the Thechemotaxis of DCs and T cells (hBD1-3), monocytes (hBD-3), and mast cells (hBD-2) by β-defensins [97]. Additionally, antibodies against CCR6 block the ability for β-defensins to exert a chemotactic effect; however, hBD-3 also induces migration of cells which do not express CCR6, such as monocytes, indicating involvement of an as of yet unidentified receptor [98]. Based on proposed activation of a shared receptor it would be anticipated that the natural chemokine ligand for CCR6, chemokine (C-C motif) ligand 20 (CCL20)/macrophage inflammatory protein-3 (MIP-3), and human β-defensin would share similar structural features. While these molecules share little sequence similarity, β-defensins adopt a similar tertiary structure as CCL20/MIP-3 in the form of an antiparallel triple-stranded β-sheet with a C-terminal α-helix [99]. Specifi-

cally the Asp4-Leu9 motif in β-defensins is a structural analog of the Asp5-Asp which is believed to be essential in the interaction with CCR6 [99]. This motif may be a crucial determinant of the ability of these HDPs to exert a chemotactic effect.

The pattern of disulfides is a critical determinant of defensins chemoattractant activity [94]. The native structure of hBD3 involves disulfide bond formation Cys1-Cys5, Cys2-Cys4, Cys3-Cys6. This configuration induces migration of CCR6 HEK293 cells at concentrations as low as 10 ng/ml [94]. Topological analogues of different disulfide bonding patterns decreased this activity 10-100 fold and the replacement of all cysteines decreased this activity by 1000-fold. This indicates that the configuration of disulfides is critical for functional interaction with the receptor [94]. As chemotactic activity requires direct interaction between the receptor and peptide it is anticipated that loss of activity within the linear analogue of hBD3 is due to peptide destabilization as chemokine receptor binding/activation requires stable ligand structures [14, 66]. However, Taylor *et al*. demonstrated that the chemoattractant properties of hBD3 are retained with substitution of five of the six cysteines found within the molecule. Interestingly, activity is not dependent on the simple introduction of a Cys residue into the peptide; introduction of Cys into position five of the six cysteine motif restores chemoattractant activity whereas a derivative with a single Cys at position one is inactive [100]. Although the overall disulfide linkages within hBD3 are indispensable for immunomodulatory activity, as evidenced by the unique chemokine activities of various hBD3 isoforms, these disulfides are notessential for antimicrobial activity. Thus, it would appear for this HDP that immunomodulatory and antimicrobial activities are independent of one another.

To add further complexity to the identification of HDP receptors there is evidence to suggest these peptidesmay also act on cytosolic receptors. A 15-residue active fragment of PR-39 selectively binds p130Cas, leading to activation of p130Cas-associated signalling pathways, as well as PI3-kinase p85-a, the regulatory subunit of PI3-kinase [101, 102]. Bao *et al*. have demonstrated PR-39-mediated blocking of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, α (IkBα) degradation by the ubiquitin-proteasome pathway through direct interaction of the peptide with the a7 subunit of the 26S proteasome [103]. It has also been noted that LL-37 has nuclear targeting sequences and can co-transport molecules which may provide yet another mechanism for induction of cellular responses [86]. For example, LL-37 can bind and promote the cellular uptake of short nucleic acid strands enhancing their ability to serve as TLR9 ligands and inducing cellular responses distinct from activation with either the peptide or nucleic acid alone [104].

A number of signal transduction pathways and mechanisms have been implicated in HDP action. In mast cells, keratinocytes, and monocytes LL-37 and β-defensins have both been shown to activate critical kinases including mitogen-activated protein kinases (MAPK) as well as extracellular-signal-regulated kinase 1 and 2 (ERK1 -2) [96]. In keratinocytes, LL37 has been linked to activation of the signal transducer and activator of transcription 3 (STAT3) pathway as well as inhibit LPS-induced nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) translocation [105]. These findings collectively highlight the ability of HDPs to initiate host responses through a diverse array of receptors and mechanisms.

4.b. Anti-Inflammatory Agents

Host responses to bacterial infection are potentially as damaging as infection itself. Unregulated inflammation in the form of systemic inflammatory syndrome or sepsis is responsible for hundreds of thousands of deaths each year [106]. While conventional antibiotics provide a mechanism to deal with microbial challenge, they are unable to address pathological inflammation which can result from excessive stimulation by bacterial effectors or disregulation of the inflammation process. A highly attractive feature of HDPs is that while many of their actions are pro-inflammatory (chemotaxis of leukocytes and induction of cytokine, chemokine and histamine release), they also modulate inflammation responses. This does not appear to be specific to any particular HDP as peptides of diverse sequences and sourcesincluding insect-derived cecropin-melittin hybrid peptide CEMA [82], LL-37 [38], BMAP-28 [56], indolicidin [107] and small synthetic cationic peptides [107] all modulate LPS-induced inflammation *in vivo* to protect against endotoxaemia [108-110].

The simplest explanation for the ability of a diverse range of HDPs to mediate an anti-endotoxin effect is through direct chelation of LPS, the principle trigger of sepsis responses, thus preventing activation of TLR4. Interaction between anionic LPS and cationic peptides is anticipated as the affinity of HDPs for this bacterial membrane component helps drives the antimicrobial action and specificity of HDPs. Interaction of various HDPs with LPS has been characterized as being moderately high binding affinity [38, 82]. The hypothesis that HDP anti-endotoxin activity is achieved exclusively through LPS binding may, however, underestimate the functional complexity of these events. Indeed, this is analogous to initial assumptions that HDP antimicrobial activity was mediated exclusively through disruption of membranes. There is evidence to suggest the protective effect afforded by HDPs in limiting excessive pro-inflammatory responses involves modulation of host responses rather than LPS chelation.

Firstly, the ability of HDPs to neutralize LPS-mediated pro-inflammatory responses does not depend on co-administration. Mookherjee *et al*. demonstrated pre-treatment of cells with a variety of HDPs 30 minutes prior to LPS stimulation effectively inhibits TNF-α induction [38, 82]. Peptides such as CEME and polymyxin B have been observed to neutralize TNF-α synthesis in mice, even when administered 60 minutes following LPS exposure. This activity was not related to direct interaction of peptide and LPS as: i) the activity was retained upon removal of LPS from the culture media, and ii) LPS is internalized by monocytes within 10-30 minutes of administration. Secondly, HDPs selectively suppress expression of subsets of LPS-induced genes in macrophages [111]. As direct binding of LPS would be anticipated to result in global suppression suggests an alternate mechanism of action. Similarly, microarray experiments of CEMA demonstrated selective modulation of

LPS-induced gene expression [93, 111]. Thirdly, HDP administration alone in macrophages up-regulates expression of a wide range of genes, including those related to pro- and anti-inflammatory responses [111]. Finally, HDPs are able to modulate cellular responses initiated by activation of other TLRs, such as TLR2 and TLR9 [38].

Recently, van Dijk *et al*. investigated the effect of truncations and substitutions on the endotoxin-neutralizing activity of CATH-2 [85]. CATH-2, as well as other members of the chicken cathelicidin family, adopts a helix-hinge-helix structure in hydrophobic environments due to a central Pro residue between N- and C-terminal helical regions. Truncation of either termini reduced CATH-2-mediated inhibition of LPS-induced cytokine induction and was postulated to be due to high affinity LPS-binding sites within each termini. While substitution of the central Pro with Gly had no significant effect on LPS neutralization, substitution with Leu significantly reduced activity indicating the LPS-neutralization activity of CATH-2 relies on a flexible peptide template. Rosenfeld *et al*. investigated the relationship between HDP sequence/structures and anti-endotoxic activities using various synthetic HDPs [112]. Using a 15-mer parent peptide, constructed from Lys and Leu residues, which folds into an ideal amphipathic α-helix, the authors investigated scrambled and segregated derivative peptides constructed from either L- or D-amino acids. All of the derivatives retained the ability to reduce LPS-induced TNF-α secretion from macrophages although the scrambled- and segregated-D amino acid derivatives were significantly less active. Indeed, at high LPS concentrations the most active detoxifying peptides were those that adopted α-helices in solution or retained amphipathicity in the all-L or the D, L forms [112]. In contrast, all of the peptide derivatives retained comparable abilities to bind LPS. Thisdemonstrates that the ability of an HDP to bind LPS may be independent of secondary structure while their ability to suppress LPS-mediated inflammation is highly dependent on secondary structure. Collectively, these observations support the conclusion that HDP modulation of inflammation is due, at least in part, to the ability to influence host cell responses. There is, however, little information on potential receptors through which the effect is mediated. For example, while the G protein-couple FPRL1 receptor is required for chemotactic activity and other aspects of activation of innate immunity by LL37 the anti-endotoxin function of this peptide is independent of the G protein-coupled receptors.

4.c. Vaccine Adjuvants

In addition to their ability to modulate innate immunity HDPs also bridge innate and adaptive immunity through recruitment of effector cells to sites of infection (or in the case of vaccines, injection) and their subsequent differentiation and activation. Specific HDPs also contribute to antigen-specific immunity by promoting differentiation of particular cell lineages, such as DCs, as well as by influencing cytokine expression to modulate polarization of adaptive immune responses between cellular Th1 and humoral Th2 responses. For example, LL-37 induces maturation of immature dendritic cells and polarizes these cells to favour Th1 responses [34]. These responses appear to be mediated through induced expression of interferon (IFN)-γ, IL-10, IL-6 as well as various co-stimulatory molecules [113]. Additionally, human defensins enhance proliferative and cytokine responses of CD4+ T cells [114] and both human α-defensins and mouse β-defensins activate antigen-specific cytotoxic T lymphocytes to promote Th1 cellular responses [113]. Presicce *et al*. recently investigated the impact of defensins on DCs [115]. The human defensins HNP-1 and hBD-1 had chemotactic activity for monocyteDCs (moDCs), promoted moDC activation and maturation, and induced the pro-inflammatory cytokines TNF-α, IL-6, and IL12p70. Collectively these activities translate into the ability to function as vaccine adjuvants as various HDPs have been shown to enhance antigen-specific responses at both humoral and cellular levels [114, 116]. Human defensins have also been co-administered as adjuvants in mice resulting in enhanced antibody responses supporting their involvement in humoral-repsonses [116]. This example also suggests that the adjuvanticity of HDPs is not limited to the species in which the peptides originated.

5. STRATEGIES FOR HDP SELECTION AND OPTIMIZATION

HDPs have yet to achieve success as systemic therapies in large part due to their prohibitive costs and potential toxicities. The expense of HDP therapies reflects both high costs of production as well as large dose quantities necessitated by their biological instability. Higher dose quantities and frequencies also promote dose-dependent toxicity. These limiting characteristics apply to both direct antimicrobial and immunotherapeutic systemic HDP applications. Strategies described in this section have been proposed to address the stability and toxicity of HDPs, often from the perspective of direct antimicrobial activity, but also offer potential for immunotherapeutic applications.

5.a. Identification of Minimal Motifs

A logical approach to reduce costs associated with HDP production is to reduce peptide lengths through identification of minimal active structures. This is an appropriate approach, provided defined and quantifiable activities are identified. Truncation of natural peptides may, however, compromise individual activities of particular sequence motifs that contribute to the overall therapeutic effect. For the more extensively studied HDPs it is becoming increasing possible to attribute particular activities to defined peptide regions (Fig. **3**). For example, an 18-mer derivative of LL-37 comprising residues 15-32 of LL-37 with E16L, Q22K, K25L, D26K and N30K substitutions, had much higher hemolytic activity than the full-length parent peptide [117]. This exemplifies the importance of these characteristics in defining microbial versus host-cell specificity. It is also of note that chemotactic activity was conserved in all three LL-37 derivatives. In contrast, a recent investigation by Sigurdardottir *et al.* demonstrated that a peptide comprised of residues 14-34 of LL-37 had reductions in hemolytic activity and apoptosis induction in human cultured smooth muscle cells [118]. In accordance with the investigation by Cirioni [110], the LL-37 fragment in the Sigurdardottir study retained chemotactic activities and inhibition of LPS-induced NO production as compared to the full-length peptide. Previously, a study by Nagoaka and colleagues of the 18-mer LL-37 derivative LLKKK

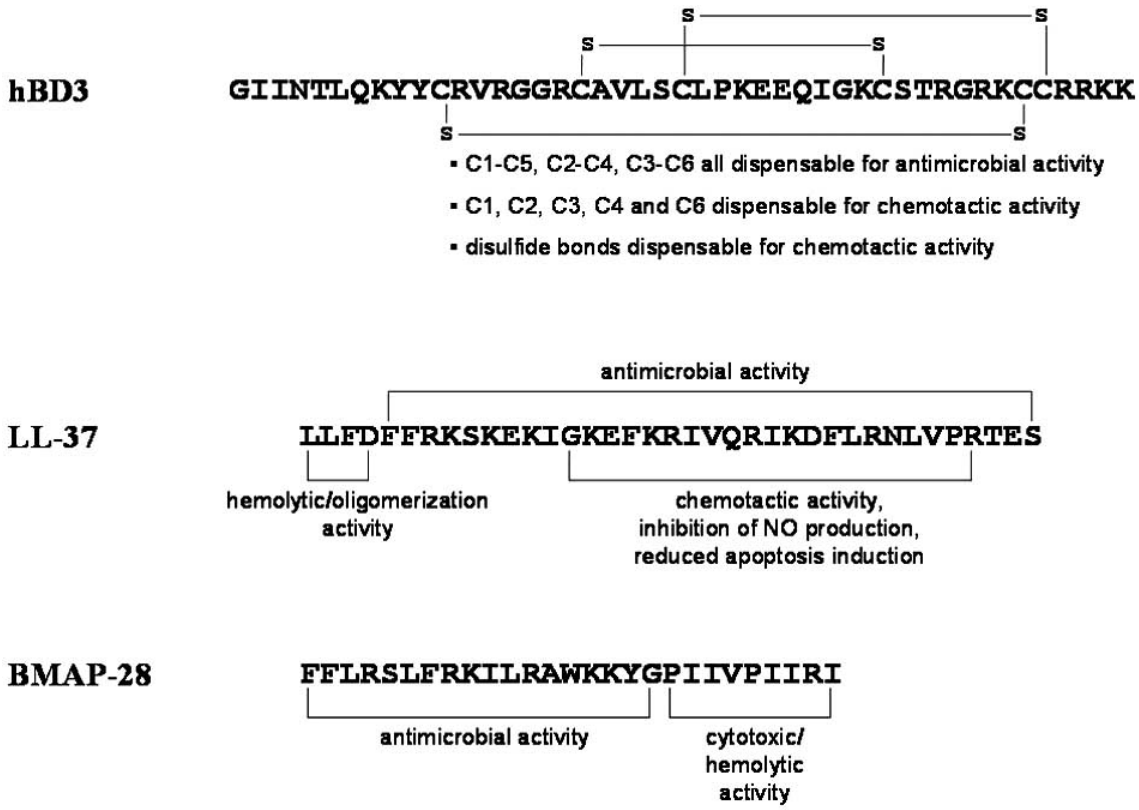

Fig. (3). Structure Activity Relationship for Select Host Defence Peptides. For a number of the more extensively characterized HDPs such as LL-37, BMAP-28 and hBD3 it is possible to attribute particular activities to defined peptide regions. In some cases these activities map to distinct peptide regions but more often overlap with other peptide activities.

showed no cytotoxicity to murine macrophages and suggests this region of the peptide cannot be attributed to overall cytotoxicity but rather only hemolytic activity [119]. It is also of note that both N-terminal truncation derivatives had reduced hemolytic activities although both peptides contain the highly hemolytic 18-mer region. Thus it can be suggested that it is not the presence of a particular sequence that determines peptide activity but rather the context of the sequence within the full-length peptide.

Furthermore, structure-activity studies of LL-37 have attributed particular immunomodulatory to particular regions or characteristics of the peptide. An investigation by Tomasinsig *et al.* demonstrated that P2X7-mediated induction of fibroblast proliferation by LL-37 is related to peptide helicity as proliferative activity was retained by an all D-LL-37 enantiomer [120]. Sigurdardottir *et al.* demonstrated that the C-terminus of LL-37 is important for chemotactic activity as GKE, a peptide corresponding to residues 14-34 of LL-37, had similar patterns of activity as the parent peptide [118]. Interestingly, a prior investigation of the 18-amino acid peptide LLKKK displayed similar patterns of chemotactic activity [121] as GKE and was postulated to reflect the similarity of LLKKK to the mid-region of GKE. In agreement with Sigurdardottir *et al.* an investigation by Braff *et al*. of an LL-37 fragment library indicated that peptide fragments truncated at the N-terminus appear to retain IL-8 inducing activities in keratinocytes whereas C-terminal truncations had diminished IL-8 induction activity [122]. These investigations highlight the importance of the C-terminal region of LL-37 to immunomodulatory activities of the peptide.

5.b. PhoQ Ligand Potential

The demonstration that HDPs activate bacterial virulence and resistance adaptations through the PhoPQ two-component system raises concerns that HDP treatments, whether from direct antimicrobial or immunotherapeutic objectives, could promote bacterial behaviours which are deleterious to the host. Thus, peptide optimization will benefit from consideration of the PhoQ activating potential in addition to other therapeutic activities of interest. That activation of PhoPQ is not equal for all HDPs indicates that variables of sequence and/or structure are associated with PhoQ activation potential [123]. It may therefore be possible to design peptides with reduced ability to activate PhoQ while maintaining or improving other desired characteristics. Peptides with reduced PhoQ-activating potential would be anticipated to have improved therapeutic potential. We have proposed a novel strategy for mathematical treatment of minimal inhibitory concentration data that permits discrimination and quantification of PhoQ activating potential (Table **2**) [123].

5.c. Stereoisomers and Retro-Inversion

A potential strategy for peptide stabilization is through incorporation of D amino acids (Fig. **4**). The key functional advantage is that peptides composed from D-amino acids are

Table 2. Calculation of PhoQ Ligand Potential Based on MIC Data

Direct Antimicrobial Activity
$dAMA = MIC_{Q-}$
$\Delta dAMA = MIC_{Q-}(PD) - MIC_{Q-}(PP)$
PhoQ Activating Potential
$PAP = MIC_{wt} - MIC_{Q-}$
$\Delta PAP = (MIC_{wt}(PD) - MIC_{Q-}(PD)) - (MIC_{wt}(PP) - MIC_{Q-}(PP))$
Peptide Efficiency
$\Delta PE = \Delta dAMA + \Delta PAP$

Direct antimicrobial activity is quantified by the MIC values against either the PhoQ- (MIC_{Q-}) strain. For any peptide derivative (PD) the relative change in direct antimicrobial activity (ΔdAMA) with respect to the parent peptide (PP) can be quantified by: $\Delta dAMA = MIC_{Q-(PD)} - MIC_{Q-(PP)}$. The ΔdAMA score provides a measure of how alterations to peptide sequence influence the ability to kill non-adapted bacteria. Negative ΔdAMA scores indicate the PD is a more effective antimicrobial than the PP. The PhoQ activating potential (PAP) is the extent to which HDPs promote bacteria to initiate phenotypic changes that decrease HDP sensitivity. For any given peptide, the PAP is calculated as the difference in MICs between the wild type and PhoQ-mutant (Q-) such that: $PAP = MIC_{wt} - MIC_{Q-}$. Changes in the ability of a PD to activate the bacterial defence mechanisms, as compared to the PP, are calculated by: $\Delta PAP = (MIC_{wt(PD)} - MIC_{Q-(PD)}) - (MIC_{wt(PP)} - MIC_{Q-(PP)})$. A negative score indicates that the PD is a more effective antimicrobial as a result of minimized induction of PhoQ-mediated adaptations. Changes in peptide efficiency can be more specifically defined in terms of the ΔdAMA and ΔPAP whereby $\Delta PE = \Delta dAMA + \Delta PAP$. Negative scores of ΔPE indicate more effective antimicrobials and the magnitude of the score reflects the degree of improvement.

poor substrates for proteolytic enzymes to improve biological stability [21]. Modifications to include D-amino acids have been shown to increase the biological half lives of peptides in serum while retaining antimicrobial activity [21]. Enantiomers of cecropin A [124], magainin 2 [124, 125], and protegrin [126] have demonstrated retained or enhanced antimicrobial activities as compared to their natural analogues. Partial D-amino acid substitutions have also been demonstrated to increase peptide stability and retain antimicrobial activity. Hong *et al.* have demonstrated that D-amino acid substitutions at the N- and/or C-terminus of the antimicrobial peptide KKVVFKVKFKK had little effect on the overall α-helical structure or antimicrobial activity; however, D-amino acid substitutions in the middle of the peptide sequence result in disruption of α-helical formation and ablation of antimicrobial activity [127]. In agreement, a recent investigation by Strömstedt *et al.* of EFK17, an LL-37 derivative, demonstrated that internal D-amino acid substitutions disrupted peptide helicity resulting in compromised antimicrobial activities and decreased adsorption to lipid membranes [128].

Retro-inversed (RI) peptides are both directional and chiral isomers of natural peptides in which the sequence of the peptide is reversed and the chirality of each amino acid is inverted [129]. Peptides modified in this manner are predicted to maintain the same three-dimensional side-chain topology as their natural counterparts and therefore have the potential to maintain biological activity. The differential ability for RI-peptides to mimic the biological activities of their natural counterparts is likely a function of both the structural complexity of the peptide and its interaction with biological effectors. Specifically, although RI-peptides are predicted to maintain a consistent topology of side chains the reversal of peptide bond orientation results in a distinct pattern of hydrogen bonding potential from the main-chain. The extent to which these main-chain groups contribute to complex formation will likely determine the extent to which the modification is tolerated. Therefore, in the absence of detailed information on the interaction between the peptide and its biological effectors it is difficult to predict the functional consequences of retro-inversion.

Retro-inversion of HDPs has been explored for a limited number of examples with the general conclusion that the modification results in retained to moderately improved antimicrobial activity [130]. Retro-inversion of lactoferricin B (LFB) increased antimicrobial activities against Gram-positive and –negative organisms and was postulated to indicate a reliance on peptide hydrophobicity. Notably, however, these investigations were limited to consideration of antimicrobial rather than immunomodulatory activity [131]. As antimicrobial activity is mediated through non-specific, non-chiral interactions with bacterial membranes it is not surprising that it is tolerant of retro-inversion. The more stringent criteria by which retro-inversion of HDPs will be evaluated are through the retained ability to influence innate immune responses through specific interactions with chiral host receptors. While some initial reports have indicated the ability for all-D and RI HDPs to influence host immune responses [122, 132] these examples likely reflect indirect mechanisms involving LPS binding [122] or disruption of host cell membranes resulting in cytokine release [132] rather than receptor-mediated effects. Interestingly, a RI derivative of indolicidin retained the antimicrobial and anti-endotoxic activities of the natural peptide [132]. As the anti-endotoxin activities of the RI derivative were conserved these results might indicate the conservation of multiple immunomodulatory activities upon retro-inversion, highlighting the potential of this modification for therapeutic applications.

5.d. Branched Peptides

Recently, branching has been investigated mechanisms for increasing the therapeutic potential of peptides. Specifically, branched HDP derivatives have been explored as a method for increasing peptide half-life [133, 134]. These molecules are constructed by the addition of peptide sequences onto radially branched lysine residues stemming from a peptidyl core [135, 136]. Pini *et al*. have constructed a tetrabranched antimicrobial peptide, M6, that had low MICs for several clinically-relevant pathogenic Gram-negative microbes, remained stable in blood and serum for greater than 24 hours (as compared to ~2 hours for natural peptides in the same study) and directly bound *E. coli* LPS *in vitro* [133].

5.e. Peptide Immobilization

Therapeutic peptide applications may also include the immobilization of peptides onto the surfaces of medical devices. Recently, surface immobilization of HDPs onto cellulose has been demonstrated to result in the retention of antimicrobial activity and reduced cytoxicity [137]. Intriguingly, the activities of the surface-immobilized HDPs were

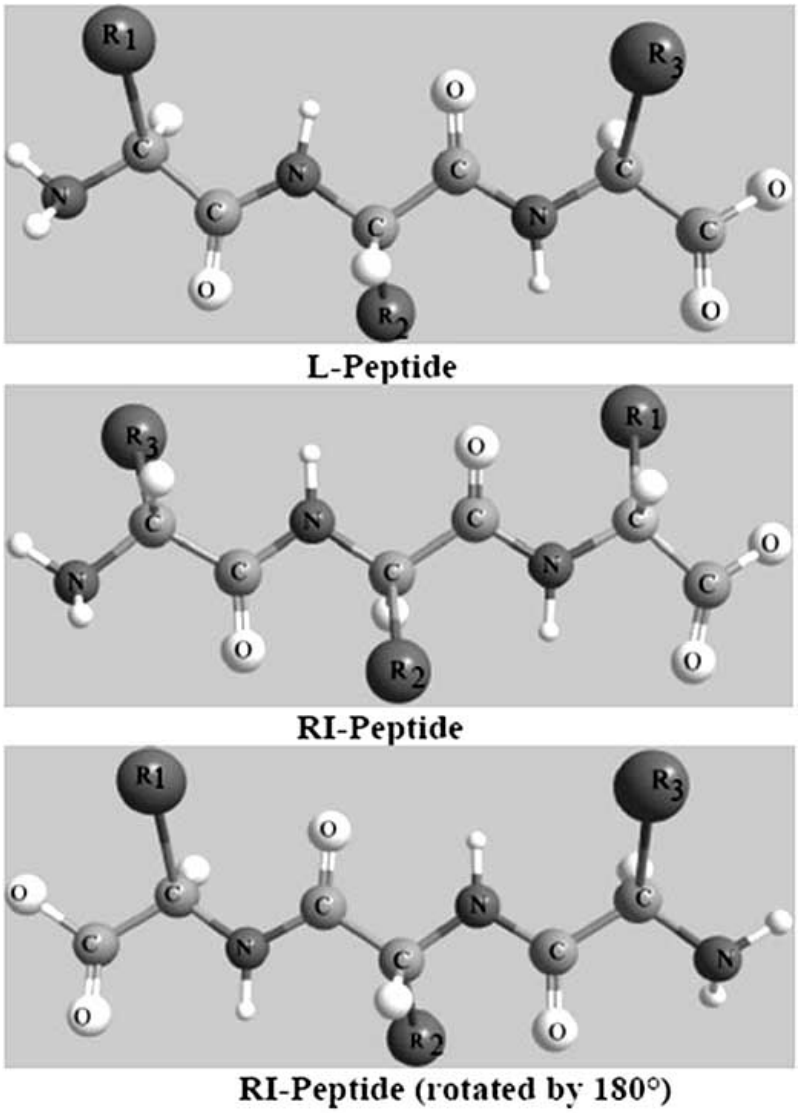

Fig. (4). Relationship between peptides and their retro-inversed isomers. A parent peptide is composed of L-amino acids in the sequence order (1, 2, 3, n) where residue 1 occupies the amino terminal position of the peptide. The corresponding RI-peptide is composed of D-amino acids in the sequence order (n, 3, 2, 1) where residue 1 now occupies the carboxy terminal position. When the retro-inversed peptide is rotated by 180 degrees in the plane of the diagram the sequence of the modified peptide now reads (1, 2, 3, n) and the shared side chains (1 to 1, 2 to 2, etc) superimpose in three-dimensional space with the parent peptide.

not directly correlated to the activities of their soluble analogs and the authors predict an alternative mechanism of direct antimicrobial action as the peptides used in this study would not be able to cross the membrane bilayer. This investigation highlights the potential for therapeutic application of HDPs that do not rely on systemic peptide administration.

5.f. Single Position Substitution Analysis

Sequential modification of HDPs through single position substitution analysis has been employed for identifying sequence patterns that contribute to superior activities. For example, Nagaoka *et al.* used substitution analysis of cationic and hydrophobic residues within LL-37 to identify residues contributing to antimicrobial activity. This analysis generated peptides with increased activity against of a broad range of Gram-positive and Gram-negative organisms [119]. Substitution analyses have also been utilized for identification of specific residues that contribute to therapeutic indices of HDPs. Lee *et al.* identified specific L- and D-amino acid substitutions within GS14, a cyclic 14-residue derivative of Gramicidin S, that impacted the overall therapeutic index of the parent peptide [61]. Similarly, Cerovsky *et al.* prepared 40 analogs of antimicrobial peptides from Polistinae wasps to identify residues and physicochemical parameters which correlate to biological activities of the peptides. These analyses have also been utilized beyond the scope of bacteria [138].

As synthesis of many peptide derivatives is time consuming and expensive, investigators have turned to alternative methodologies to identify improved peptide derivatives of parent peptide molecules through substitution analysis. Peptide array technology has proven an economical, high-throughput strategy for creation of large libraries of peptide

derivatives. Using SPOT synthesis on cellulose supports, it is possible to create peptides of up to 50 amino acids per spot and approximately 8,000 spots per cellulose sheet [139]. These peptides are not limited to gene-encoded amino acids and may be cleaved from the cellulose sheet for studies of soluble peptides or left tethered to the support. Applying SPOT synthesis to Bac2A, a linear variant of the cationic peptide bactenecin, a library of 228 derivatives was created [139]. From this screen it was found that approximately 50% of all peptide variants had improved or equivalent activity to the parent peptide Bac2A with Cys, Trp, Arg, His, and Lys representing preferred substitutions. This approach provides an economical alternative for creation of novel synthetic peptide derivatives for substitution analysis.

5.g. Quantitative Structure Activity Relationship Studies (QSAR)

Traditional structure-activity studies of HDPs have been limited due to both time- and cost-constraints. Although development of high-throughput synthesis and screening techniques increases the efficiency of this process there is still a limitation to the number of peptide derivatives that can be analyzed. The utilization of *in silico* methods focusing on rapid development of potentially superior peptide derivatives has been sought as a predictive alternative to normal prediction and synthesis procedures. Recently, QSAR studies have been employed for prediction of novel active HDPs. QSAR methodology combines computational and mathematical modeling along with unique descriptors based on structural and functional information on peptide activity [140]. Through the structural/functional peptide information unique physicochemical parameters, or descriptors, are defined and the specific nature of these descriptors dictates the quality and reliability of the QSAR model [141]. A primary advantage of QSAR models is that only the sequence of a given peptide is required although this is restricted to peptides of the same length [141]. For QSAR modeling of HDPs, peptide descriptors are defined by the physicochemical properties related to biological activities and can rely solely on experimentally derived physicochemical parameters or be combined with information from 2- and 3-D structures [141]. Thus, QSAR has been utilized for prediction of novel HDPs in a large number of studies.

Lejon *et al.* have used QSAR modeling to predict novel pentadeca peptides with antimicrobial activity based on data derived from lactoferricins [142]. Using QSAR methodology, Jenssen *et al.* recently described the use of novel descriptors of contact energies between residue neighbours to correctly predict active HDPs with 84% accuracy [140]. Similarly, QSAR modeling has been used for the 18 amino acid α-helical peptide Novispirin G10 to predict mutations that increase antimicrobial activity [141]. In the QSAR model all possible single residue mutants were tested and structural modeling and molecular dynamics optimization of all 360 mutants was performed. Based on this information, 16 analogs were created, 11 of which demonstrated increased antimicrobial activity [141]. The combination of Neural Network modeling for prediction of molecular properties with a QSAR model and Genetic Algorithms has been used for prediction of optimized peptides. Indeed, 90 out of 100 synthetic peptides in the final population were found to be acceptable [143]. Similarly, Jenssen *et al*. recently described the use of novel descriptors of contact energies between residue neighbours to correctly predict active HDPs with 84% accuracy [140]. Cherkasov *et al.* have recently combined *in vitro* data from libraries of previously synthesized HDPs, along with atomic-resolution chemical descriptors and an artificial neural net approach, to design and correctly predict the antimicrobial activity of a large library of HDP candidates [42]. An *in silico* library of 100,000 virtual peptides was created in four quartiles ranging from increased predicted antimicrobial activity (quartile 1) to suppressed antimicrobial activity (quartile 4). Through *in vitro* MIC screening it was demonstrated that 98% of the peptides in quartile 1 were more active that the control peptide and 88% of those in quartile 2 also had enhanced activities as compared to the control peptide.

5.h. Peptide Sequence Scrambling

Structure-activity relationship investigations of HDPs have attempted to elucidate the relationship between activity and residue arrangement through sequence scrambling. Sequence scrambling maintains overall physicochemical characteristics but allows for alternative sequence arrangements. If conservation of physicochemical characteristics is the only requirement for HDP activity, all scrambled peptide derivatives should have activities equal to the parent peptide. An investigation by Pag *et al.* demonstrated that sequence scrambling of a synthetic amphipathic α-helical HDP resulted in retained antimicrobial activity [144]. Hilpert *et al*. recently utilized sequence scrambling to create 49 variants of Bac2A using a nonbiased random computational methodology in which the peptide derivatives fell into six activity classes ranging from superior activity to those with a complete loss of antimicrobial activity [145]. Derivatives that maintained antimicrobial activity deviated substantially in sequence from Bac2A demonstrating that antimicrobial activity was likely related to physicochemical properties, such as overall charge and hydrophobicity, rather than particular sequence motifs. Papo *et al.* have made similar observations with a scrambled version of a synthetic amphipathic α-helical HDP wherein the scrambled derivative retained antimicrobial activity [146]. Thus, sequence scrambling may represent a methodology for rapid development of peptide derivatives with enhanced antimicrobial or immunomodulatory activities.

6. CONCLUSIONS

Induction of rapid and non-specific defensive responses is a hallmark of innate immunity. The non-specific action of this system bestows a wide range of protection against a diverse array of microbial challenges. As central components of innate immunity, HDPs appear to follow this paradigm through their broad range of action and mechanisms. At a functional level these peptides offer protection from a range of microbial challengers including Gram-negative and -positive bacteria, viruses, fungi and other parasites. At a mechanistic level, this protection is achieved through a number of potential mechanisms including direct disruption of membranes, interference of microbial biomolecules, activation of innate immunity, modulation of inflammation and the bridging of these immediate immune responses into long

term adaptive immunity. These properties make HDPs particularly important from a therapeutic perspective, but equally important from a structural perspective, in determining how such a diverse range of activities is achieved within limited structures.

The direct antimicrobial activity of HDPs, whether mediated through membrane disruption or targeting of protein or nucleic acid bacterial effectors, is characterized by low-affinity, low-specificity interactions that permit action against a wide spectrum of targets. This contrasts with the high-affinity, high specificity approach utilized for most conventional antibiotics. A considerable advantage of this "dirty drug" approach is both of the spectrum of susceptible bacteria as well as the difficulty for bacteria to develop resistance to this style of attack. The structural basis of this activity has been investigated in a large number of peptides and from these investigations a number of generally applicable guidelines for HDP design have emerged. For example, the hemolytic activities of HDPs correlate with high hydrophobicity, amphipathicity and helicity whereas antimicrobial activity is less dependent upon these factors [62]. Such information can be employed to develop rationale libraries of peptide candidates which can be screened against particular bacterial strains and then further selected with additional criteria such as PhoQ ligand potential.

While it is accepted and appreciated that direct antimicrobial offers considerable flexibility for manipulation of structure with retention of function, the traditional viewpoint of receptor-mediated events would predict that these peptides would be less tolerant of such manipulations in their roles as signalling molecules. This does not appear to be the case, however, as peptides of diverse sequence have been shown to induce innate immune responses to varying degrees of efficiency. Similarly it has been shown that HDPs can offer protective effect from microbial challenge in species distinct from that which they originated. This may arise in part from direct antimicrobial activity but does raise questions of the ability for HDPs to transcend species barriers for activation of immune responses. Most of the animal challenge experiments involving HDPs do not address the mechanism of protection.

The traditional dogma of protein structure/activity dictates that biological activity is dependent upon the formation of a precise three-dimensional structure. There is a growing appreciation, however, that this principle may not be equally applicable to all peptides and proteins. Indeed, for select proteins structural flexibility may represent an essential component of their biological function. These intrinsically unstructured peptides (IUPs) have dynamic structures which allow them to adopt active conformations upon binding effector molecules. The ability for different effectors to induce different bioactive conformations to achieve distinct physiological objectives allows these molecules to exert different biological effects through independent mechanisms which are dependent upon unique conformations. This template-driven formation of bioactive conformations allows multiple biological activities to be contained within the same length of polypeptide. Such proteins have been referred to as multitasking or moonlighting to reflect their ability to perform multiple, seemingly unrelated functions [147].

This malleability allows different functions to exist within the same molecular region; this may be of particular importance for molecules, such as the HDPs, which are of limited size. From a wider perspective it is uncertain whether a given HDP utilizes a single receptor for all cell types or multiple and distinct, cell-type specific receptors to initiate unique cellular responses at different locals. Furthermore it is not clear whether different HDPs from the same species engage a common pattern of receptors or whether individual HDPs signal through distinct mechanisms. This uncertainty may reflect a similar multi-functional mechanism that has been proposed for interaction with bacterial effectors such that the structural flexibility and cationicity of HDPs allows them to form bioactive conformations with a number of host effectors. This may offer considerable potential to use natural HDPs as flexible templates for discovery of novel immune modulating actions but also requires an appreciation that the generalized ability of such peptides to associate and influence host biomolecules will complicate considerations of toxicity and potential side effects which may be very difficult to predict or generalize across HDPs.

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